

1 **Dynamic root exudate chemistry and microbial substrate preferences drive patterns in**
2 **rhizosphere microbial community assembly**

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4 Kateryna Zhalnina^{1,2}, Katherine B. Louie¹, Zhao Hao², Nasim Mansoori^{1,3}, Ulisses Nunes da
5 Rocha^{2,4}, Shengjing Shi⁵, Heejung Cho^{2,6}, Ulas Karaoz², Dominique Loqué^{1,3,6,7}, Benjamin P.
6 Bowen¹, Mary K. Firestone^{2,8}, Trent R. Northen^{1*}, and Eoin L. Brodie^{2,8*}

7 **Affiliations**

8 ¹Environmental Genomics and Systems Biology Division, Lawrence Berkeley National
9 Laboratory, Berkeley, California, USA.

10 ²Earth and Environmental Sciences, Lawrence Berkeley National Laboratory, Berkeley,
11 California, USA.

12 ³Joint BioEnergy Institute, Biosystems Engineering Division, Lawrence Berkeley National
13 Laboratory, Emeryville, California.

14 ⁴Department of Environmental Microbiology, Helmholtz Centre for Environmental Research -
15 UFZ, Leipzig, Germany.

16 ⁵Lincoln Science Centre, AgResearch Ltd, Christchurch, New Zealand.

17 ⁶Department of Plant and Microbial Biology, University of California, Berkeley, California,
18 USA.

19 ⁷Université Claude Bernard Lyon 1, INSA de Lyon, CNRS, UMR5240, Microbiologie,
20 Adaptation et Pathogénie, Villeurbanne, France

21 ⁸Department of Environmental Science, Policy and Management, University of California,
22 Berkeley, California, USA.

23
24 (*Corresponding authors: trnorthen@lbl.gov, elbrodie@lbl.gov)

25 **Abstract**

26 Like all higher organisms, plants have evolved in the context of a microbial world, shaping
27 both their evolution and their contemporary ecology. Interactions between plant roots and soil
28 microorganisms are critical for plant fitness in natural environments. Given this co-evolution
29 and the pivotal importance of plant-microbial interactions, it has been hypothesized, and a
30 growing body of literature suggests, that plants may regulate the composition of their
31 rhizosphere to promote the growth of microorganisms that improve plant fitness in a given
32 ecosystem. Here, using a combination of comparative genomics and exometabolomics, we
33 show that pre-programmed developmental processes in plants (*Avena barbata*) result in
34 consistent patterns in the chemical composition of root exudates. This chemical succession in
35 the rhizosphere interacts with microbial metabolite substrate preferences that are predictable

36 from genome sequences. Specifically, we observed a preference by rhizosphere bacteria for
37 consumption of aromatic organic acids exuded by plants (nicotinic, shikimic, salicylic,
38 cinnamic, and indole-3-acetic). The combination of these plant exudation traits and microbial
39 substrate uptake traits interact to yield the patterns of microbial community assembly
40 observed in the rhizosphere of an annual grass. This discovery provides a mechanistic
41 underpinning for the process of rhizosphere microbial community assembly and provides an
42 attractive direction for the manipulation of the rhizosphere microbiome for beneficial
43 outcomes.

44
45

46 **Introduction**

47 The area surrounding growing plant roots in soil (the rhizosphere) represents a critical hotspot
48 for biogeochemical transformation that underlies the process of soil formation, carbon cycling
49 and the ultimate productivity of Earth's terrestrial ecosystems. Within the rhizosphere,
50 complex and dynamic interactions between plants and networks of organisms, particularly
51 microorganisms have been shaped by over 450 million years of co-evolution. With such
52 evolutionary optimization, it is not surprising that somewhat consistent patterns have
53 emerged. The 'rhizosphere effect'¹ describes the enrichment of microbial cells and activity
54 near growing roots and has been shown to involve the "selection" of phylogenetically related
55 microorganisms, with plants of different species, geographic locations, climates, and land
56 management showing distinct rhizosphere microbiomes²⁻⁹. Across systems, some consistent
57 trends have also been observed, for example an increase in bacteria of the
58 Alphaproteobacteria sub-phylum was reported in rhizosphere soil of a variety of plants^{2,10-12}.
59 Conversely, the abundance of Actinobacteria has been shown to decrease during late
60 developmental stages^{2,3,12}. Several specific traits have been identified and associated with
61 rhizosphere-enrichment, such as the presence of secretory systems, adhesion, phage defense,
62 iron mobilization and sugar transport^{11,13}. Clearly the rhizosphere microbial community
63 structure is the result of a complex series of interactions and feedbacks between plant roots,
64 microorganisms and the soil physical and chemical environment. However, despite the
65 growing number of studies demonstrating that plant development influences the composition
66 of soil microbiome and its functional capacity, relatively few studies¹⁴⁻¹⁶ have sought to
67 understand the molecular and chemical basis of the role of dynamic plant exudation in the
68 establishment of rhizosphere microbiota.

69 Plants exude a variable but substantial amount (11-40%) of photosynthesis-derived
70 carbon (C) creating a diverse chemical milieu^{17,18}. Exuded compounds include sugars, amino
71 acids, organic acids, fatty acids and secondary metabolites¹⁸⁻²⁰. The composition of root
72 exudates is not a uniform nor static property and varies depending on plant species,
73 developmental stage, root traits, environmental conditions, nutrition, soil type, etc.^{18,21-24}
74 Released compounds have been shown to attract beneficial microorganisms and influence the
75 assembly of rhizosphere microbiomes that enhance the capacity of plants to adapt to their
76 environment²⁵.

77 Many studies have demonstrated the impact of small signaling molecules (acyl-
78 homoserine lactones, flavonoids, non-proteinogenic amino acids, etc.)^{8,26-28}, polymers²⁹,
79 antimicrobials^{28,30} or plant hormones, such as salicylic acid¹⁶ on the interactions between
80 plants and microorganisms in the rhizosphere; however these compounds represent only a

81 small fraction of exuded metabolites. It is not clear how the interaction between root exudate
82 chemistry and microbial substrate preferences combine to influence rhizosphere community
83 assembly and succession. Are there relationships between the dynamics of exudate
84 composition and the growth of specific soil microorganisms? If so, can those relationships be
85 predicted and generalized?

86 To address this uncertainty we integrate information from comparative genomics and a
87 recently described exometabolomics approach^{31,32} to explore the metabolic potential of soil
88 bacteria, the composition of root exudates produced by an annual grass through its
89 developmental stages, and the substrate uptake preferences of isolated soil bacteria
90 representing groups that display distinct successional responses to growing plant roots. We
91 hypothesized that bacteria enriched in the rhizosphere have distinct substrate preferences
92 relative to those bacteria that are not enriched or decline in response to growing plant roots.
93 We demonstrate that growth responses of bacteria in the rhizosphere can be explained by their
94 predicted and observed substrate preferences and the chemical composition of root exudates,
95 thus providing evidence of direct manipulation of the soil microbiome through the specific
96 composition of exudates.

97 **Results and discussion**

98 **Succession of bacterial isolates in the rhizosphere during growth of *Avena*.** The
99 “rhizosphere effect” has been observed across countless plant species and soil types^{2,4,10,11,33}.
100 The bulk soil represents a seed bank of potential organisms that may flourish in response to
101 the resources from a growing root. Numerous studies have shown that selection in the
102 rhizosphere is non-random, with some apparent phylogenetic conservation^{2,34}. This suggests
103 that specific inherited traits are being selected by plants, potentially through their chemical
104 modification of the root zone. Here we studied the mechanisms underlying the response of the
105 soil microbiome to *Avena* root growth. Using media with a range of concentrations, nutrients,
106 anti-oxidants, vitamin and co-factor compositions, together with extended incubation times,
107 289 heterotrophic bacteria were isolated and phylogenetically characterized from the
108 Mediterranean grassland soil in which *Avena* dominates (Fig. 1). These isolates represented
109 seven phyla, coming mostly from the Actinobacteria and Alpha-proteobacteria known to be
110 dominant in this soil². Isolate recovery varied based on media composition (Fig. 1), indicating
111 selection for organisms from distinct niches. Of these isolates, 39 were selected for whole
112 genome sequencing based on their relative abundance in soil and their phylogeny
113 (Supplementary Figures 1, 2 and 3, Supplementary Data 1). Non-redundant OTUs matched to
114 these 39 isolates together represent approximately 10-12% of the total bacterial community in
115 this rhizosphere soil and 17 of these isolates had relative abundance more than 1% of the total
116 bacterial community in this environment (Supplementary Fig. 2 and Supplementary Table 1).

117
118 Those bacterial isolates with sequenced genomes were related to 16S rRNA gene
119 OTUs whose successional dynamics had been previously determined in a rhizotron
120 microcosm experiment where rhizosphere soil was sampled at 0, 3, 6, 9, and 12 weeks of
121 *Avena* growth² (Supplementary Fig. 2). Based on this analysis the isolates were classified into
122 response groups (Fig. 2). The first group (n=19) contained isolates that increased in relative
123 abundance in response to the plant growth (termed ‘positive responders’ herein), and the
124 second group (n=8) contained isolates that declined in relative abundance during plant growth

125 (termed ‘negative responders’ herein). It should be noted that although changes in relative
126 abundance can be interpreted as changes in relative fitness, they could be due to decreases or
127 increases in other community members, rather than, or in addition to, changes in absolute
128 abundance of the ‘responding’ organisms. The remainder of the isolates showed no significant
129 change in relative abundance relative to bulk soils of the 12 week period and are termed
130 ‘undefined responders’. The positive response group was comprised of isolates related to the
131 Proteobacteria (*Dongia*, *Rhodospirillales*, *Sphingomonas*, *Mesorhizobium*, *Bradyrhizobium*,
132 *Caulobacter*, *Burkholderia*, *Variovorax*, *Pseudomonas*), a limited number of Actinobacteria
133 (*Mycobacterium* and *Streptomyces*). Isolates from the negative response group were mostly
134 from the Actinobacteria and Firmicutes (*Bacillus*, *Paenibacillus*). In general, the relative
135 abundance of Actinobacteria declined while, Proteobacteria, particularly alpha proteobacteria,
136 increased during plant growth. This observation is consistent with many other successional
137 studies: including *Arabidopsis thaliana*⁴, wheat¹¹, rice¹⁰, switchgrass³³, maize⁷, demonstrating
138 some conservation in the restructuring of the rhizosphere microbiome across plant species and
139 soil types.

140
141 **Functional traits of soil isolates in the context of the life in the rhizosphere.** Based on the
142 relative abundance of 16S rRNA gene sequences, the majority of bacteria classified as
143 positive responders could be related to taxa previously known to be associated with the
144 rhizosphere, and in some cases, promote plant growth³⁵. This consistent observation of these
145 taxa as being plant-associated across numerous studies again suggests an evolutionary legacy
146 driven by inherited traits favorable to life in the rhizosphere³⁶. To identify these traits, we
147 analyzed their genomes for the presence of features that are hypothesized to be important for
148 both rhizosphere growth and soil organic matter transformation (Fig. 3a,b,c,d, Supplementary
149 Fig. 3, Supplementary Data 2). Specifically, we focused on traits associated with the
150 acquisition of carbon substrates, such as macromolecule depolymerization enzymes, monomer
151 transport, in addition to predicted generation times. We analyzed the distribution of these
152 traits across the bacterial rhizosphere response groups.

153 Conventional wisdom might suggest that the growth strategy of bacteria enriched in
154 the rhizosphere might be adapted towards rapid-growth^{17,33}, however contrary to our
155 expectations, a majority of bacteria that showed a positive response to plant growth were
156 predicted to have longer generation times based on codon-usage bias, meaning their genomes
157 bear signatures of slower growth rates (Fig. 3a, Supplementary Fig. 3). This prediction was
158 confirmed through laboratory growth rate experiments for the majority of isolates
159 (Supplementary Fig. 4). As slower growing organisms can have higher substrate utilization
160 efficiency³⁷, perhaps growth efficiency is favored over growth rate in the rhizosphere.

161 Substrate preference may confer a selective advantage in the rhizosphere and in fact
162 positive and negative responders did differ in their predicted metabolic potential to utilize
163 organic acids (Fig. 3d). Genes coding for organic acid transporters were significantly more
164 abundant in positive responders when corrected for genome size (Fig. 3d). Similarly, the
165 number of amino acid transporters was also skewed towards higher abundance in bacteria
166 with positive response to root growth. Conversely, genes coding for glycoside hydrolases
167 (GH) (primarily β -glucosidases (GH 1, 3, 5), β -xylosidases (GH 43), β -glucanases (GH 16),
168 β -galactosidases (GH 2), glucoamylases (GH 15), α -glucosidases (GH 13) and α -N-
169 acetylglactosaminidases (GH 9) were more abundant in the genomes of negative responders

170 (Fig. 3c, Supplementary Data 2) who are presumably better adapted to life outside the living
171 root zone where easily accessible and assimilable substrates (e.g. monomeric molecules) are
172 less available. Together these results suggest that positive and negative responders have
173 features in their genomes that point to differences in the potential of substrate utilization
174 between these two groups and that they occupy distinct niches within the soil. Although
175 genomics can suggest putative metabolic functions, these functions predicted by genome
176 analyses are hypotheses that require experimental confirmation. To determine whether a
177 relationship existed between the genomic potential for uptake of substrate classes found in
178 root exudates and uptake of those substrates from exudate growth medium, we used an
179 exometabolomics approach.

180
181 **Analysis of *Avena barbata* root exudate metabolite profiles during plant development.**
182 Differences in the metabolic potential for the utilization of specific components of exudates
183 suggests that traits related to this may be important to bacterial success in the rhizosphere. The
184 chemical composition of plant exudates therefore may represent a key means of shaping the
185 microbial composition of the rhizosphere. Plants secrete a cocktail of chemicals through their
186 roots that vary in composition over plant developmental stages and nutritional status^{38,39},
187 therefore we tested the composition of *A. barbata* exudates throughout its growth stages. For a
188 number of reasons, we chose to use a hydroponic system to analyze the dynamics of *Avena*
189 exudate chemistry. Using hydroponics is a trade-off³⁸, plants function differently in
190 hydroponics relative to a real soil system, however hydroponics allows precise control over
191 the chemical milieu in which the plant is growing, facilitates collection of sufficient quantities
192 of freshly produced exudates, avoids issues associated with non-uniform mineral sorption of
193 exudate components and can minimize the microbial transformation of exuded metabolites. In
194 this experiment *Avena* plants were grown *in vitro* and exudates were collected during several
195 developmental phases of *Avena* growth, which roughly proceeded along the same time scale
196 as observed in soil. After collection, exudates were analyzed for total organic carbon and a
197 peak in the quantity of organic carbon exuded was observed during the vegetative stage of 9
198 week-old *Avena* plants (Supplementary Fig. 5). We next assessed the composition of the
199 exudates across developmental stages using liquid chromatography-mass spectrometry (LC-
200 MS). This demonstrated that *A. barbata* exudates were comprised of a broad range of
201 metabolites, including sugars, sugar alcohols, nucleotides, nucleosides, amino-, organic-, fatty
202 acids, plant hormones and compatible solutes (Fig. 4, Supplementary Data 3). Metabolite
203 profiles from the early developmental stage (week 3) were distinct from those at week 6 and
204 9, and from the late developmental stage corresponding to senescence (week 12) (Fig. 4a).
205 While the exudate metabolite profiles from 6 and 9 week-old *Avena* were similar.

206 We then determined the metabolites that significantly changed in abundance with
207 *Avena* development (Fig. 4b, Supplementary Fig. 10). At the early developmental stage (week
208 3), sucrose and homoserine were at greater concentrations relative to other developmental
209 stages (Fig. 4b). Sucrose is the main sugar found in the phloem⁴⁰, it can be strongly allocated
210 to the root tip, decreasing with root maturation⁴¹. At early developmental stages of root
211 growth, sucrose has been noted as an important factor for the development of symbiotic plant-
212 microbe interactions and potential plant-defense mechanisms^{42 43}.

213 Weeks 6 and 9 correspond to the vegetative developmental stages of *A. barbata*² and
214 displayed the highest overall release of exudates compared to the other stages (Fig. 4b).

215 Between week 3 and weeks 6 and 9, amino acids and carboxylic acids with aromatic rings
216 showed the greatest increase (Fig. 4b, Supplementary Fig. 10). Plants release a variety of
217 aromatic compounds, as defense mechanisms against pathogens, as signaling molecules and
218 carbon sources for heterotrophs⁴⁴. This increased release of aromatic compounds (vanillic
219 acid, syringic acid, vanillin, ferulic acid) by *Avena fatua* to the rhizosphere soil was reported
220 earlier as a potential mechanism for allelopathy of wild oat roots²³, however it may also be
221 linked to the physiology of rhizosphere microorganisms.

222 During plant senescence (week 12), a significant increase in the abundance of
223 quaternary ammonium salts (glycine betaine, betonicine, stachydrine) and plant hormones
224 (indole-3-acetic acid, IAA; and abscisic acid, ABA) was observed. Stachydrine (L-proline
225 betaine) and IAA had the most striking change (170 and 40 times fold change, respectively)
226 during *Avena* development (Supplementary Fig. 10). Betaines are widespread in plants and
227 are produced in response to various types of environmental stress to protect to membranes,
228 enzyme activity, and regulate detoxification of reactive oxygen species⁴⁵. ABA has been
229 suggested to play a role in plant metabolism as a stress-response metabolite that promotes
230 senescence-related processes⁴⁶. IAA has been reported to both delay and promote plant
231 senescence^{47,48} and an excess of IAA and ABA are known to inhibit root growth⁴⁹. These
232 results indicate that *A. barbata* releases a variety of metabolites that change with root growth
233 and plant developmental stages in a genetically programmed manner and suggest that changes
234 in exudate metabolite composition over time may contribute to the observed successional
235 patterns in the rhizosphere microbiome (Fig. 2).

236 Although in our study we were able to detect and identify a large number of
237 metabolites present in plant exudates it is still unclear what fraction of the total exudate C is
238 reflected by individual compounds (undetected metabolites and larger water-soluble polymers
239 remain unknown). Therefore, while there may be a significant increase in a particular
240 metabolite, it is important to note that it may only represent a small fraction of the total
241 exudate C pool.

242 **Metabolism of root exudate metabolites by rhizosphere bacterial isolates.** To determine
243 whether the substrate preferences of soil bacteria interact with the chemical composition of
244 root exudates to contribute to microbial succession patterns, we selected 16 isolates as
245 representatives of the positive and negative response groups to plant growth (Fig. 2). We
246 determined differences in the substrate preferences of these isolates using an
247 exometabolomics approach³². These isolates were cultured in a medium containing pooled
248 exudates, collected across the various *Avena* developmental stages, with uptake of the specific
249 compounds from the medium measured by LC-MS. Metabolite uptake was represented as a
250 percent of a metabolite depleted from the medium by each isolate compared to the control
251 uninoculated medium (Supplementary Data 4). This exometabolomic approach specifically
252 allows substrate preferences to be evaluated as microorganisms are confronted with a choice
253 of substrates within the exudate mix, in contrast with other approaches where substrate
254 utilization is evaluated individually^{16,50-53}.

255 Root exudate metabolites were categorized into six chemical classes including amino
256 acids (organic acids containing amino group), nucleotides and nucleosides, sugars, organic
257 acids (organic acids that do not contain an amino group), quaternary amines and fatty acids.
258 Isolates favored during root growth showed significantly higher uptake of amino acids,
259 organic acids, sugars and quaternary amines (Fig. 5). Organic acids and amino acids showed

260 the most significant differences in uptake between the positive and negative responders.
261 Conversely, isolates with positive response were skewed towards lower uptake of nucleotides
262 and nucleosides compared to negative responders.

263 These observations of enhanced amino- and organic acid uptake corroborates our
264 finding that rhizosphere enriched bacteria encode a higher number of transporters for organic
265 acids and amino acids in their genomes compared to bacteria that declined in response to root
266 growth (Fig. 3d). This is compelling evidence that selective uptake of organic acids from the
267 mixture of exudates by rhizosphere bacteria interacts with enhanced release of these
268 compounds during vegetative stages of plant growth. This observation of the importance of
269 organic acids for plant-microbial interactions has been noted previously^{16,52-54}. For example,
270 addition of organic acids as single substrates significantly improved tomato root colonization
271 by *Pseudomonas*⁵¹.

272 Further global analysis of root exudate metabolite uptake by isolates (Supplementary
273 Fig. 11) showed that uptake of most root exudate metabolites was similar across isolates with
274 a large percentage of major proteinogenic amino acids, nucleotides, and sugars taken up by all
275 isolates. However, uptake of specific organic acids, fatty acids and quaternary amines was
276 highly variable across isolates (Supplementary Fig. 11). Profiles of metabolites from the same
277 class, particularly amino acids, nucleotides and aromatic organic acids formed clusters
278 showing similarity in uptake patterns of compounds within the same chemical class across
279 isolates.

280 We then tested for significant differences in root exudate metabolite uptake across
281 positive and negative responders to root growth. We found that 32 of the 101 metabolites
282 consumed by isolates showed significant ($P < 0.05$) differences across these isolate groups and
283 13 metabolites had more than 20% difference in metabolite uptake between positive and
284 negative responders (Fig. 6a and Supplementary Fig. 11). The most significant differences
285 (percentage of metabolite depletion from the medium) in substrate preferences among isolates
286 were defined by the cluster of aromatic organic acids (nicotinic, shikimic, salicylic, cinnamic,
287 and indole-3-acetic) (Fig. 6b and Supplementary Fig. 11). Isolates responding positively to the
288 root have up to 48% higher percentage of metabolite depletion from the medium for the
289 organic acids with aromatic rings compared to those isolates that responded negatively to
290 growing roots.

291 These root exudate components have been shown to influence the composition of
292 rhizosphere microbiomes. Salicylic acid for example, a key regulator of plant metabolism,
293 induces systemic resistance in plants to suppress growth of pathogenic microorganisms³⁴.
294 Although some plant pathogens have been reported to degrade it⁵⁵, salicylic acid has been
295 shown to be necessary for the assembly of a 'normal' root microbiome of *Arabidopsis*
296 *thaliana*¹⁶, and together with gamma-aminobutyric acid, salicylic acid concentration has been
297 shown to correlate with specific taxa frequently enriched in the rhizosphere¹⁴. Taken together
298 with our observations, it appears that the ability to preferentially consume salicylic acid may
299 be a distinguishing feature of rhizosphere bacteria. We also observed that several pentoses
300 showed a higher percentage of uptake by positive responders than by negative responders but
301 conversely we found that nucleosides (cytidine, guanosine, thymidine) were more
302 preferentially consumed by the negative responders (Fig. 6a). Together these data suggest that
303 root exudate chemical composition selectively enriches rhizosphere responders based on their
304 substrate utilization resulting in niche partitioning among soil bacteria in rhizosphere.

305
306 **Predicting microbial response to root growth based on exudate composition and isolate**
307 **substrate preferences.** We used data on isolate relative abundances in the rhizosphere and
308 their exudate metabolite uptake preferences to build a principal component regression model
309 in order to predict microbial response to plant development (Supplementary Fig. 12). The
310 model identified a strong relationship between exogenous metabolite resources and microbial
311 growth in response to roots. We further extracted the cumulative loading scores from the
312 optimal 11 components, and organic acids (glutaric, nicotinic, indole-3-acetic and threonic
313 acids) were found among the compounds with the highest values (Supplementary Figures 13b
314 and 14a). Despite the limited number of observations used for principal component
315 regression, the predicted top metabolites that drive separation of positive and negative
316 responders using their substrate preferences were in agreement with metabolites identified by
317 other methods in our study (Fig. 6).

318 This principal component regression model was then used to predict the response of
319 four bacterial isolates to the plant growth based solely on their measured metabolite uptake
320 preferences (Supplementary Fig. 14b). The 16S rRNA gene relative abundances of these four
321 rhizosphere isolates changed in response to plant growth, however these changes were not
322 statistically significant, these isolates were classified as “undefined” (Fig. 2). We used
323 substrate uptake preferences of these isolates as predictors to identify their behavior in
324 rhizosphere. According to the predictive model, *Nocardioides* HA20 revealed a putative
325 negative response to root growth and *Microbacterium* HA36, *Flavobacterium* HB58 and
326 *Cellulomonas* HD24 were identified as positive responders (Supplementary Fig. 14b). Three
327 out of four predicted responses corroborate the putative responses to plant development
328 suggested by their 16S rRNA gene abundance patterns. This suggests that metabolite uptake
329 traits may be particularly valuable predictors of rhizosphere colonization.

330

331 **Concluding remarks**

332 Interactions between roots, microbes and the soil matrix represents a suite of
333 extremely complex processes. Acknowledging this complexity, the work presented here
334 represents a defined suite of experiments designed to evaluate the potential for metabolic
335 plant-microbial linkages in the rhizosphere of an annual grass in the absence of soil matrix
336 effects. We show that programmed developmental processes in plants result in dynamic
337 patterns of the chemical composition of root exudates. This chemical succession in the
338 rhizosphere interacts with microbial metabolite substrate preferences that can be predicted
339 from genome sequences³². We propose that the combination of these plant exudation traits
340 and microbial substrate uptake traits contributes to a metabolic synchronization that underlies
341 microbial community assembly patterns observed in the rhizosphere².

342

343 **Methods**

344 **Bacterial isolations and genome sequencing.** This study focused on bacterial communities
345 from an annual grassland soil sampled from the University of California Hopland Research
346 and Extension Center (Hopland, CA, USA; 38° 59' 34.5768" N, 123° 4' 3.7704" W). The soil
347 is classified as a coarse-loamy, mesic Ultic Haploxeroll (USDA-NRCS web soil survey;
348 <http://websoilsurvey.nrcs.usda.gov>) and experiences a Mediterranean-type climate. *Avena*
349 *barbata* is generally the dominant annual grass present at this field site and is known to have

350 been a dominant grass in this area for the past century. *Avena fatua* is another wild oat species
351 that widely populates Mediterranean grasslands. Our previous work showed that the
352 rhizosphere microbial communities of these two closely related plants at the same
353 developmental stage are statistically indistinguishable when these species are grown in the
354 same soil⁵⁶. We selected the dominant species, *Avena barbata*, to isolate associated soil
355 bacteria and to measure plant exudation. In order to classify the dynamics of isolated bacteria
356 we used data on previously identified bacterial dynamics in response to *Avena fatua* growing
357 in the same soil. Insignificant differences between the microbiomes of both *Avena* species
358 enabled us to map the dynamics of bacterial isolates over the course of *Avena* rhizosphere
359 development. In this manuscript both of these species will be referred to as *Avena*. Other soil
360 properties are described in Shi et al. (2015)². Growth media for isolation of heterotrophic
361 bacteria were formulated with different concentrations of nutrients, different solidifying
362 agents and anti-oxidant enzymes (R2A 1/10, R2G 1/20, 1/100; OLI; VXylG) according to da
363 Rocha et al., (2015)⁵⁷ (Supplementary information). From 289 diverse isolates (Fig. 1), 39
364 were selected based on phylogeny and relative abundance within the soil to be genome
365 sequenced (Supplementary Figures 1, 2 and 3) using the Illumina HiSeq 2500 platform in
366 accordance with the standard protocols of the DOE Joint Genome Institute (Walnut Creek,
367 CA, USA). Sequences of genomes were deposited, assembled and annotated at the JGI IMG
368 portal (<https://img.jgi.doe.gov>; Supplementary Data 1, Supplementary Data 2).

369
370 **16S rRNA phylogeny and successional patterns of isolated bacteria.** The successional
371 trajectories of rhizosphere bacterial communities in this soil in response to the growth of
372 *Avena fatua* were previously reported by Shi et al (2015)². Briefly, samples were collected
373 across 12 biological replicates from the rhizosphere of *Avena* and from bulk soil over the
374 course of 0, 3, 6, 9 and 12 weeks of *Avena* growth. These samples were subsequently
375 analyzed by high-throughput sequencing of 16S rRNA genes². To relate the bacterial isolates
376 to the reported bacterial community trajectories, we mapped 16S rRNA sequences of isolates
377 to sequences obtained from bulk and rhizosphere soil during *Avena* growth² by comparing the
378 corresponding V4 regions of the isolate 16S rRNA genes (extracted using BLASTN⁵⁸ and
379 MUSCLE sequence alignment⁵⁹) with overlapping OTUs defined as having E-values <1e-10
380 and >= 97% of gene sequence homology. Isolates corresponding to OTUs detected in the soil
381 were assigned the response pattern to plant growth of those OTUs as determined by Shi et al
382 (2015)².

383
384 **Analysis of genomic features of bacterial isolates.** We analyzed genome sequences for
385 specific traits related to fitness in the rhizosphere. Traits included those related to growth
386 strategies, substrate uptake and extracellular enzyme production (Supplementary Data 2,
387 Supplementary Figures 3 and 4, Supplementary Table 2). As a proxy for growth strategies,
388 minimum generation times were predicted based on codon usage bias between all genes and a
389 set of highly expressed (ribosomal protein) genes following the linear regression model from
390 Vieira-Silva and Rocha (2010) (Equation 3)⁶⁰.

391
392
$$(\Delta ENC' = \frac{ENC'_{all} - ENC'_{ribosomal\ protein\ genes}}{ENC'_{all}})$$

393 Where ENC' is the effective number of codons given G+C composition⁶¹.

394

395 For extracellular enzyme traits we calculated gene copy numbers of glycoside hydrolases and
396 auxiliary activity enzymes using a hidden Markov model (HMM) search of protein sequences
397 against the CAZy database^{62,63}. For substrate uptake, transporters in the genomes were
398 predicted using an HMM search against TransportDB⁶⁴.

399 The relationships between isolate phylogeny and genome features were determined through
400 analysis of full length 16S rRNA following alignment using MUSCLE 3.8.31⁵⁹ and
401 construction of a maximum likelihood tree using FastTree⁶⁵ (Supplementary Fig. 3).

402 ***Avena* root exudate collection.** *Avena barbata* (*A. barbata*) wild type seeds were germinated
403 using Milli-Q water and glass wool in the dark at room temperature. Three-day-old seedlings
404 were transferred to six liter hydroponic tubs with half-strength Murashige and Skoog⁶⁶ basal
405 salt mixture M524 (Phyto Technology Laboratories, Overland Park, KS). Hydroponic tubs
406 were incubated at 24 °C on 16/8 h day/night cycle, humidity was maintained at 72% and
407 irradiance at 180 $\mu\text{E m}^{-2} \text{s}^{-1}$ in growth chambers at the Joint BioEnergy Institute, Emeryville,
408 CA, USA. We refreshed growth medium on a regular basis (every 3 days) to minimize
409 potential microbial growth. Root exudates were collected from 3, 6, 9 and 12 week-old plants
410 corresponding to different developmental stage of the plant: seedling (3 weeks), vegetative (6
411 and 9 weeks), and senescence (12 weeks) phases respectively. Four biological replicates were
412 collected at each growth stage and exudates were collected from 16 *Avena* plants. To collect
413 root exudates, roots of growing plants were washed in Milli-Q water to remove excess salts
414 from growth media and then transferred to glass cylinders containing 200 ml of sterile Milli-Q
415 water for one hour in a growth chamber⁶⁷. Milli-Q water containing exudates was
416 immediately filter sterilized using 0.22 μm filters (Corning Ink., Corning, NY, USA) and
417 frozen. This exudate collection approach has been used previously for incubation times of up
418 to 2.5 hours without any significant effect of microbial transformation of exudates³⁸. The total
419 organic carbon (TOC) concentration in samples was quantified using a Shimadzu TOC-L
420 Analyzer (Shimadzu, Japan) (Supplementary Fig. 5). Exudate samples were lyophilized using
421 a Labconoco FreeZone 2.5 lyophilizer and stored in -80 °C.

422 **Design of bacterial exudate growth medium and cultivation of bacterial isolates for**
423 **exometabolomics analysis.** Sixteen bacterial isolates were selected for metabolite profiling
424 based on their phylogeny, distributions of genomic traits related to rhizosphere carbon
425 utilization and response to plant root growth. The exudate medium was prepared from a base
426 medium (dipotassium phosphate 0.15 g/L, magnesium sulfate 0.012 g/L) to which plant
427 exudates were added. The concentration of exudates added to the growth medium was
428 selected to match the concentration of dissolved organic carbon detected in soil from
429 Hopland, CA (0.025 mg C g⁻¹ dry soil) where these isolates originated (Thea Whitman, pers.
430 comm.). When converted to a relevant concentration that bacteria would experience in soil
431 (assuming 20% v/v soil moisture and a soil bulk density of 1.6 g/ml) this corresponds to
432 approximately 125 mg-C/L. Root exudates were first lyophilized, redissolved in water, and
433 then added to growth media to achieve this final C concentration, as determined using a
434 Shimadzu TOC-L Analyzer (Shimadzu, Japan). The resulting medium was subsequently filter
435 sterilized using 0.22 μm filters and inoculated with one of each of the 16 bacterial isolates or
436 incubated as controls. The cultures inoculated at an initial OD₅₉₀=0.04 and then incubated at
437 28 °C with shaking at 200 rpm for up to 96 hours. Samples were collected at the early

438 stationary phase of each isolate, centrifuged, and spent supernatant was frozen at -80 °C. Each
439 isolate culture and uninoculated control samples had four biological replicates (n=68). The
440 concentration of TOC in the uninoculated exudate medium (control samples) and TOC in
441 spent exudate medium from isolate inoculated treatments was quantified (Supplementary Fig.
442 6). Changes in the TOC concentrations in the spent media from inoculated treatments were
443 compared to that in the uninoculated controls to determine the TOC consumed by each isolate,
444 which was used as an indicator of microbial growth. This revealed that six inoculated samples
445 failed to grow, these samples were excluded from further analysis (Supplementary Fig. 6).

446
447 **Extraction of metabolites from lyophilized exudates.** The TOC of exudates collected at
448 different time points (Supplementary Fig. 5) was measured prior to the sample lyophilization.
449 This information was used to adjust the dilution volumes for the final extracts such that all
450 samples had organic carbon concentrations of approximately 470 mg/L. Here, the necessary
451 volume of cold (-20 °C) methanol containing internal standards 1µg/mL
452 2-Amino-3-bromo-5-methylbenzoic acid (ABMBA), 5 µg/mL 13C-15N-L-phenylalanine, and
453 2 µg/mL 9-anthracene carboxylic acid (ACA) was added to the dried exudates and these were
454 sonicated for 30 min using an ultrasonic bath (VWR, Radnor PA). The resulting extracts were
455 filtered using 0.22 µm microcentrifuge PVDF filters (Merck Millipore) and aliquots of 150 µl
456 of methanol extracts were transferred to LC-MS vials for the analysis.

457
458 **Extraction of metabolites from spent bacterial growth medium.** The same starting TOC
459 concentration of growth medium (described above) was used for all treatments
460 (Supplementary Fig. 6). Spent media (1 ml) was collected and then lyophilized and extracted
461 with 150 µl of methanol containing internal standards (as described above). These were then
462 centrifuged at 6000g for one minute and filtered using 0.22 µm microcentrifuge PVDF filters
463 to remove any particles and then analyzed by LC-MS/MS.

464
465 **Mass spectrometry analysis of exudates and spent bacterial growth media.** UHPLC
466 normal phase chromatography was performed using an Agilent 1290 LC stack, with MS and
467 MS/MS data collected using a Q Exactive Orbitrap MS (Thermo Scientific, San Jose, CA).
468 Full MS spectra were collected from m/z 70-1050 at 70,000 FWHM resolution, with MS/MS
469 fragmentation data acquired using 10, 20 and 30eV collision energies at 17,500 FWHM
470 resolution. MS instrument parameters included sheath gas flow rate of 50 (arbitrary units, au),
471 auxiliary gas flow rate of 20 (au), sweep gas flow rate of 2 (au), 3 kV spray voltage and 400
472 °C capillary temperature. Normal phase chromatography was performed using a zic-pHILIC
473 column (Millipore SeQuant ZIC-pHILIC, 150 x 2.1 mm, 5µm, polymeric) at 40 °C at a flow
474 rate of 0.25 mL/min with a 2 µL injection volume. The HILIC column was equilibrated with
475 100% buffer B (90:10 ACN:H₂O w/ 5mM ammonium acetate) for 1.5 minutes, diluting buffer
476 B down to 50% with buffer A (H₂O w/ 5mM ammonium acetate) 23.5 minutes, down to 40%
477 B over 3.2 minutes, to 0% B over 6.8 minutes, and followed by isocratic elution in 100%
478 buffer A for 3 minutes. Exact mass and retention time coupled with MS/MS fragmentation
479 spectra were used to identify compounds as described below.

480
481 **Analysis of mass spectrometry data.** We first evaluated overall the quality of the dataset by
482 aligning total ion chromatograms from all LC-MS runs using MZmine version 2.26⁶⁸ they

483 demonstrated low variability across biological replicates (Supplementary Figures 7 and 8).
484 Additionally, intensities of the internal standards were assessed from each sample to ensure
485 consistency of signal intensity and retention times from sample-to-sample. Based on the
486 quality control (QC) assessment (mass accuracy within 5 ppm, retention time and peak
487 intensity of the internal standard) three samples that did not pass QC were excluded from
488 further analysis (Supplementary Figure 9). Samples that passed the QC steps were then
489 analyzed using the Metabolite Atlas (<https://github.com/biorack/metatlas>) for the metabolite
490 feature extraction and annotation⁶⁹. Briefly, metabolites were identified using Metabolite
491 Atlas by matching experimental spectra to our in-house library of authentic standards
492 (accurate mass less than 5 ppm, retention time within 0.5 min and/or match of major MS/MS
493 fragments). In order to maximize the number of compounds identified in our study, in
494 addition to our in-house library of standards we compared the MS/MS data against Metlin⁷⁰
495 and MassBank⁷¹ spectral libraries. Next, MZmine version 2.26⁶⁸ was used for manual
496 validation of identified metabolites (accurate mass less than 5 ppm, retention time within 0.5
497 min and/or match of major MS/MS fragments) to eliminate false identifications. For the
498 metabolites identified in our study (Supplementary Data 3 and 4) we provide a classification
499 of metabolite identification confidence levels recommended by the Metabolomics Standards
500 Initiative Chemical Analysis Working Group of the Metabolomics Society⁷². Briefly, a Level
501 1 identification, an ‘identified’ metabolite, requires two independent and orthogonal measures
502 relative to an authentic standard analyzed under the same experimental conditions (for
503 example, m/z and retention time; m/z and MS/MS; retention time and MS/MS). ‘Putatively’
504 annotated compounds (Level 2) and ‘Putatively characterized compound classes’ (Level 3) do
505 not have chemical reference standards and annotations are based on spectral similarity to
506 known compounds of a chemical class⁷². Although 87 of the 101 metabolites described in our
507 study were assigned to the Level 1 identifications we also include 12 level three identification
508 (Supplementary Data 3 and 4), which may be of interest. There is ambiguity for many
509 carbohydrates so in these cases we use the following notation: carbohydrate class (standard
510 matched). These data files (Supplementary Data 3 and 4) also provide metabolites peak areas
511 for both positive and negative ionization modes which were used for the statistical analysis of
512 the dynamics of the relative abundances of metabolites across treatments.

513
514 In this study we used relative quantification to compare the change in relative abundance of a
515 given metabolite across all samples. A limitation of this commonly used approach is that it
516 does not provide absolute abundances for the various metabolites. Relative comparisons of
517 metabolites were performed by comparison of integrated peak areas in uninoculated control
518 and spent medium from isolate cultivation.

519
520 **Statistical analyses.** To classify the patterns of isolate response to *Avena* growth, we first
521 compared changes in the relative abundance of isolated taxa from 3, 6, 9 and 12 week-old
522 *Avena* plants and bulk soil by using Permutational Analysis of Variance and post-hoc
523 Duncan's multiple range test. The magnitude of change (Δ of isolate abundance) for all
524 isolates was calculated by considering the maximum change in relative abundance from week
525 0 and any subsequent time point. Responses were classified as Positive, Negative and
526 Undefined according to the sign of the Δ of isolate relative abundance (positive Δ
527 demonstrating an increase in relative abundance over time, negative Δ demonstrating a

528 decrease in relative abundance over time), undefined indicating no significant change in
529 relative abundance over time (Fig. 2, Supplementary Data 1).

530 Duncan's multiple range test was also used to test for significant differences in root
531 exudate metabolite composition across the different growth stages of *Avena* (3, 6, 9, 12 week-
532 old plants) (Supplementary Fig. 10). Principal component analysis (PCA) was used to
533 evaluate and visualize relationships between root exudate metabolite profiles at different
534 growth stages of *Avena*, and also to compare the exometabolomes (substrate uptake/release)
535 of bacterial isolates. The Kolmogorov-Smirnov test (KS test) was used to test for statistical
536 differences in the distributions of uptake of specific chemical classes by the classified groups
537 of bacterial isolates (i.e. positive, negative, and undefined responders). Kruskal–Wallis one-
538 way analysis of variance was used to test for statistical differences in the substrate uptake
539 preferences and genomic traits of isolates (Supplementary Fig. 11). All statistical analyses
540 were performed within the R software environment⁷³ using the “vegan” and “agricolae”
541 packages^{74,75}.

542
543 **Principal component regression and prediction of metabolites discriminating bacterial**
544 **response to root growth.** Using exometabolomic data of substrate preferences of bacterial
545 isolates and their calculated abundance dynamics in response to root growth, we tested for
546 discriminatory root exudate metabolites that best predicted the response of these bacterial
547 isolates to growing roots. The overall analysis approach is summarized in Supplementary Fig.
548 12. Isolates were first classified as positive and negative bacterial rhizosphere responders and
549 enumerated as 1 and 0 respectively; organisms with undefined responses were not included
550 (Supplementary Data 5). Then metabolite uptake data on the biological replicates of all of the
551 isolates were split into training (45 observations) and test (12 observations) data sets. Then
552 PCA was performed and all principal components (PCs) were used to build a principal
553 component regression (PCR) model based on training data in order to predict test set isolate
554 response to root growth based solely on metabolite uptake preferences (Supplementary Data
555 5). An optimal number of components (n=11), for the PCR model was selected by iterating
556 through extracted PCs to obtain the lowest cross-validation error from 10-fold cross-validation
557 (MSEP < 0.05) (Supplementary Figures 13a and 14a)⁷⁶. Cumulative loading scores across the
558 11 components were used to identify key discriminatory metabolites whose uptake was
559 predictive of bacterial response to root growth. Using this model we predicted the response of
560 4 bacterial isolates that experimentally had shown ambiguous (termed ‘undefined’) responses
561 to root growth. R^2 values of model fit represented the accuracy of model prediction of these
562 isolates’ response to root growth. These analyses were performed using packages in the R
563 software environment, including “pls” for principal component regression⁷⁶.

564
565 **Data availability.** The genomes of Hopland isolates are publicly available in the Integrated
566 Microbial Genomes (IMG, <https://img.jgi.doe.gov>) database under IMG study name
567 Mediterranean Grassland Soil Metagenome (MGSM): Enabling a systems view of soil carbon
568 and nitrogen biogeochemistry under a changing climate. All data, including samples that we
569 excluded from the analysis, were deposited to the Global Natural Products Social Molecular
570 Networking (GNPS, <https://gnps.ucsd.edu>) data repository (*Avena* exudates [MSV000081804](https://gnps.ucsd.edu/data/nps/network/MSV000081804),
571 Bacterial isolate uptake of exudates [MSV000081808](https://gnps.ucsd.edu/data/nps/network/MSV000081808), Root exudates components library
572 [MSV000081810](https://gnps.ucsd.edu/data/nps/network/MSV000081810)).

573

574 **Acknowledgements**

575 This study was supported by the US Department of Energy, Office of Science, Office of
576 Biological Environmental Research including a Genomic Sciences program award no. DE-
577 SC0010570, DE-SC0016247 and DE-SC0014079 to MKF. Work performed at Lawrence
578 Berkeley National Laboratory including DOE Early Career Awards to DL and TRN, and work
579 performed at the DOE Joint Genome Institute (<http://www.jgi.gov>) and at the DOE Joint
580 BioEnergy Institute (<http://www.jbei.org>) is supported by the U. S. Department of Energy,
581 Office of Science, Office of Biological and Environmental Research through Contract No.
582 DE-AC02-05CH11231. DL was also supported in part by the European Union's Horizon 2020
583 research and innovation program under the Marie Skłodowska-Curie grant agreement number
584 659910. Isolate genome sequencing was conducted by the U.S. Department of Energy Joint
585 Genome Institute, a DOE Office of Science User Facility, under a Community Science
586 Program award to ELB, supported by the Office of Science of the U.S. Department of Energy
587 under Contract No. DE-AC02-05CH11231. We thank C. Castanha for background
588 information on soil temperature and are very grateful to our talented former undergraduate
589 students Badamtseteg Jargalsaikhan, Rozina Hossainkhil, Dinh Ly, Severin Ouedraogo and
590 Yen Nguyen for assistance with maintenance of the bacterial isolate collection.

591

592 **Author contributions**

593 Kateryna Zhalnina^{1,2}, Katherine B. Louie¹, Zhao Hao², Nasim Mansoori^{1,3}, Ulisses Nunes da
594 Rocha^{2,4}, Shengjing Shi⁵, Heejung Cho^{2,6}, Ulas Karaoz², Dominique Loqué^{1,3,6,7}, Benjamin P.
595 Bowen¹, Mary K. Firestone^{2,8}, Trent R. Northen¹, and Eoin L. Brodie^{2,8}
596 KZ, TN, MKF and ELB developed hypotheses. KZ, KL, NM, UNR, SS and DL performed
597 experimental analyses. KZ, HC, UK, ZH, UNR, BB analyzed data. KZ, TN, MKF and ELB
598 wrote the paper. All authors provided comments and edits on the manuscript.

599

600 **Additional information**

601 Supplementary information is available for this paper. Reprints and permissions information
602 is available at www.nature.com/reprints. Correspondence and requests for materials should be
603 addressed to TN and ELB.

604

605 **Competing interests**

606 The authors declare no competing financial interests.

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805 Figures and legends

806

807

808 **Figure 1 | Cladogram showing phylogenetic relationships between 289 soil heterotrophic**
809 **bacterial isolates, and their origin (media type).** Leaf labels indicate representative
810 sequence IDs. Rings, from the inner to the outside circles, represent: (1) genome-sequenced
811 isolates (black blocks); (2) class level taxonomy of isolate; (3) medium on which isolate was
812 originally obtained.

813

814 **Figure 2 | Growth response in soil of bacterial isolates to *Avena* growth based on changes**
815 **in 16S rRNA gene abundance.** Each bar represents the change (Δ) of isolate abundance
816 between bulk soil at week 0 and the point of maximum change over the developmental stages
817 of *Avena*. Δ of isolate abundance was normalized by the total number of 16S rRNA reads
818 identified for each isolate over all developmental stages of *Avena*. Positive responders (n=19),
819 negative responders (n=8), and * indicates undefined responders (n=11) with a non-significant
820 response relative to week 0 bulk soil relative abundance (* $P > 0.05$, Permutational Analysis of
821 Variance and post-hoc Duncan's multiple range test). Bullet points indicate isolates selected
822 for exometabolite profiling.

823

824 **Figure 3 | Distributions of select traits in the genomes of soil bacterial isolates classified**
825 **into two groups based on the response to plant root growth.** Positive responders (n=19),
826 negative responders (n=8). **a**, Minimum generation times predicted from genome sequences.
827 **b**, Genome size of isolates. **c**, Extracellular enzymes for plant polymer degradation. **d**,
828 Monomer transporters. All gene frequencies were adjusted for differences in genome size.
829 Total number of transporters shown as percent of transporters per genome. In each boxplot, a
830 point denotes a single metabolic trait or a single gene. The top and bottom of each box
831 represent the 25th and 75th percentiles, the horizontal line inside each box represents the 50th
832 percentile/median and the whiskers represent the range of points excluding outliers. Outliers
833 denoted as large points outside whiskers. Differences in the distributions of traits between the
834 two groups of isolates were evaluated using the Kruskal–Wallis one-way analysis of variance
835 and traits with significant differences ($P < 0.05$) are denoted by *.

836

837 **Figure 4 | Changes in *A. barbata* exudation through plant development (weeks 3, 6, 9,**
838 **12).** **a**, Principal component analysis of the exudate profiles of *A. barbata* at each time point
839 (n=16 exudate profiles). **b**, Changes in abundance of each exudate compound across plant
840 developmental stages (n=16 exudate profiles). All abundances were normalized to a range
841 between 0 and 1, with 1 representing sum of each metabolite released. Metabolites with the
842 highest abundance at each time point are highlighted with the same color. Duncan's multiple
843 range test was used to determine compounds with significant differences across different
844 developmental stages ($P < 0.05$).

845

846 **Figure 5 | Distributions of root exudate metabolite uptake by isolates presented as**
847 **percent of uptake from the exudate medium.** Bar colors represent positive (green) and
848 negative (purple) responders to root growth (n=12 isolates). In each boxplot, a point denotes a
849 single metabolite and its percent uptake in a single incubation. Diamond symbols in each

850 boxplot represent the mean. The box boundaries represent the first and third quartiles of the
851 distribution and the median is represented as the horizontal line inside each box. Box plot
852 whiskers span 1.5 times the interquartile range of the distribution. A significant difference
853 between distributions were determined using the Kolmogorov-Smirnov test and are denoted
854 with $** (P < 1e-08)$ and $* (P < 0.01)$.

855
856 **Figure 6 | Substrate preferences of positive and negative responders.** **a**, Metabolites with
857 significant ($P < 0.05$) differences in their uptake from *Avena* exudates by isolates (n=12
858 isolates) (Kruskal-Wallis test). **b**, Uptake of aromatic organic acids from exudate media by
859 isolates with positive (n=8 isolates) and negative (n=4 isolates) responses to plant growth. In
860 each barplot, a point denotes a biological replicate measurement of percent uptake of aromatic
861 acid by an isolate. Metabolite uptake is given as a percentage of metabolite depletion by
862 isolate from the medium, and error bars show standard error of the mean.

Legend:

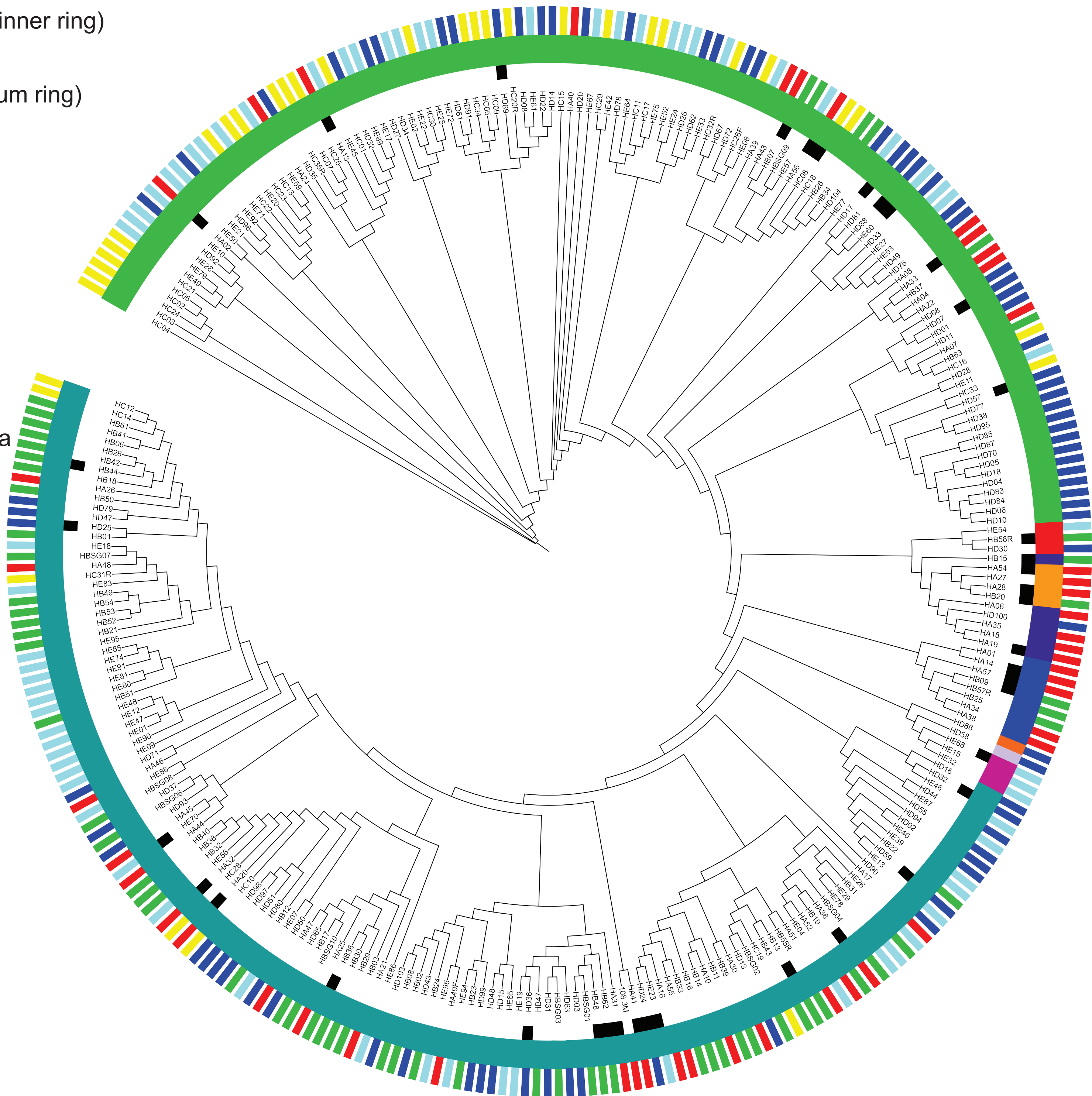
Sequenced isolates (inner ring)

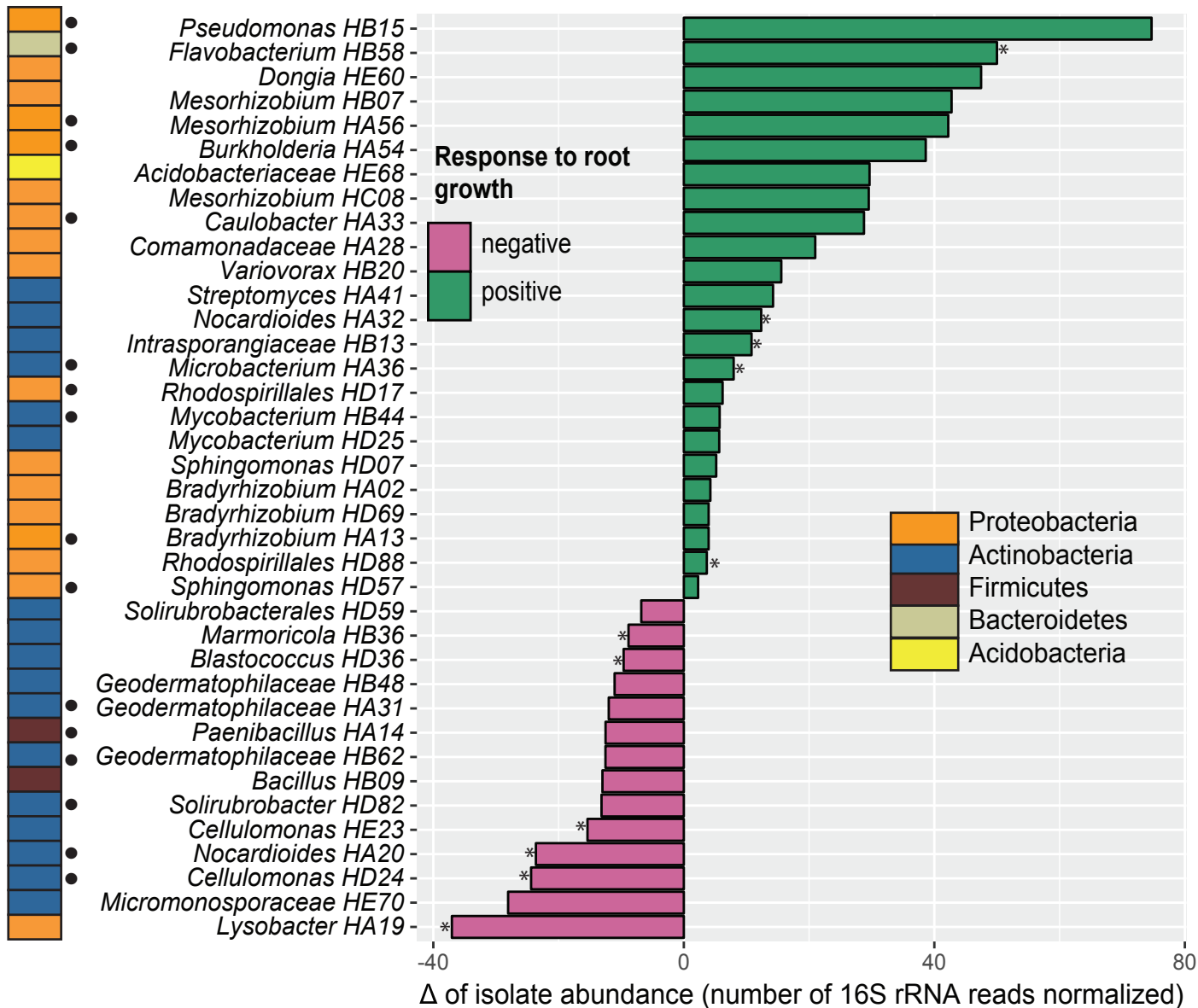
Dataset Phylogeny (medium ring)

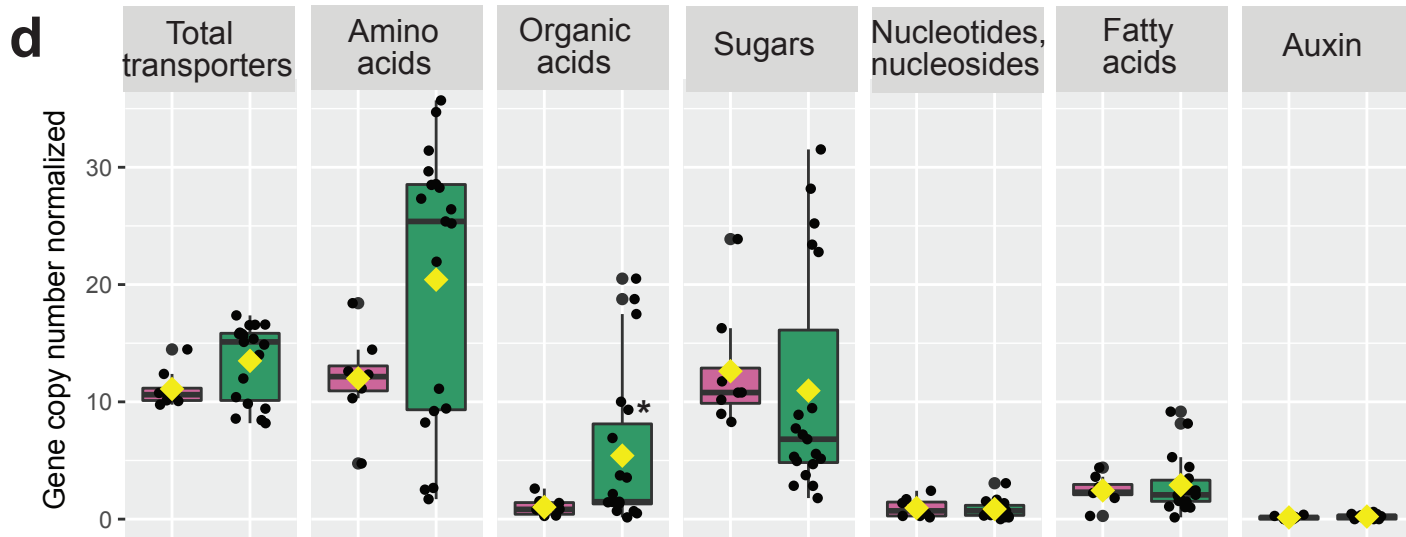
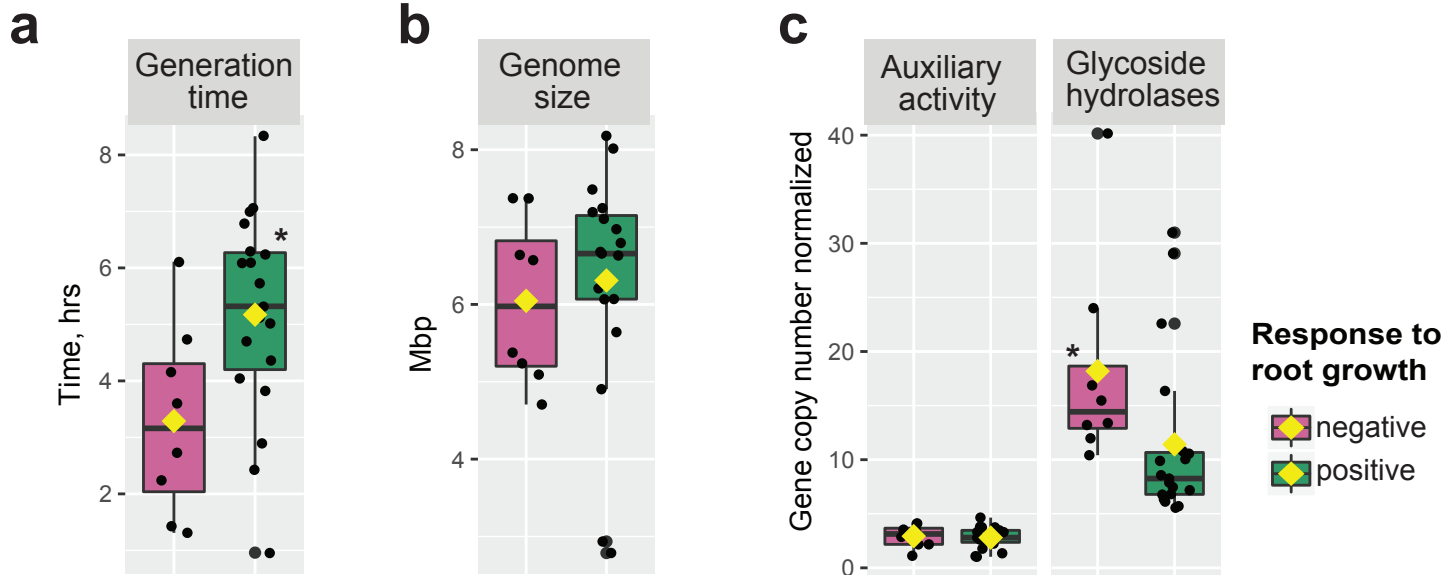
- Acidobacteria
- Actinobacteria
- Alphaproteobacteria
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- Firmicutes
- Gammaproteobacteria
- Gemmatimonadetes
- Verrucomicrobia

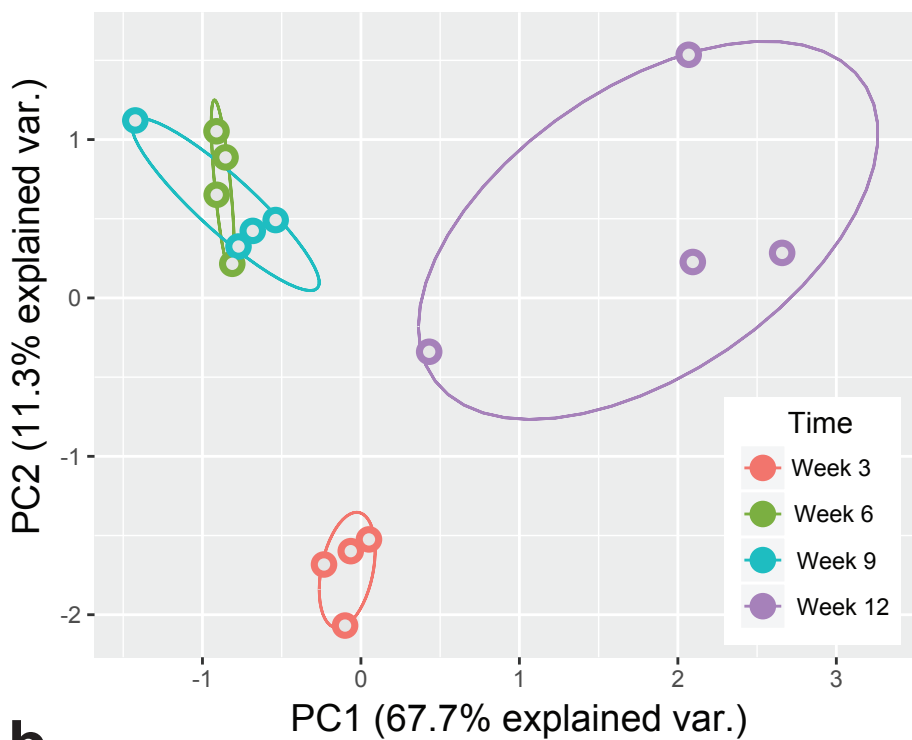
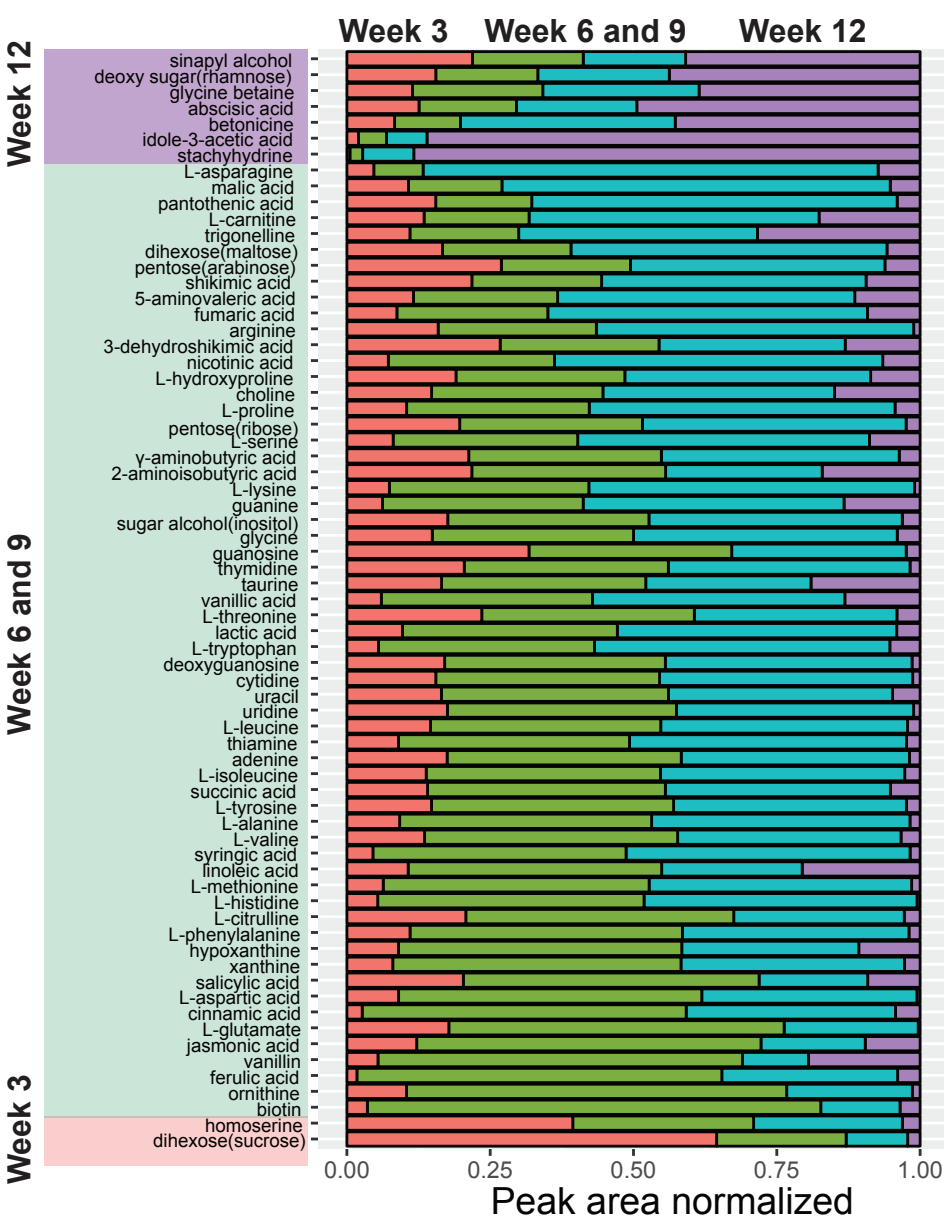
Dataset Media (outer ring)

- R2A 1/10
- R2A 1/20
- R2A 1/100
- OLI
- VXyIG

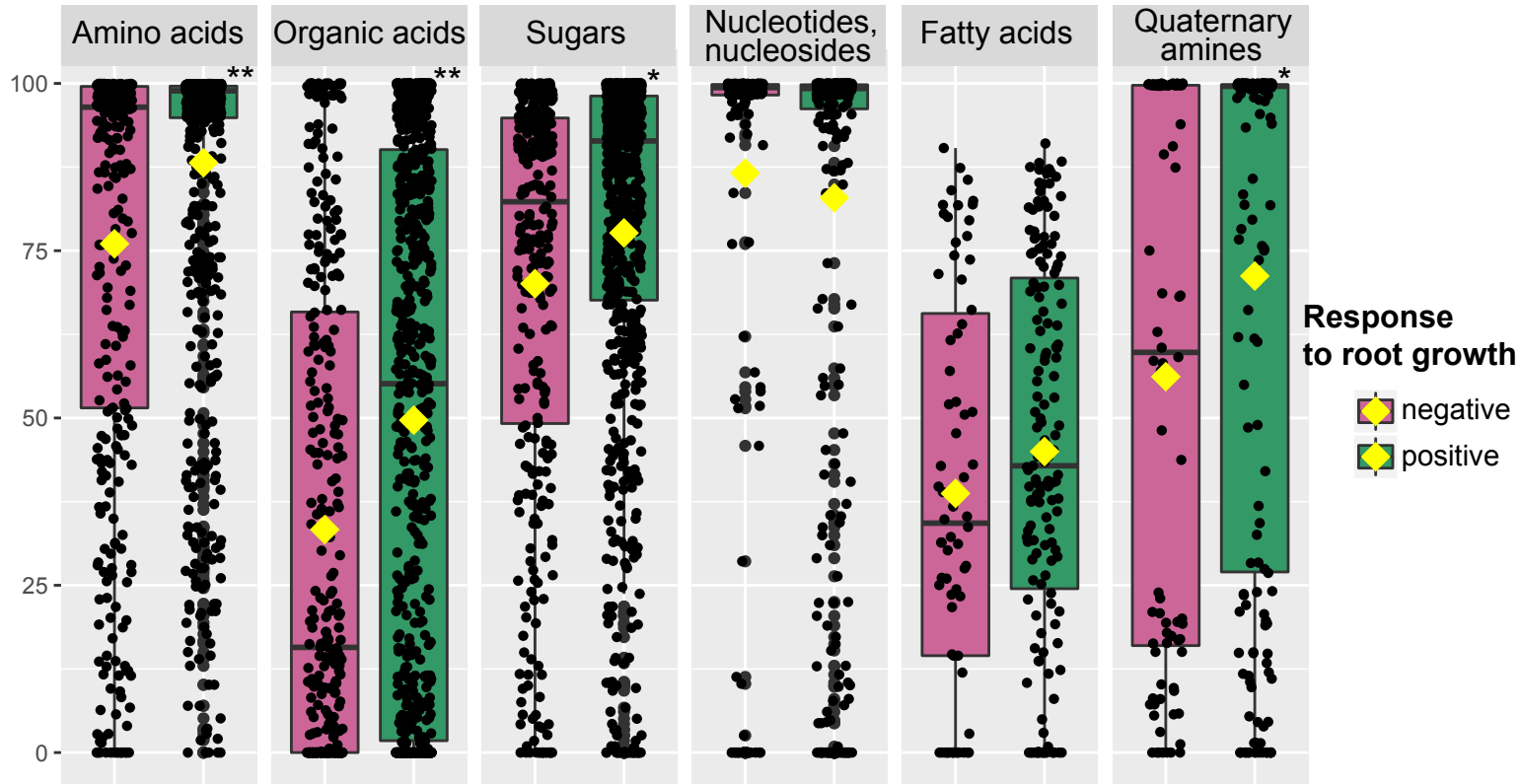


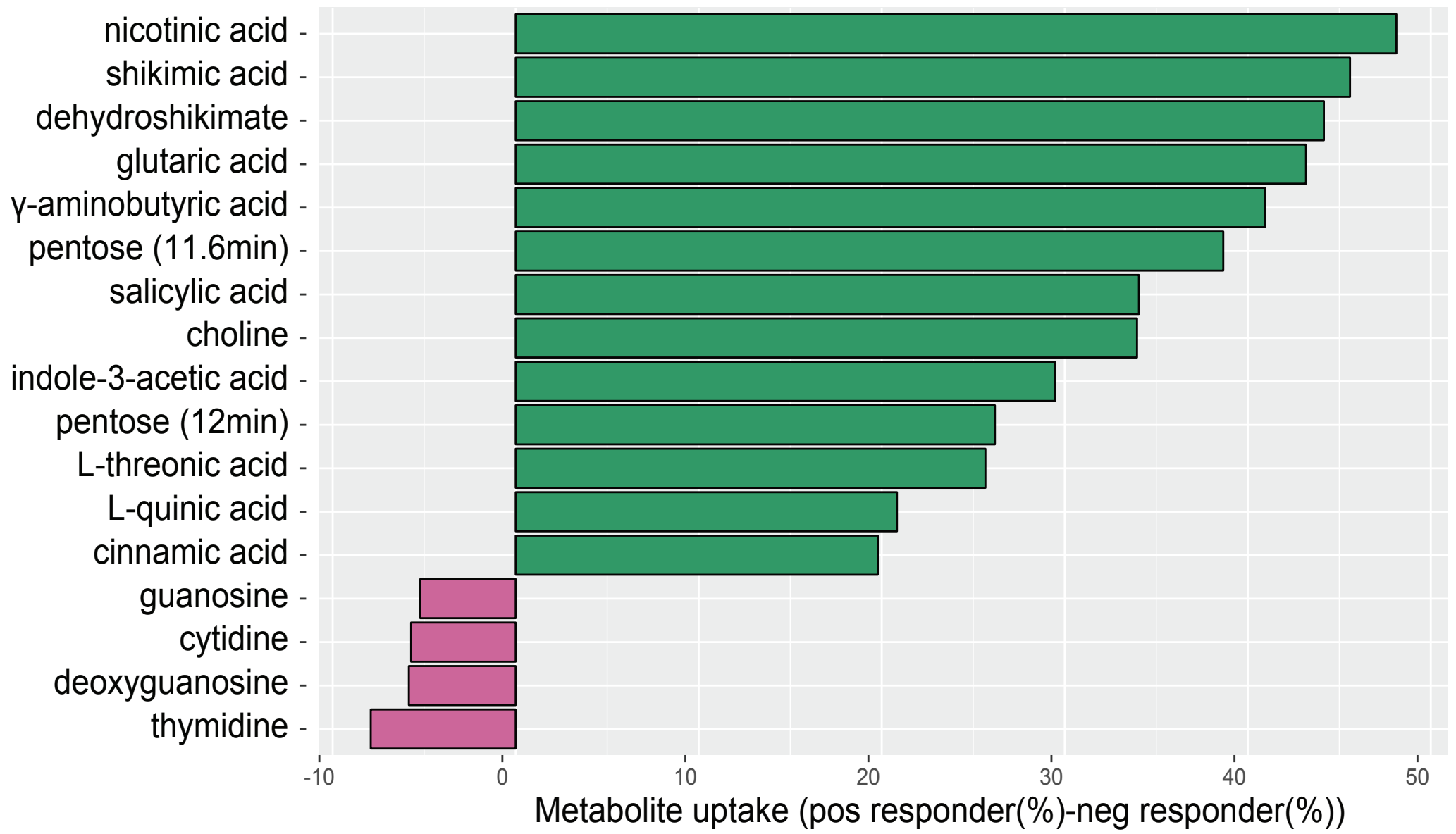




a**b**

Uptake of exudate from the medium, %



a**b**