Exometabolomic Profiling of Bacterial Strains as Cultivated Using *Arabidopsis* Root Extract as the Sole Carbon Source

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The ability of microorganisms to use root-derived metabolites as growth substrates is a key trait for success in the rhizospheric niche. However, few studies describe which specific metabolites are consumed or to what degree microbial strains differ in their substrate consumption patterns. Here, we present a liquid chromatography-mass spectrometry (MS) exometabolomic study of three bacterial strains cultivated using either glucose or *Arabidopsis thaliana* root extract as the sole carbon source. Two of the strains were previously isolated from field-grown *Arabidopsis* roots, the other is *Escherichia coli*, included as a comparison. When cultivated on root extract, a set of 62 MS features were commonly taken up by all three strains, with *m/z* values matching components of central metabolism (including amino acids and purine or pyrimidine derivatives). *Escherichia coli* took up very few MS features outside this commonly consumed set, whereas the root-inhabiting strains took up a much larger number of MS features, many with *m/z* values matching plant-specific metabolites. These measurements define the metabolic niche that each strain potentially occupies in the rhizosphere. Furthermore, we document many MS features released by these strains that could play roles in cross-feeding, antibiosis, or signaling. We present our methodological approach as a foundation for future studies of rhizosphere exometabolomics.

The rhizosphere (here widely defined to include plant roots and the immediately surrounding soil) contains a far higher concentration of microbes compared with bulk soil. This is primarily because the rhizosphere is rich in labile carbon, both within root cells and released into the soil via rhizodeposition. This stimulates the growth of soil microbes, which are usually carbon-limited (Dennis et al. 2010; Hartmann et al. 2009). However, the taxonomic breakdown of the rhizospheric microbiome is substantially different from the soil microbiome (Bulgarelli et al. 2013; Lundberg et al. 2012), indicating that certain strains are better adapted to colonize the rhizospheric niche. Studies

have investigated which particular traits are important for microbial rhizosphere competence, highlighting mechanisms such as chemotaxis, antibiosis, and biofilm formation (Barret et al. 2011; Compant et al. 2010). But at a metabolic level, perhaps the most important factor determining rhizosphere competence is the ability to effectively use root-derived metabolites as a carbon source for growth (Benizri et al. 2002; Knee et al. 2001).

The taxonomic composition of the rhizosphere microbiome has been intensively studied over the last 15 years. Using bacterial 16S pyrosequencing, studies almost invariably report a set of core bacterial taxa that are detected in the rhizosphere, including actinobacteria, flavobacteria, alpha-, beta-, and gamma-proteobacteria, firmicutes, and flavobacteria (Bulgarelli et al. 2013; Müller et al. 2016). The consistent emergence of taxonomic diversity in the rhizosphere suggests that multiple strains can coexist in the same environment, facilitated via niche differentiation processes. One aspect of niche differentiation involves strains occupying different physical locations in the rhizosphere, for instance certain strains colonizing the root surface, followed by a subset of these strains subsequently proceeding to colonize the interior of the root (Beckers et al. 2017; Edwards et al. 2015; McCully 2001). But given the huge diversity of metabolites found in plant tissue, another important process driving rhizosphere microbial diversity is resource partitioning at the metabolic level, whereby individual bacterial strains are each specialized to consume discrete subsets of root-derived metabolites (Goldfarb et al. 2011).

There is ongoing investigation as to whether different plant genotypes can reproducibly recruit different microbial strains to their rhizospheric microbiomes. Although soil and climate seem to exert dominant influences upon the taxonomic composition of the rhizosphere microbiome, there is evidence that plant genotype also exerts a significant effect (Bulgarelli et al. 2015; Peiffer et al. 2013). Mechanistically, it is particularly interesting to define the specific plant-derived molecules that are involved in the recruitment of microbial strains. There is extensive literature implicating flavonoids as a key player in plant-microbe interactions, particularly for recruitment of nitrogen-fixing rhizobium strains to legume root nodules (Hassan and Mathesius 2012). Furthermore, soil incubated with purified flavonoids exhibited an altered microbial community structure (Szoboszlai et al. 2016), indicating that flavonoids can affect the growth behavior of a wide range of soil microbes. Therefore, it can be hypothesized that flavonoids are a key player in the chemical dialogue used by plants to recruit specific microbial strains from the soil and into their rhizosphere microbiome.

In the microbiology literature, there is a long tradition of studying which carbon sources are utilized by bacterial strains as growth substrates (Harder and Dijkhuizen 1982; Monod 1966).

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Methodologically, classical studies involved providing a single substrate or a simple mixture of substrates and using biochemical methods to measure the depletion of individual metabolites from the medium. The limitation of this approach is the low throughput of measurement, meaning that relatively few substrates can be provided to the organism simultaneously. Another approach involves phenotype microarrays, where microbial strains are incubated in 96-well plates in which each well containing a single growth substrate and growth is monitored over time (Bochner et al. 2001). The disadvantage of this method is that the plates are loaded with a predefined set of growth substrates, which cannot fully represent the diverse array of metabolites present in the rhizosphere. Recently, studies investigating microbial substrate preferences are increasingly beginning to incorporate high-throughput exometabolomic approaches, in which nuclear magnetic resonance (NMR) or mass spectrometry (MS) are used to measure which metabolites are depleted from the growth medium during microbial growth (Silva and Northen 2015). The advantage of this approach is that the consumption of several hundred metabolites can be measured in complex media, meaning that the media can be formulated to reflect the potential growth substrates available to the organism in the rhizosphere.

Exometabolomic approaches have been applied to investigate metabolite uptake and release across a wide range of organisms, with an increasing tendency to design experimental scenarios that represent the metabolic milieu of the organism's natural habitat. Baran et al. (2015) presents a study of metabolic cross-feeding among soil crust isolates, reconstructing a simplified community comprised of one autotrophic primary producer and five heterotrophic consumers. Using liquid chromatography (LC)-MS exometabolomics, the study found strong evidence for metabolic resource partitioning among the heterotrophic strains, with each strain consuming a different subset of available metabolites. In the phyllosphere, Ryffel et al. (2016) inoculated *Arabidopsis* leaves with three different bacterial strains and conducted NMR metabolomics on a solution derived from rinsing the leaves as well as MS imaging of the leaves themselves. This revealed that nucleotide metabolites colocalize with bacterial colonization sites and, also, that the strains appear to modulate the plant-derived arginine pool. Regarding rhizospheric strains, Fei et al. (2016) presents an exometabolomic study of nitrogen-fixing legume symbiont *Sinorhizobium meliloti* strains grown on laboratory medium, showing that deletion of the pSymb plasmid results in slower consumption of several amino acids. However, to our knowledge there are no studies using exometabolomic approaches to investigate bacterial substrate utilization from a growth medium that includes plant root extracts.

In this study, we conducted an exometabolomic analysis of individual bacterial strains, cultivated using either glucose or *Arabidopsis thaliana* root extracts as the sole carbon source in the growth medium. We aimed to determine which root-derived metabolites are consumed by each strain and which metabolites the strains excrete into the medium. Also, we investigate the metabolomic footprint of strains grown using the flavonoid-deficient *tt4 Arabidopsis* mutant. The aim of our work was not to create a complete list of metabolites but, rather, to test the suitability of this methodological approach as a foundation for future comprehensive studies of rhizosphere exometabolomics.

RESULTS

Experimental workflow for exometabolomic analysis using root extract as the sole carbon source.

Here, we present a novel methodology for studying which root-derived metabolites are consumed by individual bacterial strains; a schematic of the experimental workflow is presented in Supplementary Figure S1. First, large quantities of *Arabidopsis*

root tissue are produced using the method of Hétyu et al. (2005). In our hands, this procedure yielded approximately 1.5 g of root material per 250-ml conical flask. Next, metabolites are extracted from ground root material and the total organic carbon concentration of this metabolite extract is determined. Our extracts contained roughly 3 mg of carbon per 1 g of fresh root tissue extracted. Bacterial growth media were then formulated, containing either glucose or root extract as the sole carbon source. The carbon concentration of all growth media was set to 0.72 mg of C per milliliter of medium. At this concentration, carbon is the stoichiometrically limiting nutrient, as determined experimentally (Egli 2015) (Supplementary Fig. S2). Next, washed bacterial cells are inoculated into these growth media on a 48-well plate and bacterial growth is monitored via measurement of optical density at 600 nm (OD₆₀₀) in a plate reader. Analysis of growth curves found that *Arabidopsis* root extract could support growth of all tested strains (Supplementary Fig. S3). After 24 h, all cultures had reached stationary phase and spent media were harvested by separating bacterial cells via centrifugation, followed by collection and filtration of supernatants. The metabolomic composition of these spent-media samples were then analyzed using LC-MS and the resulting data was analyzed using XCMS online (Tautenhahn et al. 2012).

Global analysis of exometabolomic profiles using LC-MS.

First, we aimed to characterize how the exometabolome profile can be shaped by different bacterial strains and carbon sources by conducting a global analysis of metabolite peaks in the spent-media samples, measured using LC-MS. We analyzed three bacterial strains, *Pseudomonas* sp. *Root9* and *Rhizobium* sp. *Root491* (both isolated from *Arabidopsis* roots by Bai et al. [2015]) and *E. coli* isolated from human gut and included to contrast against the rhizosphere strains. As a negative control (without bacteria), we also included samples in which the bacterial inoculation was replaced by sterile MgCl₂ and incubated side-by-side with the bacterial cultures. Alignment of MS features between samples yielded 2,786 discrete MS features, which was reduced to 2,456 when isotopic peaks were removed. To highlight features that were significantly different between samples, we filtered the data to select 1,982 features with $P < 0.01$ following t test between any two groups (Supplementary Table S1) and arranged these features by hierarchical clustering (Fig. 1). Correction for multiple comparisons was conducted using the method of Storey (2002) and showed that 1,958 MS features were significant at $q < 0.01$. By looking at the resultant heatmap, it can be clearly seen that there is a large number of MS features present in the plant root samples but absent in the glucose samples, resulting in the dendrogram splitting the eight samples into two groups according to carbon source. Looking at the different rows in the heatmap, there is clear evidence for metabolite uptake from the growth media (when a feature showed higher abundance in MgCl₂ and lower abundance in one or more bacterial inoculation) and also metabolite release into the growth media (when a feature has lower abundance in MgCl₂ and higher abundance in one or more bacterial inoculation). Also evident is a cluster of MS features that are more abundant in the glucose media compared with the Col-0 media but not taken up by any strain. We believe that these are salt adducts derived from the M9 salts, which are masked by ion suppression in the Col-0 samples, because they elute at the same retention time (RT) as many charged metabolites, including major sugars. Noticeably, there was a very large cluster of MS features that were more abundant in samples in which root extract was the sole carbon source but which were not taken up by any of the three strains tested here. It would be interesting to determine whether these MS features can serve as growth substrates for other microbial strains not included in this study.

MS features taken up by bacterial strains when *Arabidopsis* root extract is the sole carbon source.

Our next goal was to determine how many MS features are consumed by the three strains when cultivated on *Arabidopsis* root extract as the sole carbon source and also the degree of overlap in these substrate uptake patterns between bacterial strains. Therefore, we filtered for MS features that were significantly depleted in the Col-0 medium inoculated with bacteria compared with Col-0 medium incubated with MgCl_2 (details of filtering provided below). This resulted in 422 MS features that were implicated as root metabolites consumed by bacterial uptake, presented as a heat map and Venn diagram in Figure 2. From this analysis, we observed a core set of 62 MS features taken up by all three strains. It was clear that *Rhizobium* sp. *Root491* consumes a much larger set of metabolites than the other two strains, with 372 MS features being consumed by this strain, compared with 187 in *Pseudomonas* sp. *Root9* and only 70 in *E. coli*. There was also a set of 76 MS features commonly taken up by the two root-inhabiting strains but not taken up by *E. coli*.

MS features released by bacterial strains on either glucose or root extract as the sole carbon source.

We were also interested in characterizing the MS features that were excreted into the media by the different bacterial strains when either glucose or Col-0 root extract were provided as the sole carbon source. To select these features, we filtered for MS features that were substantially more abundant in the inoculated media compared with the media incubated with sterile MgCl_2 control (details of filtering are provided below). The 448 MS features that met filtering criteria are presented as a heatmap and several Venn diagrams in Figure 3. From these analyses, it is evident that there is relatively little overlap in the MS features released by an individual strain when grown on the two carbon sources. For instance, *Rhizobium* sp. *Root491* releases 135 MS features uniquely when grown on Col-0 root extract and 65 only when grown on glucose, but the overlap between these two conditions contained just 19 MS features. For *E. coli*, only 48 MS features were released under both conditions, whereas 147 were uniquely released under glucose nutrition.

Assignment of candidate metabolite identity to MS features.

Next, we wished to ascertain which particular metabolites correspond to the MS features that were taken up or released by the analyzed bacterial strains. Therefore, we used the Metabosearch program (Zhou et al. 2012) to assign candidate identities for the MS features that were taken up or released by the individual strains (Supplementary Table S2 for uptake, Supplementary Table S3 for release). For the 422 MS features that were taken up from Col-0 medium, Metabosearch gave candidate identities for 300, while for the 448 MS features that were released into either medium, Metabosearch gave candidate identities for 323. As a quality check for this approach, we began by assessing whether we could reliably detect the consumption of glucose in the cultures in which it was present as the sole carbon source. Therefore, we prepared a standard solution of 10 μM glucose and measured this on our LC-MS system, to determine the best-responding reporter ion for this molecule (Na^+ adduct of glucose, m/z 203.053, RT 1.6 min). Then, we checked the abundance pattern of this particular MS feature in our spent-media samples, with the result shown in Figure 4. The huge depletion of glucose during bacterial growth can be observed by the low abundance of this MS feature in the three bacterial inoculations of the media in which glucose is the sole carbon source, compared against its very high abundance in the MgCl_2 incubation. Although Metabosearch correctly identi-

fied the molecular formula of this metabolite ($\text{C}_6\text{H}_{12}\text{O}_6$), our data filtering strategy led to this MS feature being assigned to fructose, an isomer of glucose. Furthermore, we also observed several other MS features exhibiting a very similar abundance pattern, eluting at the same RT, which almost certainly derive from different adducts and fragments of glucose. This highlights two caveats of our approach. i) Although the molecular formula assignment of a given MS feature is likely to be accurate due to the high mass accuracy of the MS system and the stringent search parameters used, the molecular structure of these assignments is uncertain because many isomers can derive from the same molecular formula. ii) The number of MS features detected may be higher than the number of discrete molecules in the sample, because one single molecule can be detected in multiple adducted or fragmented states.

Metabolites of particular interest.

Upon inspecting the derived data, we observed a number of candidate metabolites that warranted particular interest. First, we inspected the candidate identity of the 62 MS features commonly taken up by all three strains, which revealed that

Fig. 1. Overview of exometabolomic differences observed between three bacterial strains cultivated on two carbon sources. The heatmap depicts the mean intensity values for the 1,982 mass spectral (MS) features that had a $P < 0.01$ following t test between any two sample groups. Each row corresponds to a MS feature and each column corresponds to a strain $\times$ medium combination. Rows and columns were hierarchically clustered using Pearson's correlation coefficient. Labels above the heatmap indicate carbon source, labels below the heatmap indicate bacterial inoculation, with MgCl_2 as the sterile control.

many of these features had m/z values matching fundamental components of central metabolism, such as amino acids (including valine, tryptophan, threonine, and glutamine), and also purine or pyrimidine derivatives (including adenosine, cytidine, guanine, and uridine). In Figure 5, we show the abundance patterns for three of these metabolites that were taken up by all strains, annotated as tryptophan, uridine, and malate. Malate (Fig. 5C) is an interesting case, as it is consumed from the

medium when Col-0 root extract is the carbon source but released into the medium when glucose is the carbon source. Regarding metabolites that were released into the medium, we observed that *E. coli* excretes a large amount of orotate into the medium when grown under glucose as C source (Fig. 6A), which matches established literature (Enjalbert et al. 2013; Womack and O'Donovan 1978). For *Pseudomonas* sp. Root9, we detected the release of several nucleotides into the medium

Fig. 2. Mass spectral (MS) features taken up by bacterial strains cultivated on medium containing *Arabidopsis* Col-0 root extract as the sole carbon source. **A**, A heatmap depicting the mean intensity values for the 422 MS features that were taken up from Col-0 medium by any bacterial strain. Each row corresponds to a MS feature and each column corresponds to a bacterial strain. Rows were hierarchically clustered using Pearson's correlation coefficient. Labels below the heatmap indicate bacterial inoculation, with $MgCl_2$ as the sterile control. **B**, A Venn diagram showing the number of MS features that were taken up commonly and uniquely by the three strains.

Fig. 3. Mass spectral (MS) features released by the bacterial strains. Data were filtered to select 448 MS features that were released into either glucose or Col-0 medium by at least one strain. **A**, A heatmap depicting the mean intensity values for the 448 MS features, in which each row corresponds to a MS feature and each column corresponds to a strain $\times$ medium combination. Rows and columns were hierarchically clustered using Pearson's correlation coefficient. $MgCl_2$ was the sterile control. **B**, Venn diagrams showing the degree of overlap between MS features released by each strain across the two carbon sources.

under both glucose and Col-0 nutrition, including m/z values matching AMP, CMP, GMP, and UMP (Fig. 6B); these nucleotides could potentially play roles in signaling (Grundling and Lee 2016). Although we did not detect release of the active auxin indole-3-acetic acid by any of these strains, we did detect that *Rhizobium* sp. *Root491* releases an m/z value matching an auxin conjugate, indole-3-acetyl-myoinositol, under Col-0 nutrition but not glucose (Fig. 6C). It would be interesting to determine whether this bacterial-derived auxin conjugate can be taken up by the plant. For *Rhizobium* sp. *Root491* grown under Col-0 root extract, we observed a particularly interesting phenomenon, whereby this strain took up MS features matching scopolin and sinapoyl glucose and released MS features matching scopoletin and sinapate (Fig. 7). We postulate that this strain can cleave and metabolize the labile sugar from these molecules but is unable to metabolize the more recalcitrant core structures.

Application of our exometabolomic profiling approach to the flavonoid-deficient *tt4* mutant.

Finally, we investigated whether our methodological approach could be used to profile an *Arabidopsis* mutant. There is extensive literature implicating flavonoids as a key player in plant-microbe interactions (Hassan and Mathesius 2012; Szoboszlay et al. 2016), so we prepared root extract from the flavonoid-deficient *tt4* mutant and used this as a carbon source for bacterial growth. The absence of flavonoid-derived pigments in the *tt4* root extracts was confirmed by photometric measurements across the UV range (Supplementary Fig. S4). When analyzing growth phenotypes using Growthcurver, we observed no significant differences between Col-0 and *tt4* root extracts, suggesting that flavonoids are not major metabolic substrates for the growth of any bacterial strains assayed here. Next, we compared exometabolomic profiles between Col-0 medium versus the *tt4* medium, filtering for MS features that were significantly different between the two samples (Fig. 8A). This comparison yielded 62 MS features, with the predominant grouping being 41 MS features that were highly abundant in all four Col-0 samples but low abundance in all four *tt4* samples. Upon inspecting the candidate identities of these features derived from Metabosearch, we found that 16 of these features were matched to H^+ adducts of flavonoid glycosides and, also, eight features matched to Na^+ adducts of flavonoid glycosides.

Fig. 4. Uptake of glucose by bacterial strains. White-filled columns represent mean intensity of the mass spectral (MS) feature when glucose was the sole carbon source, black-filled columns represent mean intensity of the MS feature when Col-0 root extract was the sole carbon source. X axis labels indicate bacterial inoculation, with $MgCl_2$ as a sterile control. Error bars represent standard deviation ($n = 3$). Different letters indicate significant difference at $P < 0.01$ (Tukey's highly significant difference test). Metabolite identity was confirmed by comparison with a reference glucose standard.

Looking at the abundance of these features in the samples where Col-0 root extracts had been inoculated with bacteria, we observed that none of the bacteria took up these candidate flavonoid glycosides (Fig. 8B and C). From this evidence, we conclude that flavonoid glycosides are not major metabolic substrates for these particular bacterial strains and, also, that exometabolomic profiles of these strains are not strongly influenced by flavonoid-linked signaling.

DISCUSSION

In the rhizosphere, there is a bidirectional exchange of nutrients between plants and microbes, but our mechanistic understanding

Fig. 5. Selected mass spectral features taken up by all three strains. Candidate metabolite identities (IDs) were matched to **A**, tryptophan, **B**, uridine, and **C**, malate. White-filled columns represent mean feature intensity when glucose was the sole carbon source, black-filled columns represent mean feature intensity when Col-0 root extract was the sole carbon source. X axis labels indicate bacterial inoculation, with $MgCl_2$ as a sterile control. Error bars represent standard deviation ($n = 3$). Different letters indicate significant difference at $P < 0.01$ (Tukey's highly significant difference test).

of this phenomenon is incomplete (Sasse et al. 2018). To gain new insights into this process, it will be important to develop new methodological approaches that can directly measure the metabolic activity of rhizospheric microbial strains. Exometabolomics is increasingly being used to profile metabolite uptake and release by various microbes (Pinu and Villas-Boas 2017), and here, we present the first report of MS exometabolomics applied to rhizospheric microbes using plant root extract as the sole carbon source. The presented approach is highly versatile, with the potential to analyze root extracts from any plant species or genotype and, also, to measure the metabolic footprint of any microbial population, including bacteria,

fungi, or even synthetic microbial communities. Furthermore, future studies could combine this general approach with a wide range of other analytical possibilities, including alternative chromatography methods, other MS systems, or NMR detection of metabolites. We present this study as a first investigation of rhizosphere exometabolomics, to serve as a foundation for future work.

We analyzed three bacterial strains, two root-inhabiting (*Pseudomonas* sp. *Root9* and *Rhizobium* sp. *Root491*) recently isolated from field-grown *Arabidopsis* roots by Bai et al. (2015) and *E. coli*, isolated from human gut and included as a comparison. Comparing the substrate uptake profiles between the three strains, we observed a small set of MS features commonly taken up by all three strains but found that the two root-inhabiting strains took up a much larger set of MS features that were not taken up by *E. coli*. Regarding metabolite release, we found that each individual strain profile of released metabolites was dramatically influenced by the available carbon source (glucose versus *Arabidopsis* root extract), for instance, with *Rhizobium* sp. *Root491*, a large number of MS features was released only when root extract was provided as the carbon source. Furthermore, we applied our methodological approach to the flavonoid-deficient *tt4* mutant, finding that *tt4* root extracts were deficient in several MS features likely to be flavonoid glycosides. However, we observed no discernible uptake of these MS features when the strains were grown on Col-0 root extract, indicating that none of the three studied strains take up flavonoid glycosides as growth substrates.

A major priority of this study was to define which specific root metabolites are consumed as growth substrates by various bacterial strains, thus defining the metabolic niche that these strains can potentially occupy in the rhizosphere. We found a core set of 62 MS features that all three strains deplete from the growth medium when Col-0 root extract was the sole carbon source. Assigning candidate identities to these MS features showed that many were matched to molecules of central metabolism, such as amino acids and purine or pyrimidine derivatives. Extrapolating to the rhizosphere, we can infer that there would be a strong competition for these shared metabolic substrates and that a strain such as *E. coli* would find it difficult to compete in the rhizospheric niche, because it can only take up this limited set of substrates. We were particularly interested in identifying root-derived metabolites that were specifically consumed by individual strains, which could contribute to rhizospheric niche differentiation via metabolic resource partitioning. We observed that *Rhizobium* sp. *Root491* took up 233 MS features that were not taken up by the other two strains, and, inspecting the candidate identities (IDs) for these MS features, we observed several *m/z* values matching plant-specific metabolites such as scopolin, coniferin, sinapoyl glucose, and xylobiose. Although the *Rhizobium* taxon is best-known for the nitrogen-fixing symbionts associated with legumes, there is increasing evidence that strains from this taxon are highly successful at colonizing a wide range of plant species (Chi et al. 2005; García-Fraile et al. 2012). Therefore, perhaps the wide range of plant-specific metabolites taken up by *Rhizobium* sp. *Root491* observed in this study reflects its metabolic specialization to consume phytochemicals as growth substrates. For *Pseudomonas* sp. *Root9*, we observed that this strain took up 43 MS features that were not taken up by the other two strains, including *m/z* values matching the purine breakdown products allantoin acid and uric acid. There is significant literature describing bacterial strains that can catabolize allantoin acid and uric acid (Vogels and Vander Drift 1976), and it is known that plants accumulate significant amounts of these molecules, increasing under stress (Werner and Witte 2011). So, although most of the literature on bacterial consumption of

Fig. 6. Selected mass spectral features released by individual strains. Candidate metabolite identities (IDs) matched to **A**, orotate, **B**, uridine monophosphate, and **C**, indol-3-acetyl-myoinositol. White-filled columns represent mean feature intensity when glucose was the sole carbon source, black-filled columns represent mean feature intensity when Col-0 root extract was the sole carbon source. X axis labels indicate bacterial inoculation, with MgCl₂ as a sterile control. Error bars represent standard deviation ($n = 3$). Different letters indicate significant difference at $P < 0.01$ (Tukey's highly significant difference test).

allantoic acid and uric acid focuses on the cycling of nitrogenous waste from animals, our data indicate that these molecules could also be involved in nutrient exchange processes in the rhizosphere.

There is growing awareness that bacterial metabolite excretion plays a key role in shaping microbial community structure, via processes such as cross-feeding, signaling, and antibiosis (Foster et al. 2017; Widder et al. 2016). In this study, we

Fig. 7. Mass spectral features postulated to be reciprocally taken up and released by *Rhizobium* sp. *Root491*. Candidate metabolite identities (IDs) matched to **A**, scopolin, **B**, sinapoyl-glucose, **C**, scopoletin and **D**, sinapate. White-filled columns represent mean feature intensity when glucose was the sole carbon source, black-filled columns represent mean feature intensity when Col-0 root extract was the sole carbon source. X axis labels indicate bacterial inoculation, with MgCl₂ as a sterile control. Error bars represent standard deviation ($n = 3$). Different letters indicate significant difference at $P < 0.01$ (Tukey's highly significant difference test).

Fig. 8. Comparison of exometabolomic profiles between Col-0 versus *tt4*. **A**, A heatmap depicting the mean intensity values, for 62 mass spectral (MS) features, after data were filtered to select for MS features that were differentially abundant between Col-0 versus *tt4* in either the MgCl₂ incubation or in any bacterial inoculation. Each row corresponds to a MS feature, and each column corresponds to a strain $\times$ medium combination. Rows and columns were hierarchically clustered using Pearson's correlation coefficient. **B**, and **C**, Selected MS features likely to be flavonoid glycosides that differed significantly between Col-0 versus *tt4*. Candidate metabolite identities (IDs) matched to isoorientin 2'-O-rhamnoside (**B**) and violanthin (**C**). White-filled columns represent mean feature intensity when Col-0 root extract was the sole carbon source, black-filled columns represent mean feature intensity when *tt4* root extract was the sole carbon source. X axis labels indicate bacterial inoculation, with MgCl₂ as a sterile control. Error bars represent standard deviation ($n = 3$). Different letters indicate significant difference at $P < 0.01$ (Tukey's highly significant difference test).

document a large number of MS features that the three bacterial strains release into two media, finding discrete patterns according to whether glucose or Col-0 root extract were provided as the sole carbon source. We found that *E. coli* gave out a large set of metabolites only under glucose nutrition, perhaps indicating that a metabolic bottleneck occurs under this single carbon substrate. Conversely, *Rhizobium* sp. *Root491* released a large set of metabolites only under Col-0 nutrition, some of which had *m/z* values matching degradation products derived from consumption of specific root-derived metabolites. We also observe that *Pseudomonas* sp. *Root9* releases a large array of nucleotides, which are likely to have roles in signaling. Our detection of these excreted metabolites shows that exometabolomic approaches applied to the rhizosphere have a strong potential to deepen our knowledge of microbial community function.

Confidently assigning identity to MS features detected via LC-MS is a longstanding problem in metabolomics research (Bowen and Northen 2010; Watson 2013). Here, we used the MetaboSearch program to assign candidate identities to many of the MS features that were taken up or released by the studied strains, allowing us to postulate molecular mechanisms rather than simply comparing MS features. However, there are clear limitations to this annotation strategy, as it does not account for all possible fragments and adducts and, also, it does not differentiate between the many isomers that can be derived from a molecular formula. Obviously, it would be desirable to validate the identity of these MS features using reference compounds and tandem MS, but this approach is unfeasible for the large number of MS features detected in this analysis (1,982 features significant at $P < 0.01$ between any two groups), particularly because our metabolite pool derives from the combined complexity of the plant plus bacterial metabolomes. Therefore, we refer to all our molecular assignments as candidate IDs and are designing future studies to focus on a smaller number of specific MS features with more analytical precision.

In the metabolomics literature, a wide variety of LC-MS methods have been applied to profile chemically diverse metabolites, with each method having various strengths and weaknesses (Gika et al. 2014). For instance, hydrophilic interaction liquid chromatography (HILIC) is better suited to resolve polar metabolites, whereas reverse-phase LC (RPLC) is better suited to resolve hydrophobic metabolites (Tang et al. 2016). Positive-mode ionization is better suited to detect a wide range of polar and nonpolar metabolites, whereas negative-mode ionization is better suited to detect specific metabolite classes, such as tricarboxylic acid cycle intermediates and phosphorylated metabolites (Theodoridis et al. 2012). Here, our methodology involved RPLC chromatography followed by positive-mode MS and, although this gave good coverage of many diverse metabolites, there were still several metabolite classes that are not optimally resolved and detected by this method. For instance, sugars were poorly resolved with our chromatography setup and applying an alternative chromatography technique such as HILIC would yield higher-quality insights into sugar metabolism in the rhizosphere. Also, we did not detect molecules that only ionize in negative mode, such as glucosinolates, showing that alternative ionization conditions would be required to study microbial glucosinolate metabolism. Furthermore, organic acids are highly abundant in plant roots and exudates, and the microbial consumption of plant-derived organic acids is considered to be a major nutrient flux in the rhizosphere (Oburger et al. 2009; van Hees et al. 2005). However, our method seems poorly suited to detect organic acids, with only malate being observed in this study. Due to these limitations therefore, we encourage further studies that build on the general approach presented here, particularly those using other analytical methodologies to detect a wider range of molecules.

There is a large volume of literature studying how root exudation is involved in plant-microbe nutrient transfer (Hartmann et al. 2009; Massalha et al. 2017), and one potential criticism of our approach is that we have used root extracts rather than root exudates as the carbon source for bacterial growth. Here, we give four reasons to justify this choice. First, many rhizosphere microbes are root endophytes (Gaiero et al. 2013), and root metabolite extracts are probably a reasonable approximation of the endophytic substrate pool. Indeed, the root-inhabiting bacterial strains investigated in this study may, in fact, be endophytes, because the sampling procedures used by Bai et al. (2015) to generate these bacterial isolates did not discriminate between endophytes and epiphytes. Second, although root exudation is often used as a convenient shorthand to describe the belowground release of plant metabolites, rhizodeposition can occur through a range of mechanisms, such as i) lysis of sloughed-off root cells, ii) production of mucilage, iii) diffusion of volatiles, and iv) exudation of metabolites from living root cells (Dennis et al. 2010; Jones et al. 2009). Although estimates vary, results from several studies indicate that the amount of carbon deposited via root lysates can significantly outweigh what is deposited via root exudates (Farrar et al. 2003; Kaštovská et al. 2017). Indeed, the root metabolite extracts used in this study are probably a reasonable approximation of the metabolite pool that would occur upon initial lysis of sloughed-off root cells. Third, it is technically challenging to collect *Arabidopsis* root exudates in a representative, sterile, and reproducible manner (Jacoby et al. 2017; Vranova et al. 2013), particularly in the large quantities needed for this study. Fourth, there is considerable overlap between the molecules we observed in this study compared with the list of molecules reported to be present in root exudates, such as sugars, amino acids, organic acids, vitamins, nucleosides, and phenolics (Dakora and Phillips 2002; Mönchgesang et al. 2016). This seems reasonable, as any molecule that will eventually be exuded into the soil must first be present within the root cell. But despite these justifications for our approach, we accept that root exudates are still an important nutrient flux in the rhizosphere, particularly around rapidly proliferating meristematic tissue, and we encourage future studies that attempt to modify our approach to use root exudates as a sole carbon source.

It is well-documented that many microbial strains mediate plant growth promotion via mechanisms such as nutrient mobilization, pathogen suppression, and hormonal signaling (Bulgarelli et al. 2013), and there is increasing research interest in engineering the rhizospheric microbiome to promote these functions and thus replace synthetic agricultural inputs (Bender et al. 2016). However, any efforts to rationally design rhizospheric microbiomes are hampered by the fact that we have very little mechanistic understanding of the principles that govern microbial community formation in the rhizosphere (Busby et al. 2017). Here, we present an approach that could provide new mechanistic insights into rhizospheric microbial communities, by identifying the root-derived metabolites that are consumed by individual strains as carbon substrates and, also, the metabolites that are excreted by individual strains and, therefore, have possible roles in cross-feeding, signaling, or antibiosis. These insights have the potential to guide the rational design of high-functioning rhizosphere communities. For instance, plant varieties could be selected to contain root metabolite profiles that are tailored to nourish targeted microbial strains with desired functional benefits. To rationally guide microbial community formation, one possibility involves combining complementary strains with divergent substrate uptake profiles, which can coexist in the rhizosphere via niche differentiation. Another possibility involves the promotion of cross-feeding, by matching the metabolite release of one strain to the metabolite uptake of another strain.

MATERIALS AND METHODS

Plant accessions and bacterial strains.

Plant genotypes used in this study were *Arabidopsis thaliana* ecotype Columbia (Col-0) and a *tt4* mutant derived from a T-DNA insertion in the chalcone synthase gene At5g13930, stock number SALK_020583. Both were purchased from the Nottingham Arabidopsis Stock Centre. Bacterial strains used in this study were *Pseudomonas* sp. *Root9* (National Center for Biotechnology Information [NCBI] taxonomy ID 1736604), *Rhizobium* sp. *Root491* (NCBI taxonomy ID 1736548), both isolated from field-grown *Arabidopsis* roots (Bai et al. 2015) and provided by P. Schulze-Lefert, Max Plank Institute for Plant Breeding Research, Cologne, Germany. Also used was *Escherichia coli* strain *K12 MG1655* (NCBI taxonomy ID 511145), purchased from DSMZ.

Plant cultivation, root metabolite extraction, and total organic carbon (TOC) measurement.

Arabidopsis plants were cultivated using anchored shaking liquid culture, according to the method of Héty et al. (2005). First, seeds were surface-sterilized for 2 min in 70% ethanol, followed by 5 min in 50% bleach, followed by rinsing five times in sterile Milli-Q water. Then, 15 to 20 seeds were pipetted onto steel mesh discs lying on agar plates (0.7% agar, 1% sucrose, 0.5× Murashige and Skoog, pH 5.8) and were incubated at 4°C in darkness for 2 days. Next, plates were transferred to a growth cabinet, operating at 22°C, with a 16-h photoperiod, 120 µE per square meter of light intensity for 7 days. Then, steel disks containing the anchored seedling plants were transferred to 250-ml Erlenmeyer flasks, containing 15 ml of growth medium (3% sucrose, 0.5× Murashige and Skoog, pH 5.8), and were placed in a shaking incubator at 100 rpm in the growth chamber. After 10 days on the sucrose-containing medium, the medium was swapped to a medium without sucrose (0.5× Murashige and Skoog, pH 5.8), and plants were left shaking for 7 days. During shaking liquid cultivation, the medium was exchanged every three or four days. Plants were harvested by rinsing the plants twice in 10 mM MgCl₂ and separating roots from shoots and immediately snap-freezing the root tissue in liquid N₂. Root tissue was ground in liquid N₂, using a mortar and pestle, and metabolites were extracted by adding 1 ml of 90% MeOH to 200 mg of ground tissue, followed by a 30-min incubation at 60°C with 1,500-rpm shaking. Extracts were then centrifuged at 20,000 × g for 10 min and 850 µl of supernatant was dried, using a vacuum centrifuge. Next, metabolite extracts were dissolved in Milli-Q water and were filter sterilized (0.22 µm pore size) (Millipore). TOC was measured using a Dimatoc 2000 (Dimatec).

Formulation of bacterial growth media.

Bacterial growth media was based on M9 formulation (Cold Spring Harbor Laboratory 2010). Nutrient concentrations were: 24 mM Na₂HPO₄, 20 mM NH₄Cl, 11 mM KH₂PO₄, 4 mM NaCl, 350 µM MgSO₄, 100 µM CaCl₂, 50 µM Fe-EDTA, 50 µM H₃BO₃, 10 µM MnCl₂, 1.75 µM ZnCl₂, 1 µM KI, 800 nM Na₂MoO₄, 500 nM CuCl₂, and 100 nM CoCl₂. To this, one carbon source was added, either glucose (10 mM, equivalent to 0.72 mg of C per milliliter) or *Arabidopsis* root extract (0.72 mg of C per milliliter, as measured by TOC).

Bacterial cultivation and spent media collection.

Bacterial strains were precultivated by streaking glycerol stocks onto trypticase soy agar plates (0.5× trypticase soy broth [TSB], 1.2% agar) and incubation at 28°C for between 1 and 4 days. After visible growth, single colonies were picked from

plates and inoculated into TSB medium (0.5× TSB) and were incubated for 24 h at 28°C with 200-rpm shaking. Next, cells were harvested by centrifuging 800 µl of culture at 5,000 × g for 2 min at room temperature. These cells were then rinsed three times in sterile 10 mM MgCl₂ and resuspended at a final OD₆₀₀ of 1.0 in sterile 10 mM MgCl₂. For growth assays, 20 µl of this bacterial suspension was inoculated into 380 µl of growth medium, in individual wells of a sterile 48-well plate (Corning). These plates were then transferred to a plate reader (Tecan Infinite Pro 100) and were incubated at 28°C for 24 h, with shaking (3 min continuous orbital shaking followed by 7 min stationary, shaking amplitude 3 mm). Culture density at OD₆₀₀ was measured once per 10-min cycle, without correcting for path length, and derived growth curves were analyzed using the Growthcurver program (Sprouffske and Wagner 2016). For spent media collection, bacterial cultures were centrifuged at 10,000 × g for 10 min at room temperature; then, 250 µl of supernatant was filtered, using a spin filter (Corning, 0.22 µm pore size), and the flow-through was stored at -80°C. Three independent biological replicates were conducted, in which each replicate derived from an independent plant cultivation and also an independent bacterial cultivation. As a negative control (without bacteria), we included samples for which growth media were incubated with 20 µl of sterile 10 mM MgCl₂ instead of being inoculated with bacteria.

LC-MS analysis of spent media.

For spent media analyses, 2 µl of sample was loaded onto a C18 column (XSelect HSS T3, 2.5 µm particle size, 10-nm pore size, 150 mm length by 3.0 mm width; Waters), using a high-pressure LC (HPLC) (Dionex Ultimate 3000, Thermo Scientific). Buffer A was 0.1% FA in HPLC-grade water, buffer B was 0.1% FA in HPLC-grade methanol, and flow rate was 500 µl/min. Samples were eluted using the following gradient: hold at 1% B between 0 to 1 min, linear increase to 40% B until 11 min, linear increase to 99% B until 15 min, hold at 99% B until 16 min, linear decrease to 1% B until 17 min, and finally, hold at 1% B until 20 min. MS was conducted using a quadrupole-time-of-flight MS (maXis 4G; Bruker Daltonics), following ionization via an electrospray ionization source. The MS was operated in positive-ion mode, using N₂ as drying gas at a flow rate of 8 liters/min, dry heater set to 220°C, nebulizer pressure of 1.8 bar, capillary voltage of 4,500 V, and collision radio frequency voltage of 370 V (corresponding to a mass range of 50 to 1,300 *m/z*). Scan rate was 1 Hz.

Data analysis.

Raw .D files were centroided and converted to .MZXML using the MSConvert program (Kessner et al. 2008). Files were then uploaded to XCMS online (Tautenhahn et al. 2012) and were aligned using the following parameters: *m/z* tolerance of 15 ppm, minimum peak width of 10 s, maximum peak width of 60 s, signal/noise threshold of 10, overlapping peaks split when *m/z* difference was greater than 0.01 *m/z*, features only considered if they occurred in at least five consecutive scans with an intensity greater than 5,000. Also, features were only considered if they occurred in at least two of three replicates from any sample group. The CAMERA algorithm was implemented to detect isotopes and adducts. Following export of files from XCMS online, data were filtered to remove isotopic peaks and to only include MS features with RT between 1 to 16 min. Samples were compared between groups using Student's *t* test, and the resulting *P* values were corrected for multiple comparisons using the method of Storey (2002) to yield *q* values. To calculate significant differences shown in Figures 4, 5, 6, 7, and 8, data were compared using analysis of variance, followed by posthoc Tukey's highly significant difference test to determine

significant differences between groups at $P < 0.01$. To select the MS features that were taken up from Col-0 media, we filtered for features with intensity $>10,000$ in Col-0 incubated with MgCl_2 , where $\log_2$ fold change was >2 between Col-0 incubated with MgCl_2 versus Col-0 inoculated with bacteria and where the t test of this comparison gave $P < 0.01$. In instances in which the MS feature had an abundance of zero in the inoculated sample and, thus, no fold change was calculated, we imposed a filter that the feature must have a relative standard deviation of $<25\%$ in the Col-0 MgCl_2 sample. To select the MS features that were released into the media, we again filtered for features where $\log_2$ fold change was >2 between medium inoculated with bacteria versus medium incubated with MgCl_2 and where the t test of this comparison gave $P < 0.01$. In instances in which the MS feature had an abundance of zero in the uninoculated sample and, thus, no fold change was calculated, we imposed a filter that the feature must have a relative standard deviation of $<25\%$ in the sample inoculated with bacteria. To select the MS features that were differentially abundant between Col-0 and the *tt4* mutant, we filtered for MS features with a $\log_2$ fold change > 2 and a P value < 0.01 , either in the MgCl_2 incubations or in any of the bacterial inoculations. To assign candidate metabolite identities for the MS features of interest, we used the Metabosearch program to search the m/z values against three databases—MMCD, HMDB, and Lipidmaps (Zhou et al. 2012). We searched for possible H^+ , Na^+ , K^+ , and NH_4^+ adducts of each MS feature, imposing a ± 2 ppm m/z error tolerance. In many cases, Metabosearch output multiple candidate identities per single MS feature, so to filter these assignments into a manageable list, we sorted for the metabolite hit with the lowest m/z error and then for the metabolite hit with the lowest entry number in the KEGG database, and we present only the first result from this list.

Data availability.

All LC-MS data files have been uploaded to the Metabolights database (Haug et al. 2013).

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AUTHOR-RECOMMENDED INTERNET RESOURCE

Metabolights study: <http://www.ebi.ac.uk/metabolights/MTBL555>