

The lactonase BxdA mediates metabolic adaptation of maize root bacteria to benzoxazinoids

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Author contributions

L.T., M.E., and K.S. designed research; L.T. performed microbial plating assays, metabolomic assays, *in vitro* growth assays, performed the transcriptome experiment, and selected candidate genes. M.K. performed comparative genomics and analysed transcriptomic data. M.F. expressed the candidate genes in *E. coli* and tested purified proteins. P.M. performed NMR analyses, P.M, T.Z. and E.K. performed metabolomic analyses. L.R. tested Arabidopsis isolates for AMPO-formation, V.G. cultivated plants, and M.N.D. conducted the anaerobic experiments. S.H., C.B., N.S, C.A.M.R., and T.G.K. provided technical infrastructure and R.B. provided new analytic tools. L.T. and M.K analysed the data and L.T., M.E. and K.S. wrote the manuscript. All authors revised the paper.

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1 Abstract

2 Root exudates contain secondary metabolites that affect the plant's root microbiome.
3 How microbes cope with these bioactive compounds, and how this ability shapes root
4 microbiomes remain largely unknown. We investigated how maize root bacteria
5 metabolise benzoxazinoids, the main specialised metabolites of maize. Diverse and
6 abundant bacteria metabolised the major compound (6-methoxy-benzoxazolin-2-one,
7 MBOA) in the maize rhizosphere to 2-amino-7-methoxyphenoxazin-3-one (AMPO). By
8 contrast, bacteria isolated from *Arabidopsis*, which does not produce benzoxazinoids,
9 were unable to metabolise MBOA. Among *Microbacteria* strains, this differential
10 metabolism allowed to identify a conserved gene cluster containing the lactonase
11 *bxdA*. BxdA converts MBOA to AMPO in vitro and we show that this capacity provided
12 bacteria a growth benefit under carbon-limiting conditions. Together these results reveal
13 that maize root bacteria - through BxdA - are metabolically adapted to the benzoxazinoids
14 of their host. We propose that metabolic adaptation to plant-specialised compounds
15 shapes root bacterial communities across the plant kingdom.

16 *150/150 words*

17

18

19 **Keywords:**

20 Benzoxazinoids, maize microbiome, root bacteria, *Microbacteria*, adaptation

21

22 Introduction

23 Plant microbiomes fulfil key functions for plant and ecosystem health. Root-
24 associated microbes promote plant growth, provide nutrients, and protect plants from
25 pathogens^{1,2}. While some root microbes are ubiquitous, many microbes form specific
26 relationships with their host plants, and host plants often exert substantial control over
27 the structure and function of their microbiome. Plants primarily shape their root-
28 associated microbiome through the secretion of root exudates, which can account for up
29 to one-fifth of the plant's assimilated carbon³. Root exudates may attract, nourish, or repel
30 soil microbes and contain primary metabolites including sugars, amino acids, organic
31 acids and fatty acids, as well as secondary metabolites. The latter, also called specialised
32 metabolites, govern the plant's interactions with the environment, and among other
33 functions, they increase biotic and abiotic stress tolerance⁴. A key function of exuded
34 specialised metabolites is to shape the root microbiomes⁵⁻⁷, documented with examples
35 including glucosinolates, camalexins, triterpenes, and coumarins from *Arabidopsis*
36 *thaliana*⁵, the saponin tomatine from tomato⁸, and benzoxazinoids⁹⁻¹³, diterpenoids¹⁴,
37 zealexins¹⁵ and flavonoids¹⁶ from maize.

38 Benzoxazinoids are multifunctional indole-derived metabolites produced by
39 *Poaceae*, including crops such as wheat, maize, and rye¹⁷. These compounds accumulate
40 in leaves as chemical defences against insect pests and pathogens¹⁷ and are exuded from
41 the roots as phytosiderophores¹⁸ and antimicrobials¹⁹⁻²¹. Benzoxazinoids directly shape
42 the root and rhizosphere microbiomes^{9-11,22}, and when metabolised to
43 aminophenoxazinones by soil microbes, they also become allelopathic, inhibiting the
44 germination and growth of neighbouring plants¹⁷. DIMBOA-Glc is the main root-exuded
45 benzoxazinoid of maize¹¹, and its chemical fate in soil is well understood. In [Fig. S1](#) we
46 document full names, structures and relatedness of all compounds relevant to this study.
47 Upon exudation, plant- or microbe-derived glucosidases¹⁷ cleave off the glucose moiety
48 to form DIMBOA, which spontaneously converts to more stable MBOA²³. In soil, MBOA
49 has a half-life of several days and can be further metabolised to reactive aminophenols
50 by microbes¹⁷. Three routes to different metabolite classes are known: route (I), favoured
51 under aerobic conditions²⁴, forms aminophenoxazinones such as AMPO and AAMPO;
52 route (II) results in acetamides such as HMPAA through acetylation²⁵, or alternatively,
53 route (III) yields malonic acids such as HMPMA through acylation²⁵. Route I is certainly
54 relevant for the rhizosphere as the AMPO can be detected in soils of cereal fields over

55 several months²³. While the chemical pathways of benzoxazinoid metabolism are
56 well-defined, the responsible microbes and enzymes remain largely unknown (but see
57 below).

58 Benzoxazinoids and their metabolism products have antimicrobial properties.
59 Yet, it remains poorly understood, how microbes cope with these bioactive plant
60 metabolites^{19–21,26,27}. We discriminate metabolite-microbe interactions as ‘native’ or
61 ‘non-host’, the latter referring to context where root microbes and root metabolites do
62 not originate from and occur in the same host. Recently, we demonstrated that ‘native’
63 root bacteria (isolated from maize) tolerated the maize-originating benzoxazinoids
64 better compared to ‘non-host’ bacteria isolated from *Arabidopsis*¹⁹. This suggested that
65 native maize bacteria were adapted and have evolved strategies to tolerate these
66 compounds. Evolution of tolerance could either involve reduced sensitivity of molecular
67 targets in the bacteria or improved and/or specialised strategies for metabolic
68 detoxification. Adapted bacteria may metabolise plant-derived compounds either by
69 conversion to less toxic compounds, or by degrading them entirely. Metabolism of
70 plant-derived compounds may not only reduce toxicity but also have added benefits for
71 bacterial growth. *Pseudomonas* or *Sphingobium* bacteria for instance use exuded
72 triterpenes or tomatine as carbon sources, respectively^{28,8}. These examples suggest that
73 native bacteria have evolved specialized adaptations to metabolise secondary
74 metabolites in root exudates of their host – this hypothesis remains untested.

75 Several soil microbes have been found to metabolise benzoxazinoids. Examples of
76 compound conversions include APO formation from BOA (non-methoxylated form of
77 MBOA, [Fig. S1](#)) by *Acinetobacter* bacteria²⁹, formation of the acetamide HPA from BOA
78 by the fungus *Fusarium sambucum*²⁵, or accumulation of APO from BOA upon co-culture of
79 *Fusarium verticillioides* with a *Bacillus* bacterium³⁰. Testing different soil microbes from
80 various environments revealed that they differed strongly in their metabolic activities
81 but that degradation resulted in the expected sequence of compounds from DI(M)BOA-
82 Glc to DI(M)BOA to (M)BOA¹³. First insights into the molecular mechanisms include the
83 identification of the metal-dependent hydrolase CbaA from *Pigmentiphaga* bacteria that
84 degrade modified benzoxazinoids³¹, and of a metallo- β -lactamase (MBL1) from the maize
85 seed endophytic fungus *Fusarium verticillioides* that degrades BOA to the malonamic acid
86 HPMA³². Benzoxazinoid metabolism by microbes has commonly been studied with
87 diverse microbes isolated from different soil environments. Microbial metabolism of

88 benzoxazinoids and its genetic basis have not yet been investigated in the native context
89 of root microbes from benzoxazinoid-exuding plants.

90 To uncover ecological context and biochemistry of microbial benzoxazinoid
91 metabolism, we systematically screened native maize and non-host *Arabidopsis* root
92 bacteria. Using metabolite analyses, genetics, comparative genomics, and biochemical
93 validation, we characterised benzoxazinoid-metabolising maize root bacteria and
94 identified the underlying genetic mechanisms. We found a conserved gene cluster for
95 benzoxazinoid metabolism with a lactonase that catalyses the degradation of MBOA.
96 Our work thereby uncovered a metabolic adaptation of root bacteria to host-exuded
97 specialised metabolites.

98 Results

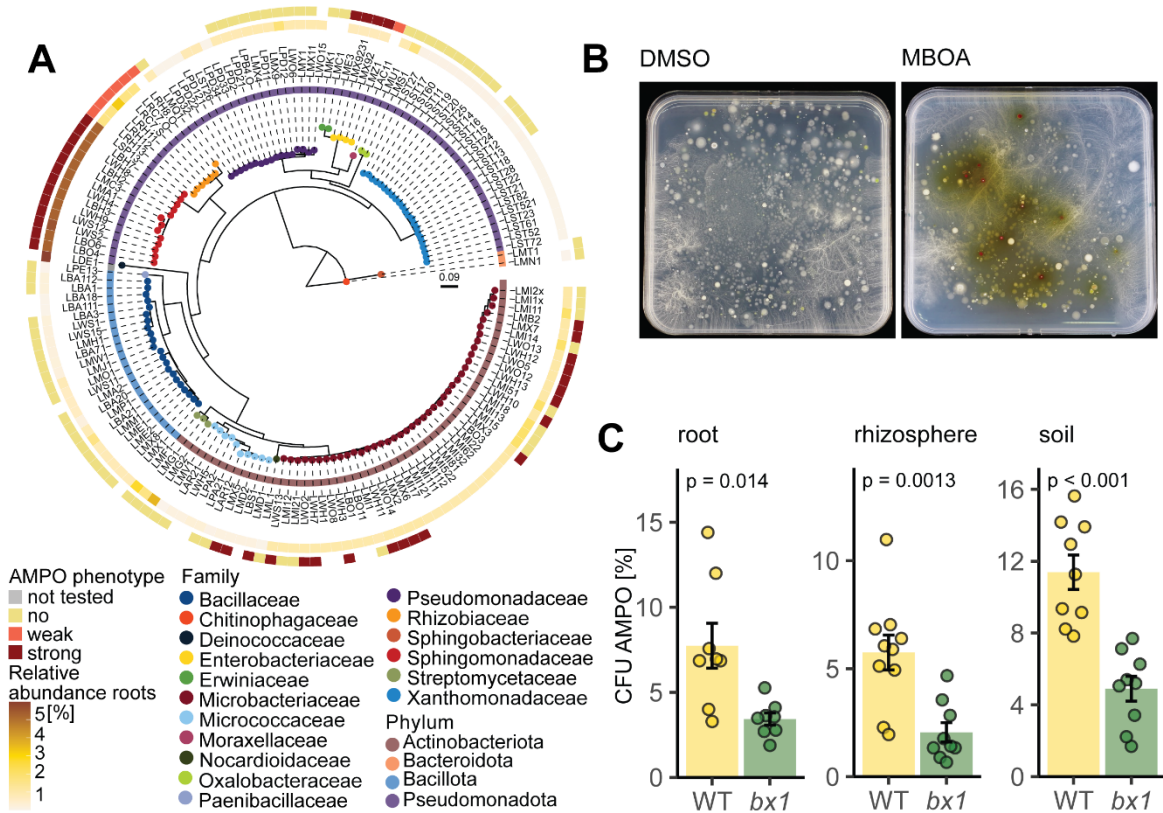
99 Taxonomically widespread and abundant maize root bacteria form AMPO

100 Screening maize root bacteria (i.e., the ‘MRB collection’) for MBOA tolerance¹⁹, we
101 observed that some liquid cultures, including *Sphingobium* LSP13 and *Microbacterium*
102 LMB2, turned red (Fig. S2). Analysis of the liquid media by UPLC-MS revealed that these
103 bacteria degraded MBOA, and NMR analysis with comparison to an analytical standard
104 confirmed the formation of AMPO, which has a dark red colour. The colour change to red
105 also manifests on MBOA-containing agar plates (Fig. S3) and thus, served as visual screen
106 for AMPO-formation in subsequent experiments.

107 To define the distribution of AMPO-forming bacteria, we screened our culture
108 collection¹⁹ on MBOA-containing plates and classified the strains as *non*, *weak* or *strong*
109 AMPO formers (see Fig. S3). We identified 44/151 strains belonging to six genera from
110 two phyla with colour changes to light or dark red (Fig. 1a). Strong AMPO-formers were
111 strains from *Microbacteria* (17/28 tested) and *Pseudoarthrobacter* (3/3), both of the
112 phylum Actinobacteriota. Among Pseudomonadota, *Sphingobium* (13/13) and
113 *Enterobacter* (4/4) strains were strong AMPO-formers too, while *Rhizobium* (6/7) and
114 *Acinetobacter* (1/1) isolates were weak AMPO-formers. Metabolite profiling confirmed
115 AMPO-formation in liquid cultures with MBOA (see below). We concluded that AMPO-
116 formation is a taxonomically widespread trait among maize root bacteria.

117 To approximate the abundance of AMPO-forming bacteria in microbiomes, we
118 mapped the identified strains to maize root microbiome datasets. First, mapping them to
119 roots from which they were isolated from¹¹, revealed that they accounted for 9% of the
120 community, with *Sphingobium* contributing most (5.3%, strong AMPO-former), followed
121 by *Rhizobium* (1.6%, weak), *Microbacteria* (1%, strong) and *Enterobacter* (0.7%, strong;
122 Fig. 1a). Second, in maize root microbiomes from field data⁹, community abundances
123 ranged from 2.9% (Changins, CH), to 6.7% (Aurora, US) and 14.9% (Reckenholz, CH; Fig.
124 S4). Then, to confirm the abundance of AMPO-formation in natural microbial
125 communities, we plated extracts of maize roots, rhizospheres, and soil of a pot
126 experiment with wild-type and benzoxazinoid-deficient *bx1* mutant plants on MBOA-
127 containing agar plates and determined the proportion of red colonies (Fig. 1b). In extracts
128 from wild-type plants, ~7.7 % of the root bacteria, ~5.8% of the rhizosphere bacteria and
129 ~11.4% of the soil bacteria formed AMPO (Fig. 1c). In extracts from *bx1* mutants, the

130 proportion of AMPO-forming bacteria decreased by more than 50%. Together with the
 131 mapping, these results suggest that AMPO-forming bacteria are abundant and enriched
 132 by benzoxazinoids in root microbiomes of maize.



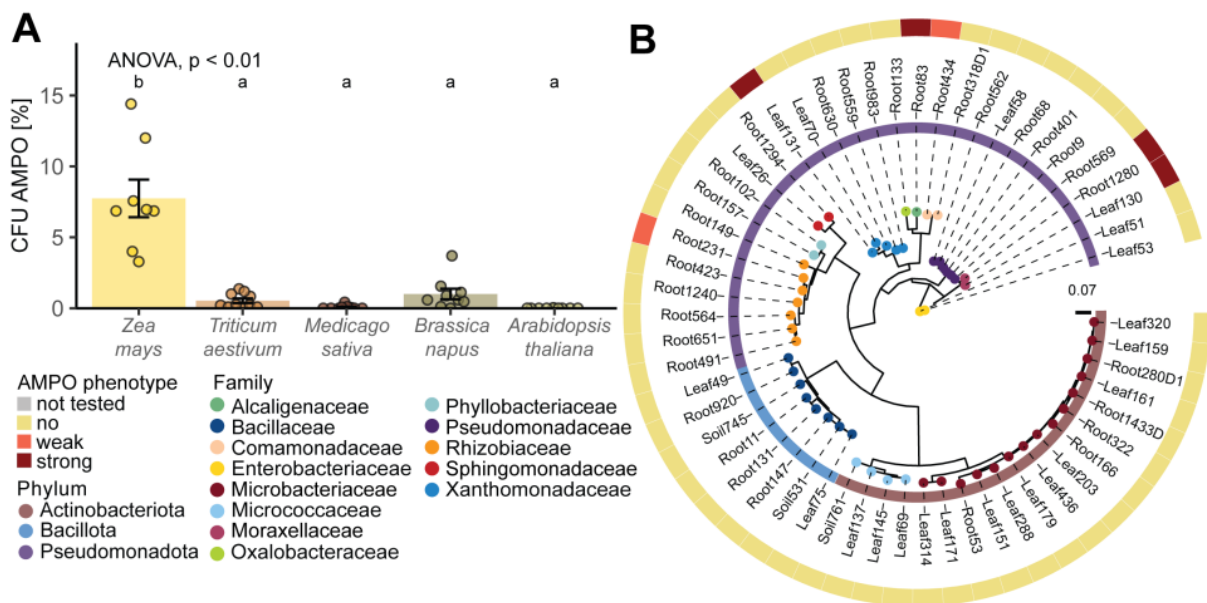
133
 134 **Figure 1: AMPO-forming colonies are abundant on benzoxazinoid-exuding maize roots.** **A)** Maximum
 135 likelihood phylogeny, constructed from the alignment of 16S rRNA gene sequences of maize root bacteria
 136 Leaf nodes are coloured by family taxonomy and the ring next to the strain IDs reports phylum taxonomy.
 137 The inner ring is coloured according to the relative abundance (%) of the corresponding sequence in the
 138 microbiome profile of the roots, from which the isolates were isolated. The outer ring displays the
 139 phenotype of the strains in the plate assay classified as “strong AMPO-former” based on a strong red
 140 colouring of the agar plate, “weak AMPO-former” for strains colouring the media to lighter red or “no
 141 AMPO-former” for strains not showing a colour change compared to the control root extracts plated on
 142 bacterial growth medium supplemented with DMSO (left) and MBOA (right) grown for 10 days. AMPO-
 143 forming colonies appear red on the MBOA-supplemented medium. **C)** Percentage of total colony forming
 144 units (CFU) that form AMPO on wild-type (WT) or benzoxazinoid-deficient *bx1* mutant roots, in
 145 rhizosphere and soil. Means ± SE bar graphs and individual data points are shown (WT n = 8, *bx1* n = 9).
 146 Results of pairwise t-tests are shown inside the panels.

148 AMPO-formation is specific for maize root bacteria

149 Using the same plate assay, we tested for AMPO-formation among root bacteria
 150 from different host plants. We compared root extracts from maize with wheat (*Triticum*
 151 *aestivum*), which accumulate less and predominantly non-methoxylated benzoxazinoids
 152 (i.e., BOA instead of MBOA)^{33–35}, and with lucerne (*Medicago sativa*), oilseed rape
 153 (*Brassica napus*) and Arabidopsis (Fig. 2a), all of which do not produce benzoxazinoids.
 154 We found the highest proportion of AMPO-forming colonies on maize roots (~7.7%),

155 followed by *Brassica* (~1 %), *Triticum* (~0.5 %), *Medicago* (~0.07 %) and *Arabidopsis*
 156 (~0.002 %). These findings highlight that AMPO-forming bacteria are specifically
 157 enriched on roots of maize plants.

158 To confirm this finding, we screened a collection of *Arabidopsis* bacteria³⁶ for
 159 AMPO-formation using the classification approach. On MBOA-containing plates 2/57
 160 strains classified as weak and 4/57 as strong AMPO-formers (Fig. 2b). A subset of
 161 *Arabidopsis* bacteria was further tested in liquid culture alongside two strong and two
 162 weak AMPO-formers from maize (Fig. S5a). None of the *Arabidopsis* strains efficiently
 163 degraded MBOA compared to the strong AMPO formers of maize. Only *Acinetobacter*
 164 (Root1280 and Leaf130) and *Variovorax* (Root434) formed low amounts of AMPO. This
 165 screening of *Arabidopsis* bacteria confirmed the results from plating root extracts (Fig.
 166 2a) and revealed that efficient AMPO-formation is a specific trait of bacteria isolated from
 167 benzoxazinoid-exuding maize roots.



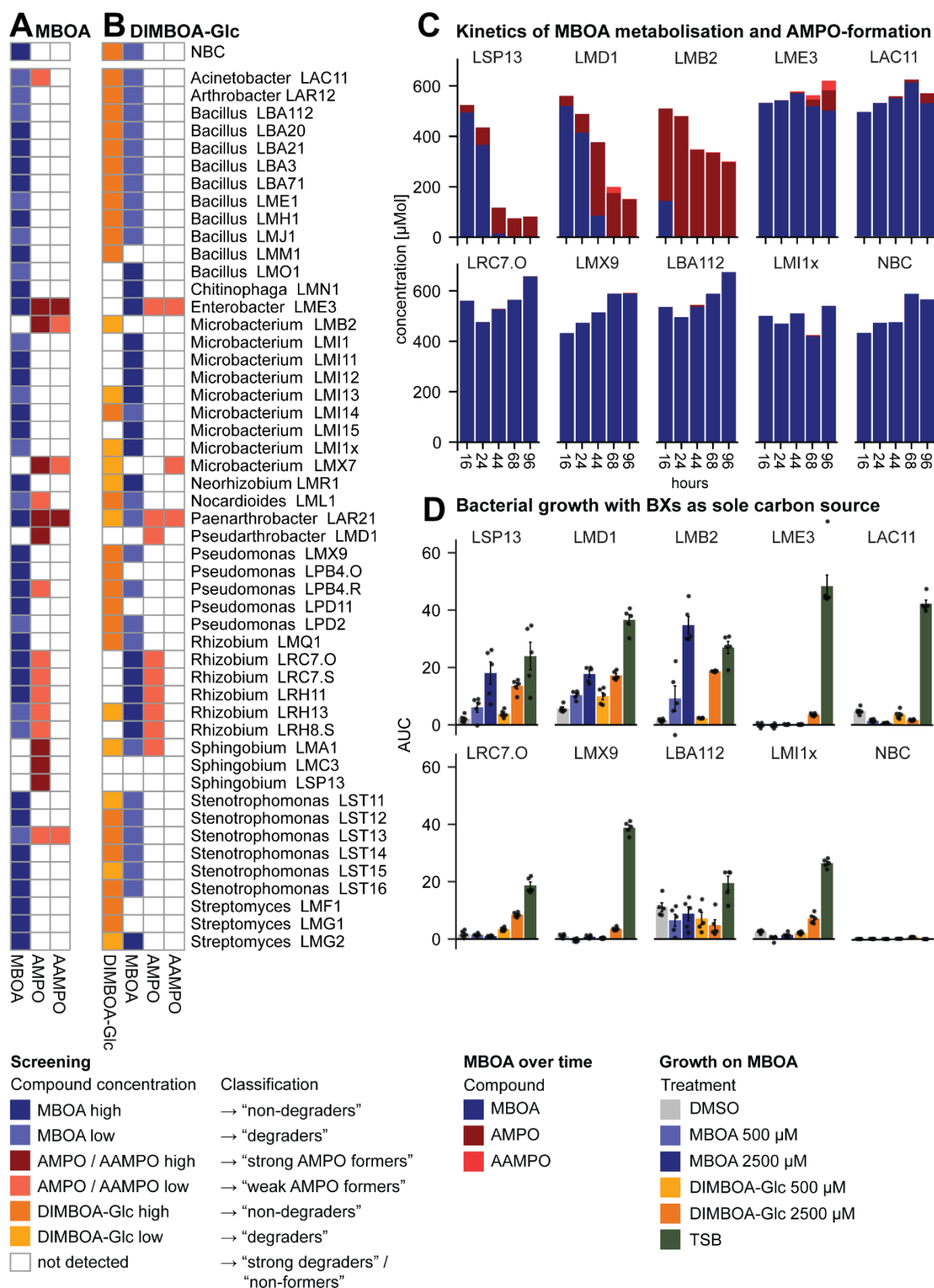
168
 169 **Figure 2: AMPO-formation in root bacteria from other host plants. A)** Percentage of colony forming
 170 units (CFU) of AMPO-forming colonies in root extracts of benzoxazinoid producing plants *Zea mays* (maize),
 171 *Triticum aestivum* (wheat) and non-benzoxazinoid producing plants *Medicago sativa* (lucerne), *Brassica*
 172 *napus* (oilseed rape) and *Arabidopsis thaliana*. Means \pm SE and individual data points are shown (n = 10,
 173 except maize n = 8) ANOVA and compact letter display of all pair-wise comparisons (Significance-level:
 174 FDR-corrected $p < 0.05$) of estimated marginal means are shown. **B)** Maximum likelihood phylogeny,
 175 constructed from the alignment of 16S rRNA gene sequences of *Arabidopsis* bacteria (AtSphere). Leaf nodes
 176 are coloured by family taxonomy and the ring next to the strain IDs reports phylum taxonomy. The ring
 177 displays the phenotype of the strains in the plate assay classified as “strong AMPO-former” based on a
 178 strong red colouring of the agar plate, “weak AMPO-former” for strains colouring the media to lighter red
 179 or “no AMPO-former” for strains not showing a colour change compared to the control.

180

181 Strong MBOA-degradation is required for AMPO-formation

182 For chemical validation of the AMPO formers and to investigate whether maize root
183 bacteria also degrade MBOA without forming AMPO, we exposed 50 strains to 500 μ M
184 MBOA in liquid cultures and quantified MBOA metabolisation using UPLC-MS. Because
185 the metabolite data (Fig. S5B) is not normalized by bacterial growth, we report
186 qualitative classifications in Fig. 3a. We classified 30/50 strains not to degrade MBOA
187 ($\pm 10\%$ of the control), while 14 strains partially degraded MBOA ('weak MBOA-
188 degraders'; $>30\%$ degraded compared to the control) and 6 strains were 'strong MBOA-
189 degraders' ($>90\%$ degraded). 8/50 strains were classified as 'strong AMPO-formers', 9
190 strains forming lower amounts of AMPO ('weak AMPO-formers', $<10\%$ of max. AMPO-
191 former) while most strains were non-AMPO formers ($<0.1\%$ of max. AMPO-former) and
192 we noticed 5 strains that metabolised AMPO further to AAMPO. We also noticed that the
193 initial amount of MBOA disappeared in the cultures of the strong MBOA-degraders while
194 low amounts of AMPO formed (Fig. S5B). This screening allowed the following
195 conclusions: the liquid assay confirmed the AMPO-formers previously classified on plates
196 (Fig. 1a), many bacteria degraded MBOA without forming AMPO, which is consistent with
197 the existence of alternative MBOA degradation pathways (Fig. S1), and importantly,
198 strong MBOA-degraders were strong AMPO-formers. Exceptions were only *Enterobacter*
199 LME3 and *Paenarthrobacter* LAR21, which formed (A)AMPO without a strong decrease
200 in MBOA, suggesting the existence of multiple ways to form AMPO from MBOA.

201 Since maize bacteria are not first exposed to MBOA on roots, we analysed
202 metabolisation of DIMBOA-Glc, the main compound in maize exudates. Of note, maize
203 exudates also contain DIMBOA (Fig. S1), we did not analyse it because of spontaneous
204 conversion to MBOA in absence of bacteria in the assay (Fig. S5C). DIMBOA-Glc is
205 commercially not available, thus we purified it from maize (traces of other co-purified
206 benzoxazinoids, Fig. S5C). Half of the strains were classified as 'non-degraders' of
207 DIMBOA-Glc ($\pm 10\%$ of the control; Fig. 3b). The DIMBOA-Glc metabolising strains were
208 classified as 'weak degraders' (11 strains; $>30\%$ degraded compared to control) and
209 'strong degraders' (14 strains, $>90\%$ degraded). These DIMBOA-Glc degrading strains
210 generally accumulated MBOA in their cultures, while only a few strains subsequently
211 formed low amounts of (A)AMPO. Importantly, strong degraders of DIMBOA-Glc were not
212 necessarily strong MBOA-degraders, revealing that these are two uncoupled traits in
213 maize root bacteria.



214

215 **Figure 3: Metabolisation of benzoxazinoids and use as sole carbon source by maize root bacteria. A)**

216 Heatmap displaying qualitative classifications for MBOA and its metabolisation products AMPO and

217 AAMPO and for **B)** DIMBOA-Glc and its metabolisation products MBOA, AMPO and AAMPO in liquid cultures

218 of 50 tested maize root bacteria. “No bacteria control” NBC only contains the medium supplemented with

219 the respective chemicals. **C)** Metabolisation of MBOA to AMPO and AAMPO over time (16h, 24h, 44h, 68h,

220 96h) for selected single strains: strong AMPO-formers *Sphingobium* LSP13, *Pseudoarthrobacter* LMD1,
221 *Microbacterium* LMB2, *Enterobacter* LME3 and weak AMPO-formers *Acinetobacter* LAC11 and *Rhizobium*
222 LRC7.0 and *Pseudomonas* LMX9, *Bacillus* LBA112 and *Microbacterium* LMI1x as negative controls. All
223 measurements were made from three independently grown cultures, which were pooled in equal ratios
224 before metabolite analysis. **D)** Testing MBOA as sole carbon source reported as bacterial growth during 68
225 h (area under the curve, AUC) of the same single strains in minimal medium supplemented with DMSO
226 (negative control), MBOA, or DIMBOA-Glc each in two concentrations (500 μ M or 2'500 μ M) and in TSB as
227 positive growth control. Means in bar graphs and individual data points are shown (n = 5).
228

229 To characterize the kinetics of MBOA-degradation and AMPO-formation, we
230 performed a time-series experiment with four strong (*Sphingobium* LSP13,
231 *Pseudoarthrobacter* LMD1, *Microbacterium* LMB2, and *Enterobacter* LME3) and two weak
232 AMPO-formers (*Acinetobacter* LAC11 and *Rhizobium* LRC7.0) alongside three non-AMPO
233 formers (*Pseudomonas* LMX9, *Bacillus* LBA112 and *Microbacterium* LMI1x). Rapid and
234 strong AMPO-formation was coupled with a strong decrease of MBOA (LSP13, LMD1 and
235 LMB2) while low amounts of AMPO formed with time and without much decrease of
236 MBOA (LME3 and LAC11; [Fig. 3c](#)). Neither MBOA-degradation nor AMPO-formation was
237 detected in LRC7.0 and the negative controls. Together with [Fig. 3a](#) these experiments
238 indicate at least two ways to form AMPO from MBOA: (i) AMPO is formed slowly and most
239 likely as the only product from MBOA or (ii) AMPO is rapidly formed in course of a fast
240 and strong degradation of MBOA.

241 Literature suggests that MBOA degrades to the reactive intermediate AMP ([Fig.](#)
242 [S1](#)), of which two molecules spontaneously form AMPO in the presence of oxygen²⁴. To
243 confirm the requirement for oxygen in AMPO formation, we cultivated the bacteria LMB2,
244 LMD1 and LSP13 both under aerobic and anaerobic conditions. Much less MBOA was
245 degraded and much less AMPO formed in anaerobic conditions ([Fig. S6](#)). While this result
246 should be interpreted with caution, as bacterial growth was also reduced in absence of
247 oxygen, it is in line with spontaneous AMPO formation from AMP in presence of oxygen²⁴.

248 AMPO-formation benefits bacterial growth

249 Because the degradations of DIMBOA-Glc and MBOA are key steps to form AMPO,
250 ([Fig. 3ab](#)), we tested whether these metabolic traits provided AMPO-forming strains a
251 growth benefit. We grew the same strains of the kinetic analysis ([Fig. 3c](#)) in minimal
252 media with DIMBOA-Glc or MBOA as sole carbon source. All strains grew well in the
253 positive growth control with TSB medium and the non-degraders of both DIMBOA-Glc
254 and MBOA (LAC11 and LMX9, LBA112) did not grow on either carbon source ([Fig. 3d](#)).
255 LME3, LRC7.0 and LMI1x, being capable to degrade DIMBOA-Glc but not MBOA, partially

256 benefited in minimal medium with the high concentration of DIMBOA-Glc but not MBOA.
257 In contrast, the strong DIMBOA-Glc- and MBOA-degraders (LSP13, LMD1, LMB2) strongly
258 increased their cell numbers compared to the controls. Together, these results reveal that
259 the capacities to degrade DIMBOA-Glc and MBOA are directly associated with growth
260 benefits under carbon-limiting conditions.

261

262 *AMPO-formation varies within Microbacteria*

263 To identify the genetic basis of AMPO-formation, we took advantage of the
264 phenotypic diversity in *Microbacteria* (Fig. 1a) and chose all isolates from maize¹⁹ (n=18),
265 Arabidopsis³⁶ (n=17), and other plants we had available in the laboratory (n=4, [Dataset](#)
266 [S1](#); see [methods](#)). We tested this set of 39 *Microbacteria* for AMPO-formation using the
267 plate assay and confirmed MBOA-degradation and AMPO-formation in liquid cultures
268 (Classifications in [Fig. 4](#), metabolite data in [Fig. S7](#)). MBOA was degraded and AMPO
269 accumulated in cultures of most *Microbacteria* classified as AMPO-formers in the plate
270 assay. Exceptionally, no AMPO was detected for three genomically similar strains (LTA6,
271 LWH12, LWO13). The testing of MBOA metabolisation uncovered four partially related
272 strains (LWH10, LBN7, LWH11 and LWO12) that also accumulated HMPAA, an
273 alternative degradation product of MBOA ([Fig. S1](#)). Finally, testing metabolisation of
274 DIMBOA-Glc revealed that also non-AMPO formers degraded DIMBOA-Glc and that most
275 AMPO-forming strains were strong DIMBOA-Glc degraders. An exception of the latter
276 observation was a group of four genomically similar strains that formed AMPO following
277 weak DIMBOA-Glc degradation (LM3X, LMB2, LMX7 and LWO14). For all 39
278 *Microbacteria*, we further quantified growth in minimal media containing MBOA as sole
279 carbon source, corroborating that AMPO-forming strains have a growth benefit from this
280 trait. Our chemical validation provides a robust basis for comparative genomics of 16
281 AMPO-forming and 23 AMPO-negative *Microbacteria* strains.

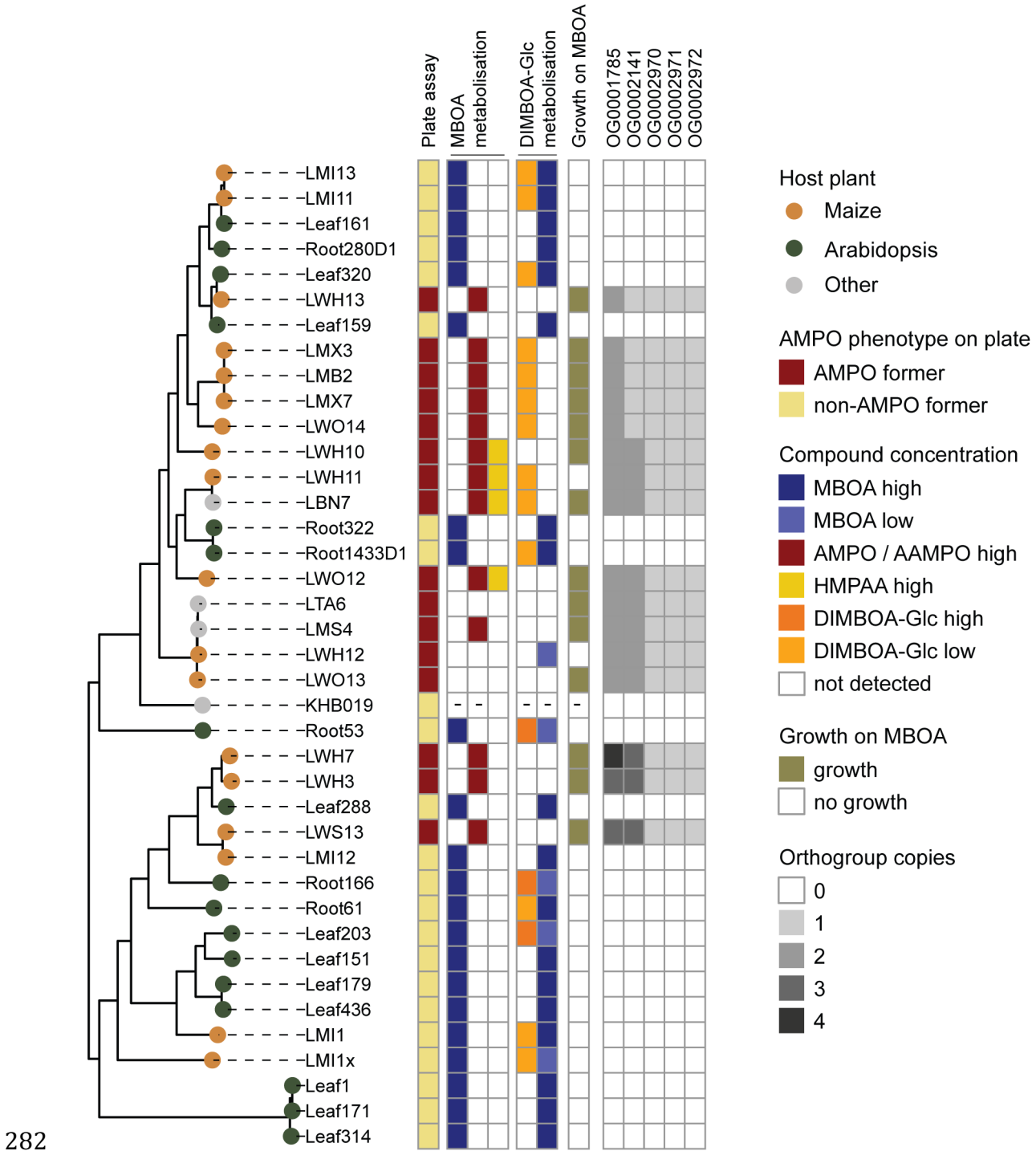
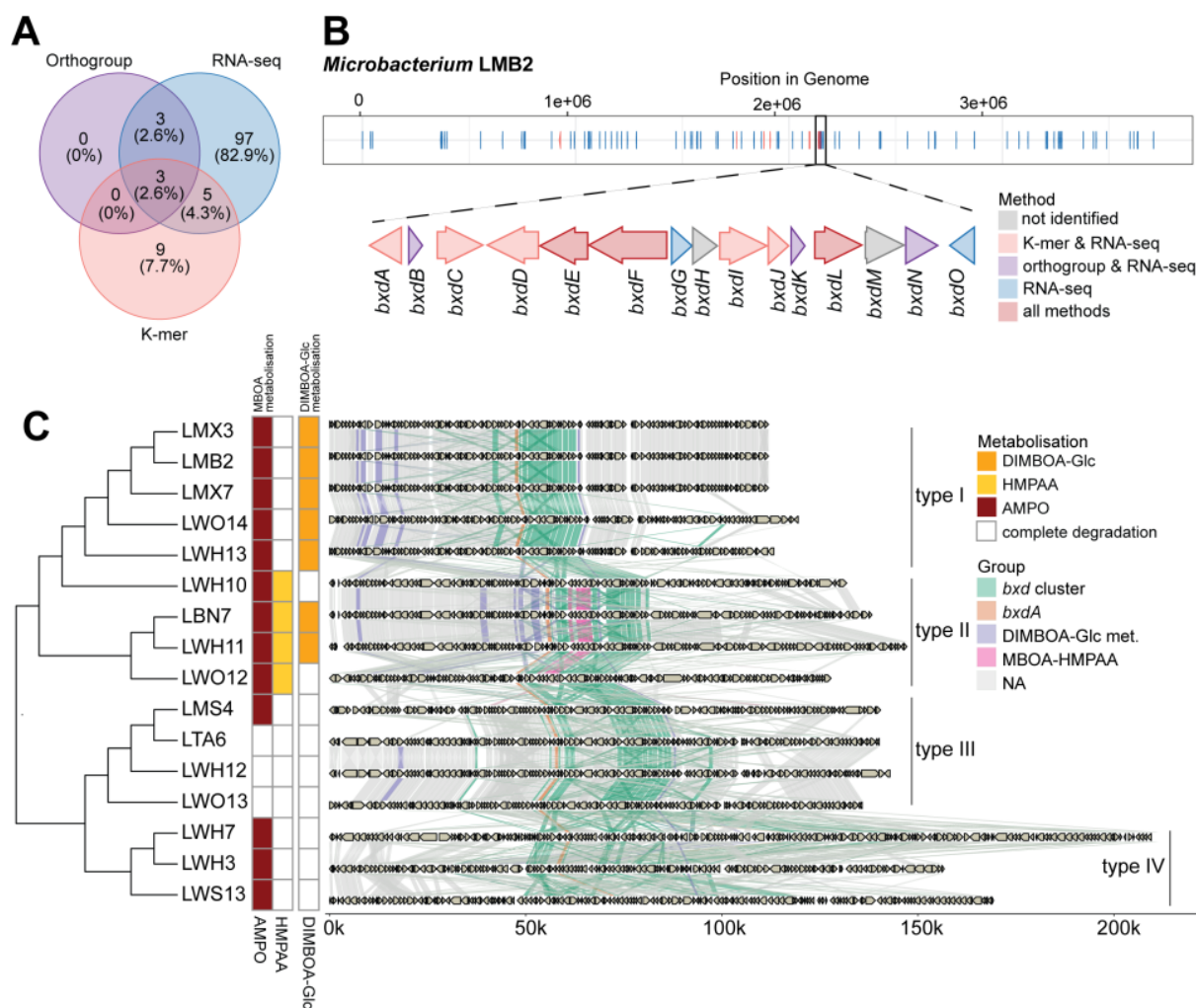


Figure 4: Phenotypic diversity of AMPO-formation in *Microbacteria*. Phylogenetic tree constructed from whole genome alignment of *Microbacteria*. Tips are coloured by host plant from which the strains were isolated from. The first row shows the AMPO classification (AMPO-former or non-AMPO former) of the strains based on the visual plate assay. The adjacent columns display the qualitative classifications of metabolite analyses (MBOA, AMPO, HMPAA and DIMBOA-Glc) of liquid cultures. The binary scale in column six indicates if the strain grew in minimal medium supplemented with MBOA as a sole carbon source. Mean results of 12 independent replicates grown in two independent runs. Columns seven to eleven report the results from the comparative genomic analysis, representing the copy number of the orthogroups found in each strain.

292 Identification of a gene cluster for AMPO-formation in *Microbacteria*

293 To identify candidate genes for AMPO-formation, we used the AMPO-phenotype
294 of the plate assay and combined three comparative genomic approaches ([Supplementary](#)
295 [results](#)). The orthogroup method identified 6 candidate genes ([Fig. 4, Dataset S2](#)), the
296 kmer approach 17 ([Dataset S3](#)) and the transcriptome analysis 108 ([Fig. S8, Dataset S4](#));
297 their overlaps are displayed in [Fig. 5a](#). Mapping the resulting candidates to the genome
298 of LMB2 revealed 15 genes that were located adjacently, pointing to a gene cluster for
299 AMPO-formation ([Fig. 5b](#)). This gene cluster contained all 6 genes of the orthogroup
300 analysis, and all 8 genes detected by the kmer approach. Transcripts of the entire gene
301 cluster were significantly upregulated in presence of MBOA, corroborating an active role
302 in AMPO-formation ([Fig. S8](#)). We termed this cluster *benzoxazinoid degradation* and
303 named the 15 genes in sequence *bxdA* to *bxdO*. The *bxd* gene cluster encodes 13 enzymes
304 and two transcriptional regulators ([Table S1](#)).

305 We performed in-depth analysis of the *bxd* gene cluster on closed long-read
306 genomes of all AMPO-forming *Microbacteria*. High resolution alignments revealed four
307 types of cluster architectures ([Fig. 5c](#)). Interestingly, they largely agreed with the
308 different metabolisation phenotypes of the strains ([Fig. 4](#)). Gene cluster type I was
309 present in five strains (LMX3, LMB2, LMX7, LWO14 LWH13), all 'weak DIMBOA-Glc
310 degraders' that fully degraded MBOA and accumulated AMPO as the only metabolisation
311 product. Type II was found in four strains (LWH10, LWH11, LBN7, LWO12), contained
312 five additional genes in the *bxd* gene cluster, and these strains uniquely formed HMPAA
313 besides accumulating AMPO. Cluster type III, present in four strains (LMS4, LTA6,
314 LWH12, LWO13), corresponded to bacteria that efficiently metabolised DIMBOA-Glc and
315 MBOA without accumulating AMPO. Finally, the cluster type IV, containing many gene
316 duplications, was found in three strains (LWH7, LWH3, LWS13) that all formed AMPO
317 after efficient metabolisation of DIMBOA-Glc or MBOA. This fine-grained genome analysis
318 revealed multiple variants of the *bxd* gene cluster, possibly representing multiple
319 metabolic pathways of benzoxazinoid degradation in *Microbacteria*.



320

321 **Figure 5: *Bxd* gene cluster in *Microbacteria*.** **A)** Overlap of three approaches used to identify candidate
 322 genes in AMPO formation: orthogroups, kmers and in RNA-seq. **B)** Position of all candidate genes identified
 323 with the three approaches in the genome of *Microbacterium* LMB2 with a zoom-in of the *bxd* gene cluster,
 324 annotated with its gene architecture including all genes named *bxdA* to *bxdO*. **C)** Across *Microbacteria*, the
 325 *bxd* gene cluster consists of four types (type I, type II, type III and type IV) that differ in gene order and
 326 content that correspond to their chemical phenotypes.

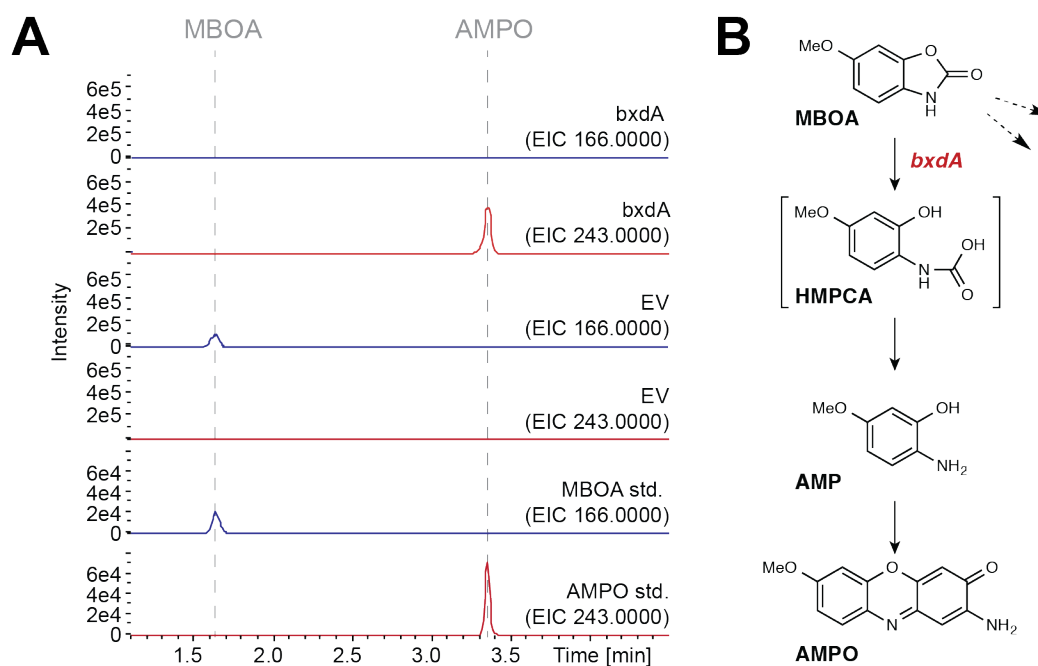
327

328 *BxdA* converts MBOA to AMPO *in vitro*

329 To identify the gene(s) responsible for MBOA breakdown and AMPO-formation,
 330 we selected the four candidates *bxdA*, *bxdD*, *bxdG*, and *bxdN* based on the functional
 331 annotation of their proteins as N-acyl homoserine lactonase family protein (*BxdA*),
 332 aldehyde dehydrogenase family protein (*BxdD*), VOC family protein (*BxdG*), and NAD(P)-
 333 dependent oxidoreductase (*BxdN*). We chose heterologous expression in *E. coli* as
 334 *Microbacteria* remain genetically unamenable. While neither purified *BxdD*, *BxdG* and
 335 *BxdN* nor the empty vector control showed MBOA degrading activity (Fig. S9), purified
 336 *BxdA* degraded MBOA and led to the accumulation of AMPO (Fig. 6a). Hence, the
 337 *Microbacteria* gene *bxdA*, encoding a ~34 kDa protein annotated as an N-acyl homoserine

338 lactonase family protein, has *in vitro* activity to degrade MBOA and form AMPO. We
339 propose that BxdA functions as a lactonase, opening the lactone moiety of MBOA to form
340 2-amino-5-methoxyphenol (AMP) via the corresponding carbamate (HMPCA) as
341 potential intermediate (Fig. 6b).

342 To elucidate whether BxdA is a *Microbacterium*-specific adaptation or a
343 widespread strategy for MBOA degradation, we conducted homology searches
344 ([Supplementary results](#)). In brief, besides being present in all our AMPO-forming
345 *Microbacteria*, *bxdA* was also found in the other strong AMPO-formers (3 gene copies in
346 *Sphingobium*, 1 in *Pseudoarthrobacter*) but not in all other taxa ([Fig. S9](#)). In wider
347 homology searches, BxdA was rarely found in other bacteria and unrelated to previously
348 described benzoxazinoid-degrading proteins. Together this highlights the importance of
349 BxdA for AMPO-formation by maize root bacteria and suggests it to be a novel enzyme
350 for microbial metabolism of benzoxazinoids.



351

352 **Figure 6: BxdA converts MBOA to AMPO. A)** Purified recombinant BxdA was incubated with the substrate
353 MBOA and product formation was monitored using high-pressure liquid chromatography-mass
354 spectrometry (HPLC-MS) operated in positive mode (full-scan, EIC = extracted ion chromatogram). An
355 empty vector (EV) control showed no activity. Authentic MBOA and AMPO were used as standards. **B)**
356 Proposed reaction sequence from MBOA to AMPO catalysed by BxdA. The dashed arrows refer to possible
357 alternative MBOA degradation pathways. The potential intermediate HMPCA is proposed but was not
358 confirmed experimentally. HMPCA = (2-hydroxy-4-methoxyphenyl)carbamic acid.

359 Discussion

360 Plants recruit distinct root microbial communities from the soil by exuding
361 bioactive specialised metabolites⁵. Thus, they shape species-specific microbiomes⁵, but
362 the mechanisms are not well understood. Here, we show that many maize root bacteria
363 can metabolise host-exuded benzoxazinoids, the main specialised metabolites of maize.
364 This trait is specific to native root bacteria from maize and is present among
365 taxonomically diverse and abundant members of the root microbiome. Metabolisation of
366 benzoxazinoids was rare in ‘non-host’ Arabidopsis bacteria, i.e., strains isolated from a
367 plant that does not produce benzoxazinoids. Maize bacteria benefitted from metabolising
368 MBOA because they can use it as carbon source in nutrient limiting conditions. Of the
369 different known chemical routes to degrade MBOA (Fig. S1), we have identified *bxdA*,
370 which encodes a novel lactonase enzyme converting MBOA to AMPO. Through *bxdA*,
371 maize root bacteria are metabolically adapted to benzoxazinoid exudates of maize.
372 Below, we discuss metabolic adaptation, the biochemistry of BxDa, and the biological
373 context of these findings.

374 Root microbes metabolise specialised plant metabolites. For example, Arabidopsis
375 root bacteria degrade host-synthesized triterpenes²⁸; several soil microbes metabolise
376 benzoxazinoids^{17,37}. Here, we investigated if maize root bacteria are adapted - defined by
377 a heritable trait improving an organism’s fitness - to maize-exuded benzoxazinoids. We
378 found support for this hypothesis by uncovering that benzoxazinoid metabolisation is
379 enriched in maize bacteria, whereas it is missing in non-host bacteria. This differential
380 metabolisation was seen comparing maize¹⁹ (Fig. 1a) and Arabidopsis strains³⁶ (Fig. 2b)
381 and again plating natural root microbiomes of different plant species (Fig. 2a). The
382 finding that AMPO-forming bacteria were less abundant in the wheat root microbiomes
383 may appear surprising but is consistent with the MBOA levels in the rhizosphere of this
384 wheat variety. More than 10x less MBOA (~5 ng/mL) was found in the rhizosphere of CH
385 Claro compared to maize (~60 ng/mL)³⁸. Hence, we think that the much lower levels of
386 MBOA in the rhizosphere of CH Claro resulted in a much lower selection of AMPO formers.

387 The fact that roots of benzoxazinoid-deficient *bx1* mutants harboured 50% less
388 AMPO-forming bacteria highlighted a direct link between benzoxazinoid exudation from
389 maize and bacterial MBOA metabolisation (Fig. 1b). Metabolite profiling of *Microbacteria*
390 from maize and Arabidopsis revealed that only maize-derived isolates metabolised
391 benzoxazinoids (Fig. 4), and genomic comparisons uncovered the *bxd* gene cluster, which

392 was only present in AMPO-forming *Microbacteria* (Fig. 5). The key gene *bxDA* was also
393 present in other MBOA-metabolising maize bacteria but not in Arabidopsis-derived
394 bacteria (Fig. S9), which reveals metabolic adaptation of maize root bacteria to host-
395 specialised metabolites at the genomic level. Further research, for instance comparing
396 Arabidopsis and maize root bacteria for metabolisation of specialised compounds of
397 Arabidopsis such as coumarins²⁶, is required to broaden this conclusion. Given the high
398 degree of host-species specific microbiomes⁵ and the widespread nature of plant species-
399 specific specialised metabolites, we propose that metabolic adaptation may structure
400 root microbiomes across the plant kingdom.

401 To complement previous studies²⁰, we specifically investigated the genetic basis
402 of benzoxazinoid metabolisation in the native context of root bacteria isolated from
403 benzoxazinoid-exuding maize plants. We focused on MBOA, the most abundant¹¹ and
404 most selective¹⁹ benzoxazinoid in the maize rhizosphere. The phenotypic and genomic
405 screening of maize- and Arabidopsis-derived *Microbacteria* (Fig. 4) permitted the
406 identification of BxDa, an N-acyl homoserine like lactonase. Gene homologs were only
407 found in AMPO-forming *Pseudoarthrobacter* and *Sphingobium* strains from maize but not
408 in Arabidopsis bacteria (Fig. S9). We detected only weak similarity (<43% amino acid
409 level) with known enzymes such as CbaA³¹ or MBL³⁹, both involved in metabolisation of
410 benzoxazinoids. Thus, the lactonase BxDa represents a novel enzyme for benzoxazinoid
411 metabolisation pointing to a highly specific adaption restricted to root microbiome
412 members of benzoxazinoid-producing plants.

413 We confirmed BxDa to catalyse the metabolisation of MBOA to AMPO *in vitro* (Fig.
414 6a). We chose this approach as *Microbacteria* are genetically unamenable, but future
415 experiments, e.g. with *bxDA* mutants in genera like *Sphingobium*, could allow *in vivo*
416 confirmation. The biochemistry of BxDa is consistent with its annotation as a lactonase
417 that hydrolyses the ester bond of a lactone ring⁴⁰. With MBOA as a substrate, this reaction
418 yields AMP that spontaneously dimerizes to AMPO in the presence of oxygen²⁴.
419 Lactonases occur in various bacteria⁴¹ and typically degrade N-acyl homoserine lactones,
420 which are signalling metabolites of bacterial quorum sensing⁴². This supposedly similar
421 biochemical function opens a range of novel questions, including on the evolutionary
422 origin of BxDa or its impact on quorum sensing that warrant further investigation.

423 Metabolisation of specialised metabolites has multiple biological consequences.
424 Generally, bacteria aim at detoxification, suppression of other microbes, or utilization as

425 carbon source^{43,44}. Our analyses suggested that the bacteria primarily degrade MBOA
426 (Fig. 3c), which is consistent with all AMPO-forming bacteria using MBOA as a carbon
427 source (Fig. 3d & 4). It is conceivable that AMPO is rather formed as a side product of
428 incomplete or inefficient bacterial catabolism of the intermediate AMP. Nevertheless,
429 AMPO-formation will affect the microbial and plant ecology. It confers advantages to
430 AMPO-tolerant bacteria to expand their niche by preferentially suppressing Gram-
431 positive bacteria, which are generally less tolerant to aminophenoxazinones compared to
432 Gram-negative bacteria¹⁹. Alternatively, AMPO may promote rhizosphere health through
433 its suppressive activity against phytopathogenic fungi^{30,45}. Plants, on the other hand, may
434 benefit from recruiting A(M)PO-forming bacteria as they convert (M)BOA to strongly
435 allelopathic compounds that suppress weeds⁴⁶, thereby improving host fitness. Overall,
436 AMPO-forming bacteria contribute to microbiome traits that benefit their host plant.

437 We had originally discovered benzoxazinoid-dependent and microbe-driven
438 feedbacks on plant performance in controlled conditions¹¹ and recently, we show that
439 they also operate in field conditions increasing wheat yield in certain soils³⁸. The latter
440 suggest that microbial feedbacks are agriculturally relevant and highlights that plant
441 specialised metabolites present a strong tool for leveraging microbiome functions. In
442 combination, our previous work on tolerance¹⁹ and the present study demonstrate that
443 metabolic adaptation to plant specialised metabolites are key determinants for root
444 colonisation by bacteria. Regarding possible agricultural applications, our data implies
445 that effective biocontrol or biofertilizer strains should be tolerant and/or metabolically
446 adapted to the specialised metabolites produced by the target crop. Hence,
447 understanding how specific specialized plant metabolites shape and stabilize their
448 microbiomes will be important to harness microbiome functions to improve plant health
449 in sustainable agricultural systems⁴⁷.

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462 Materials and Methods

463 Plating experiment

464 To assess the number of AMPO-forming colonies on roots, we grew wild-type
465 maize plants and BX-deficient *bx1*(B73) maize, wheat (CH Claro), *Medicago sativa* (Sativa,
466 Rheinau, Switzerland), *Brassica napus* (Botanik Saemereien AG, Pfaeffikon, Switzerland)
467 and *Arabidopsis thaliana* (Col-0) in field soil. The soil was collected in Winter 2019 from
468 the field in Changins¹¹. We grew the plants for 7 weeks in a walk-in growth chamber with
469 the following settings: 16:8 light/dark, 26/23 °C, 50 % relative humidity, ~550 $\mu\text{mol m}^{-2}\text{s}^{-1}$
470 light. We fertilized the plants in the following regime: Weeks 1 – 4: 100 mL; 0.2 %
471 Plantactive Typ K (Hauert HBG Duenger AG, Grossaffoltern, Switzerland), 0.0001 %
472 Sequestrene Rapid (Maag, Westland Schweiz GmbH, Dielsdorf, Switzerland); weeks 5
473 onwards: 200 mL; 0.2 % Plantactive Typ K, 0.02 % Sequestrene Rapid. To account for
474 different need of *Arabidopsis* growth, all seeds were stratified for three days in the dark
475 at 4 °C and then grown in growth cabinets (Percival, CLF Plant climatics) at 60 % relative
476 humidity, 10 h light at 21 °C and 14 h dark at 18 °C. *Arabidopsis* were fertilized two times
477 during the experiment by watering with 2/3 water and 1/3 of half-strength Hoagland
478 solution⁴⁸. To harvest the roots, we shake off loose soil and prepared 10 cm long root
479 fragments (corresponding to the depth of -1 to -11 cm in soil) which we then chopped
480 into small pieces with a sterile scalpel. We transferred them into a 50 mL Falcon tube
481 containing 10 mL sterile magnesium chloride buffer supplemented with Tween20
482 (MgCl_2 Tween, 10 mM MgCl_2 + 0.05 % Tween, both Sigma-Aldrich, St. Louis, USA). We
483 homogenized the roots with a laboratory blender (Polytron, Kinematica, Luzern,
484 Switzerland; 1 minute at 20'000 rpm) followed by additional vortexing for 15 seconds.
485 For the rhizosphere fraction, we resuspended the pellet from the washing step in 5 mL
486 MgCl_2 Tween. For the soil fraction, we mixed 5 g of soil from the pot with 5 mL
487 MgCl_2 Tween and vortexed it for 15 s.

488 To quantify bacterial community size, we plated root, rhizosphere, and soil
489 extracts. We serially diluted the extracts and plated 20 μL on 10 % TSB agar (3 g/L tryptic
490 soy broth and 15 g/L agar, both Sigma-Aldrich, St. Louis, USA) plates (12 x 12 cm, Greiner
491 bio-one, Kremsmünster, Austria) containing filter-sterilized cycloheximide (10 mg/L,
492 Sigma-Aldrich, St. Louis, USA) and filter-sterilized DMSO (2 mL/L, Sigma-Aldrich, St.
493 Louis, USA). To spread the drops for counting we tilted the plates and incubated them for
494 6 days at room temperature. We counted colony-forming units (CFU), multiplied them by

495 the dilution factor and normalized them with the sample's fresh weight. Before statistical
496 analysis, we transformed CFU counts by log₁₀.

497 To count the number of AMPO-forming colonies in the extracts, we spread one
498 dilution on a square agar plate containing MBOA. Depending on the plant species and the
499 compartment, we selected a dilution between 1:10⁻¹ and 1:10⁻⁴ to reach a colony density
500 which is countable. We spread the 50 µl of the sample with a delta cell spreader on square
501 agar plates with 10% TSB supplemented with filter-sterilized cycloheximide and filter-
502 sterilized MBOA (200 mg/L, Sigma-Aldrich, St. Louis, USA). For 10 days we incubated the
503 plates at room temperature (21 - 25 °C). We photographed the plates and counted the red
504 colonies on the pictures. To get the proportion of AMPO-forming colonies per sample, we
505 divided the count of AMPO-forming colonies by the total CFU.

506

507 Bacterial strains and cultures

508 Maize root bacteria (i.e., MRB collection)¹⁹ and Arabidopsis bacteria (i.e.,
509 AtSPHERE collection)³⁶ were routinely grown on TSA (30 g/L tryptic soy broth and 15
510 g/L of agar, both Sigma-Aldrich) at 25 °C – 28 °C or TSB liquid medium (30 g/L tryptic
511 soy broth). To screen for AMPO-formation of single isolates, we plated a loop of pure
512 bacterial cultures on TSA plates supplemented with MBOA (200 mg/L) or DMSO (2 mL/L)
513 as control. We incubated the plates for 10 days at room temperature, assessed the
514 phenotype by eye and photographed the plates.

515

516 *In vitro* growth & metabolisation assays

517 To screen plant root bacteria for their capacity to metabolise benzoxazinoids we
518 deployed the custom, high-throughput, in vitro liquid culture based-growth system
519 reported previously^{19,49}. This system makes it possible to culture many bacterial strains
520 in parallel in a replicated manner using many 96-well plates which are handled with a
521 stacker (BioStack 4, Agilent Technologies, Santa Clara, United States), so that the
522 connected plate reader (Synergy H1, Agilent Technologies) records bacterial growth via
523 optical density (OD₆₀₀, absorbance at 600 nm) over time. The assay is set up by
524 inoculating pre-cultures to culture media supplemented with the respective chemical
525 compounds at different concentrations.

526 Pre-cultures were prepared by transferring isolate colonies with inoculation
527 needles (Greiner bio-one, Kremsmünster, Austria) to 1 mL of liquid 50% TSB (15 g/L
528 tryptic soy broth, Sigma-Aldrich) in 2 mL 96-well deep-well plates (Semadeni,
529 Ostermundigen, Switzerland). These pre-culture growth plates were covered with a
530 Breathe-Easy membrane (Diversified Biotech, Dedham, USA) and grown until stationary
531 phase for 4 days at 28°C and 180 rpm.

532 Then 4 μ L of the pre-cultures were inoculated to 200 μ L fresh liquid 50% TSB in
533 96-well microtiter plates (Corning, Corning, USA) containing the compounds and
534 concentrations to be tested: DIMBOA-Glc, MBOA (500 or 2'500 μ M) and BOA (500 μ M).
535 These treatments were prepared by mixing their stock solutions into liquid 50% TSB.
536 DIMBOA-Glc was isolated from maize seedlings as described previously¹⁹ while synthetic
537 MBOA and BOA were commercially available (Sigma-Aldrich). Stock solutions were
538 prepared in the solvent DMSO (Sigma-Aldrich) depending on the solubility of the
539 compounds: DIMBOA-Glc at 500 mM (186.55 mg/mL), MBOA at 606 mM (100 mg/mL)
540 and BOA at 500 mM (67.55 mg/mL). The DMSO concentration was kept constant in each
541 treatment including the control.

542 All reactions and replicated plates were pipetted using a liquid handling system
543 (Mettler Toledo, Liquidator 96™, Columbus, USA). All plates had lids and were piled up
544 and inserted to a stacker (BioStack 4, Agilent Technologies, Santa Clara, United States),
545 which was connected to a plate reader (Synergy H1, Agilent Technologies, Santa Clara,
546 United States). Using this system, OD₆₀₀ of every culture was recorded every 100 min over
547 68 h. Prior to each measurement, the plates were shaken for 120 s. In each plate, wells
548 with 50% TSB were included as no bacteria controls (NBC) and in each run one plate
549 containing only media was included to monitor potential contaminations. This procedure
550 applies to the time-series experiment, the Microbacteria screen, the carbon source and
551 BOA assays. To measure MBOA metabolism over time, we removed plates from the
552 stack after 16 h, 24 h, 44 h, 68 h and 96 h.

553 For the growth assays with benzoxazinoids as sole carbon source, we followed the
554 same procedure as described above but using minimal media instead of 50% TSB. The
555 minimal media was prepared as described previously⁵⁰ and complemented with defined
556 amounts of stock solutions of either MBOA or DIMBOA-Glc to reach a final concentration
557 of 500 or 2'500 μ M. As positive growth controls we grew the bacteria in glucose at
558 different concentrations (500, 2'500 and 30'000 μ M) and in 50% TSB.

559 For the initial metabolite screen of all MRB and the transcriptome experiment, we
560 incubated the plates on a laboratory shaker at 28 °C instead of using the stacker. To avoid
561 evaporation, we sealed the plates with a stripe of Breathe-Easy membrane. We recorded
562 the optical density of the cultures at the end of the experiment in a plate reader (Tecan
563 Infinite M200 multimode microplate reader equipped with monochromator optics, Tecan
564 Group Ltd., Männedorf, Switzerland). The initial metabolite screen ended after 68 h and
565 the transcriptome experiment after 16 h. We exported bacterial growth data from the
566 software (Gen 5, Agilent Technologies, Santa Clara, United States) to excel. We used R
567 statistical software (version 4.0, R core Team, 2016) to analyze growth data. First we
568 calculated the area under the growth curve (x-axis for time and y-axis for OD₆₀₀ AUC)
569 using the function *auc()* from package MESS⁵¹ and normalized growth in a treatment
570 relative to the control. Such normalized bacterial growth data of a given concentration
571 was statistically assessed (compound vs control) using one-sample t-tests (p-values
572 adjusted for multiple hypothesis testing). Further details on statistical analysis are
573 described below.

574

575 **Assessing MBOA metabolisation in anaerobic conditions**

576 To test the requirement of oxygen for AMPO-formation, we performed a
577 metabolisation experiment in anaerobic conditions. As described above, we prepared
578 treatment solutions with 500 µM or 2'500 µM MBOA in 15 mL Falcon tubes. Before the
579 experiment, we pre-incubated the treatments over three days in a sealed jar under an
580 anaerobic environment to remove oxygen from the TSB medium. To start the experiment,
581 we inoculated a loop of bacteria from fresh plates. An anaerobic environment was created
582 for half of the samples with an environment generator according to the manufacturer's
583 instructions (TRILAB, Jenny Science, Rain, Switzerland). We grew the cultures either
584 under anaerobic or aerobic conditions in an incubator at 28 °C (Memmert, Schwabach,
585 Germany). After 68 h of growth, we measured the optical density of the cultures.

586

587 **Metabolite extraction from bacterial cultures**

588 At the end of the experiment, we examined colour changes in the cultures by eye.
589 To fix bacterial cultures, we added 150 µL bacterial cultures to 350 µL of the extraction
590 buffer (100 % Methanol + 0.14 % formic acid) in non-sterile round bottom 96-well plates
591 (Thermo Fisher Scientific, Waltham, USA). We stored the fixed samples with a final

592 concentration of 70 % methanol and 0.1 % formic acid at -80 °C. To reduce the number
593 of samples, we pooled three replicates of the same culture. For the transcriptome
594 experiment (n = 5) and the anaerobic experiment (n = 3), we did not pool samples. We
595 diluted the pooled sample by mixing 50 to 700 µL MeOH 70% + 0.1 % FA. We filtered the
596 cultures through regenerated cellulose membrane filters (CHROMAFIL RC, 0,2 µm,
597 Macherey-Nagel, Düren, Germany) by centrifugation (6'200 rpm for 2 min) to remove
598 bacterial debris. To avoid any residual particles, we centrifuged the extracts at 13'000
599 rpm for 10 min at 4 °C. We aliquoted the supernatants in glass vials (VWR, Dietikon,
600 Switzerland) and stored the samples for a few days at 20 °C until analysis.

601

602 Profiling benzoxazinoid degradation products in bacterial cultures

603 Using an Acquity I-Class UHPLC system (Waters, Milford, US) coupled to a Xevo
604 G2-XS QTOF mass spectrometer (Waters, Milford, US) equipped with a LockSpray dual
605 electrospray ion source (Waters, Milford, US) we quantified benzoxazinoids in samples
606 of filtered bacterial cultures. Gradient elution was performed on an Acquity BEH C18
607 column (2.1 x 100 mm i.d., 1.7 mm particle size (Waters, Milford, US) at 98–50% A over
608 6 min, 50-100% B over 2 min, holding at 100% B for 2 min, re-equilibrating at 98% A for
609 2 min, where A = water + 0.1% formic acid and B = acetonitrile + 0.1% formic acid. The
610 flow rate was 0.4 mL/min. The temperature of the column was maintained at 40 °C, and
611 the injection volume was 1 µL. The QTOF MS was operated in sensitivity mode with a
612 positive polarity. The data were acquired over an m/z range of 50–1'200 with scans of
613 0.1 s at a collision energy of 6 V (low energy) and a collision energy ramp from 10 to 30
614 V (high energy). The capillary and cone voltages were set to 2 kV and 20 V, respectively.
615 The source temperature was maintained at 140°C, the desolvation temperature was 400
616 °C at 1'000 L/hr and the cone gas flow was 100 L/hr. Accurate mass measurements (<2
617 ppm) were obtained by infusing a solution of leucine encephalin at 200 ng/mL at a flow
618 rate of 10 µL/min through the Lockspray probe (Waters, Milford, US). For each expected
619 benzoxazinoid compound, four standards with concentrations of 10, 50, 200, and 400
620 ng/mL were run together with the samples (DIMBOA-Glc, DIMBOA, HMBOA, MBOA-Glc,
621 MBOA, BOA, AMPO, APO, AAMPO, HMPMA) or 40, 200 ng/mL, 1 and 10 µg/mL for HMPAA
622 and AMP.

623

624 NMR identification of AMPO

625 To confirm the presence of AMPO in the liquid cultures of *Sphingobium* LSP13 and
626 *Microbacterium* LMB2, we analysed them by ¹H NMR spectroscopy (Bruker Advance 300,
627 1H: 300.18 MHz, Bruker Corp., Billerica, MA, USA). Briefly, liquid cultures were
628 centrifuged (20 min, 13'000 rpm) and the supernatants extracted twice with Et₂O, dried
629 with Na₂SO₄ and filtered in a glass funnel with cotton wool. During cultivation a red
630 precipitate formed towards the neck of the Erlenmeyer flasks, i.e. at the edge of the
631 shaking cultures (Fig. S2). This red precipitate left was collected from the Erlenmeyer
632 flasks with acetone. The two extracts were combined, concentrated under reduced
633 pressure, and dried over P₂O₅. The ¹H NMR spectrum of the red residue obtained was
634 recorded in DMSO-*d*₆ and compared to an analytical AMPO standard^{23,52}, confirming its
635 presence in our bacterial cultures.

636

637 Phylogenetic tree construction

638 The phylogenetic tree of all MRB and AtSphere bacteria was computed as
639 described previously¹⁹. The species tree estimation for *Microbacteria* was obtained from
640 OrthoFinder v. 2.3.8⁵³. The 16S trees were reconstructed as follows: First, the 16S
641 sequences were combined into a single FASTA file and then aligned using MAFFT v.
642 7.475⁵⁴ with default options. The aligned sequences were then used as input to RAxML v.
643 8.2.12⁵⁵. The multi-threaded version `raxmlHPC-PTHREADS` was used with the options
644 `-f a -p 12345 -x 12345 -T 23 -m GTRCAT` with 1'000 bootstrap replicates. The
645 phylogenetic tree was visualized and annotated in R using the package ggtree⁵⁶.

646

647 Comparative genomics

648 To find genes that are involved in the transformation of MBOA to AMPO we built
649 an extended collection of 39 *Microbacteria* (MicroE) strains. We selected all *Microbacteria*
650 from maize¹⁹ (n=18) and from the *AtSphere* collection³⁶ isolated from Arabidopsis (n=17)
651 and one strain isolated from clover⁵⁷. Additionally, we selected three strains which we
652 isolated, from root extracts of *Brassica napus* (LBN7), *Triticum aestivum* (LTA6) and
653 *Medicago sativa* (LMS4) due to their red colony phenotype on MBOA plates. For those
654 strains we sequenced the genome by PacBio as described for the MRB collection¹⁹. The
655 39 *Microbacteria* were phenotypically divided into AMPO-forming (n = 16) and AMPO-

656 negative *Microbacteria* (n = 23) strains based on the MBOA plate assay. Two approaches
657 were investigated independently. The first consisted of grouping the genes into
658 orthogroups with OrthoFinder v. 2.3.8⁵³ and estimating significant associations between
659 the phenotype and orthogroups by applying Fisher's Exact Test using the gene trait
660 matching tool in OpenGenomeBrowser⁵⁸. In the second approach, a kmer-similarity
661 search strategy was conducted. The scaffolds of the assemblies were first divided into
662 unique kmers of size 21 base pairs and counted using the tool Kmer Counter v. 3.1.1⁵⁹.
663 The resulting kmer libraries per sample were then merged into a single matrix using
664 custom python scripts. In the next step, the kmers were scored based on their occurrence
665 in AMPO-positive or negative strains. Specifically, the score of a kmer was increased by
666 1, if the kmer is present in a sample with AMPO-forming phenotype and was decreased
667 by 1 if the kmer is present in a sample with AMPO-negative phenotype. This score can
668 thus be seen as a correlation between genetic sequence and phenotype. The highest
669 scoring kmers were then used to filter genes containing those kmers using custom python
670 scripts. Since this approach relies on exact matches of kmers, the gene sequences
671 containing high-scoring kmers were clustered with a 70% similarity cut-off using vsearch
672 v. 2.17.1⁶⁰. The obtained centroid sequences were then searched with BLAST v. 2.10.0⁶¹
673 against a database of all genes from all *Microbacteria* strains using 'blastn'. The BLAST
674 output was filtered for matches with an e-value < 1e50 which resulted in a list of genes
675 for each centroid sequence. These gene lists were then statistically assessed for their
676 association with the phenotype using Fisher's Exact Test in R (v. 4.2.1). The p-values were
677 corrected using the Benjamini-Hochberg method.

678

679 Transcriptome analysis

680 For the transcriptome experiment, bacterial cultures which were grown for 16 h
681 in six individual wells were pooled, and immediately stabilized by the addition of
682 RNeasy Protect Bacteria Reagent (Qiagen, Hilden, Germany). Bacterial cells were lysed by
683 enzymatic lysis and proteinase K treatment and total RNA was extracted using the
684 RNeasy Mini Kit (Qiagen, Hilden, Germany) with subsequent DNase treatment using the
685 RapidOut DNA removal kit (Thermo Fisher Scientific, Waltham, USA) following
686 manufacturer's instructions. The quantity and quality of the purified total RNA were
687 assessed using a Thermo Fisher Scientific Qubit 4.0 fluorometer with the Qubit RNA BR
688 Assay Kit (Thermo Fisher Scientific, Waltham, USA) and an Advanced Analytical

689 Fragment Analyzer System using a Fragment Analyzer RNA Kit (Agilent, Basel,
690 Switzerland), respectively. One hundred ng of input RNA was first depleted of ribosomal
691 RNA using an Illumina Ribo-Zero plus rRNA Depletion Kit (Illumina, San Diego, US)
692 following Illumina's guidelines. Thereafter cDNA libraries were made using an Illumina
693 TruSeq Stranded total Library Prep Kit (Illumina, San Diego, US) in combination with
694 TruSeq RNA UD Indexes (Illumina, San Diego, US) according to Illumina's reference guide
695 documentation. Pooled cDNA libraries were sequenced paired end using an Illumina
696 NovaSeq 6000 SP Reagent Kit v1.5 (100 cycles Illumina, San Diego, US) on an Illumina
697 NovaSeq 6000 instrument. The run produced, on average, 14 million reads/sample. The
698 quality of the sequencing run was assessed using Illumina Sequencing Analysis Viewer
699 (Illumina version 2.4.7) and all base call files were demultiplexed and converted into
700 FASTQ files using Illumina bcl2fastq conversion software v2.20. The quality control
701 assessments, generation of libraries and sequencing were conducted by the Next
702 Generation Sequencing Platform, University of Bern.

703 The quality of the RNA-Seq data was assessed using fastQC v. 0.11.7⁶² and RSeQC
704 v. 4.0.0⁶³. The reads were mapped to the reference genome using HiSat2 v. 2.2.13⁶⁴. The
705 reference genome of strain LMB2 was prepared before the mapping step as follows: The
706 General Features Format (GFF) file obtained from the assembly was transformed to the
707 Gene Transfer Format (GTF) using AGAT v0.8.0⁶⁵ and subsequently transformed to
708 Browser Extensible Data (BED) format using BEDOPS v. 2.4.39⁶⁶. The HiSat2 index from
709 the reference FASTA file was created using the `hisat2-build` command. FeatureCounts v.
710 2.0.14⁶⁷ was used to count the number of reads overlapping with each gene as specified
711 in the genome annotation. The Bioconductor package (DESeq2 v1.32.0 5)⁶⁸ was used to
712 test for differential gene expression between the experimental groups. To annotate the
713 genes with Gene Ontology (GO) terms, the genes from the reference assembly were
714 translated to amino acid sequences using the `esl-translate` command in HMMER3 v.
715 3.3.2⁶⁹. Pfam domains were then searched using `hmmsearch`. GO terms were then mapped
716 to genes and their pfam domains using the pfam2go mapping file
717 (<http://current.geneontology.org/ontology/external2go/pfam2go>). GO term analysis
718 was performed using the R Bioconductor package TopGO⁷⁰.

719

720 Heterologous expression of candidate genes and protein purification

721 Plasmids for expression of *bxdA* (N-acyl homoserine lactonase family protein),
722 *bxdD* (aldehyde dehydrogenase family protein), *bxdG* (VOC family protein), and *bxdN*
723 (NAD(P)-dependent oxidoreductase) were ordered from Twist Bioscience. The DNA
724 sequences of the genes were used to generate codon-optimized nucleotide sequences for
725 expression in *E. coli*, applying the default settings. Sequences were introduced to
726 expression plasmid pET28a(+) with *Bam*HI and *Hind*III restriction sites (Twist
727 Bioscience HQ, San Francisco, US). All genes were amplified with Platinum Superfi
728 polymerase II (Thermo Fisher Scientific, Waltham, USA) according to the manufacturer's
729 instructions by using the following primers for *bxdA* forward
730 AAGTTCTGTTTCAGGGCCCGATGAGTGAGCGTAAAACGGAT and reverse
731 ATGGTCTAGAAAGCTTTACTAAGTTAACAAAATCCCGGC, for *bxdD* forward
732 AAGTTCTGTTTCAGGGCCCGATGGCCATAATGCGGTCCG and reverse
733 ATGGTCTAGAAAGCTTTATTAGGCCACCCAGACAGT, for *bxdG* forward
734 AAGTTCTGTTTCAGGGCCCGATGGCTGACGCTGTACG and reverse
735 ATGGTCTAGAAAGCTTTATTAGCGCTCCGGATGG, for *bxdN* forward
736 AAGTTCTGTTTCAGGGCCCGGTAACACTACAGTAGGCTTCTTAG and reverse
737 ATGGTCTAGAAAGCTTTATCAGGACTGGCGGCG and for expression vector pOPINF
738 forward TAATACGACTCACTATAGGG and reverse TAGCCAGAAGTCAGATGCT. Then
739 candidate genes were cloned in the expression vector pOPINF (N-terminal His tag)
740 digested with *Hind*III-HF and *Kpn*I-HF. Cloning was performed with In-Fusion (Takara
741 Bio, Shiga, Japan) according to manufacturer protocol and transformed in chemically
742 competent *E. coli* Top10 (NEB, Ipswich, US) and plated on LB plates (25 g/L Luria-Bertani
743 agar, Carl Roth, Karlsruhe, Germany) supplemented with carbenicillin 100 µg/mL
744 (Sigma-Aldrich, St. Louis, USA). Plasmids were isolated from recombinant colonies and
745 the identity of the inserted sequences was confirmed by Sanger sequencing. Next, the
746 constructs were used to transform chemically competent *E. coli* BL21 (DE3) (NEB,
747 Ipswich, US). Correct uptake of the plasmids was verified through colony PCR with vector
748 specific primers (see above). Positive colonies were inoculated in 5 mL LB with
749 carbenicillin 100 µg/mL and grown overnight at 37 °C, 220 rpm. 100 µL of the preculture
750 were inoculated in 100 mL 2xYT media with carbenicillin 100 µg/mL and incubated at
751 37°C, 220 rpm until they reached OD₆₀₀ = 0.5-0.6. At this point, cultures were incubated
752 for 15 min at 18 °C, 220 rpm and then induced with IPTG 0.5 mM and incubated at 18 °C,

753 220 rpm for 16 h. For purification, the cultures were harvested by centrifugation at 3'200
754 g, 10 min and resuspended in 10 mL of buffer A1 (50 mM Tris-HCl pH 8, 50 mM glycine,
755 500 mM sodium chloride, 20 mM imidazole, 5% v/v glycerol, pH 8) supplemented with
756 0.2 mg/mL Lysozyme and EDTA free protease inhibitor cocktail (cOmplete, Roche, Basel,
757 Switzerland) and incubated for 30 min on ice. Cells were disrupted by sonication using a
758 Sonics Vibra Cell at 40% amplitude, 3s ON, 2s OFF, and 2.5 min total time. The crude
759 lysates were centrifuged at 35'000 g for 30 min and the cleared lysates incubated with
760 200 μ L Ni-NTA agarose beads (Takara Bio, Shiga, Japan) for 1h at 4 °C. The beads were
761 then sedimented by centrifugation at 1'000 g for 1 min and washed 4 times with buffer
762 A1 before eluting the proteins with buffer B1 (50 mM Tris-HCl pH 8, 50 mM glycine, 500
763 mM Sodium Chloride, 500 mM imidazole, 5% v/v glycerol, pH 8). Dialysis and buffer
764 exchange were performed using buffer A4 (20 mM HEPES pH 7.5; 150 mM NaCl) in
765 centrifugal concentrators (Amicon Ultra – 10kDa, Merk Millipore Cork IRL). Proteins
766 were aliquoted in 50 μ L and stored at -20 °C. Protein concentration was determined
767 spectrophotometrically at 280 nm on a NanoPhotometer N60 (Implen, Munich, Germany)
768 considering the molecular weight and extinction coefficient. Protein purity and size were
769 checked trough SDS-Page on Novex WedgeWell 12% Tris-Glycine Gel (Invitrogen,
770 Waltham, US). The protein ladder used was Colour Protein Standard Broad Range (NEB,
771 Ipswich, US).

772

773 Enzyme assays and product analysis

774 All reactions were performed in a total volume of 100 μ L, in 25 mM potassium
775 phosphate buffer, pH=7.5 with 5 μ g protein. AMPO biosynthetic activity was tested by
776 supplementing the enzyme with 1 mM MBOA (30 mM stock in MeOH, Sigma-Aldrich, St.
777 Louis, USA). In addition, BxdD was supplemented with NADP⁺ and BxdN with NADP⁺ and
778 NADPH. Reactions were initiated by protein addition and incubated at 30 °C, 300 rpm for
779 2 h in the dark. Reactions were quenched by the addition of 100 μ L MeOH, incubated on
780 ice for 15 min and then centrifuged at 15'000 g for 15 min. The reactions were filtered
781 through 0.22 μ m PTFE syringe filters and then transferred to LC-MS glass vials.

782 LC-MS analysis was performed on a Dionex UltiMate 3000 UHPLC (Thermo Fisher
783 Scientific, Waltham, USA) equipped with Phenomenex Kinetex XB-C18 column (100 x 2.1
784 mm, 2.6 μ m, 100 Å, column temperature 40 °C) coupled to a Bruker Impact II Ultra-High-
785 Resolution Quadrupole-Time-of-Flight mass spectrometer (Bruker Daltonics) equipped

786 with EVOQ Elite electrospray ionization. Analytical conditions consisted of A: H₂O + 0.1
787 % FA and B: ACN, 0.6 mL/min flow with the following gradient: 0-1 min, 15 % B, 1-6 min,
788 15-35 % B, 6.1-7.5 min, 100 % B, 7.6-10 min, 15 % B. Mass spectrometry data were
789 acquired through ESI with a capillary voltage of 3500 V and end plate offset of 500 V,
790 nebulizer pressure of 2.5 bar with a drying gas flow of 11.0 L/min and a drying
791 temperature of 250 °C. The acquisition was performed at 12 Hz with a mass scan range
792 from 80 to 1'000 m/z. For tandem mass-spectrometry (Ms²) collision energy, the
793 stepping option model (from 20 to 50 eV) was used.

794

795 Statistical analysis

796 We used R version 4.0 (R core Team, 2016) for statistical analysis and visualization of the
797 data. All code used for statistical analysis and graphing is available from
798 https://github.com/PMI-Basel/Thoenen_et_al_BX_metabolisation. For the analysis of
799 bacterial colonisation, we used log transformed data. We checked for normality using
800 Shapiro-Wilk-test. Using t-test or ANOVA we tested for variance. Raw chromatogram data
801 were peak integrated using MassLynx 4.1 (Waters, Milford, US), using defined properties
802 for the reference compounds in the standards. We used the following packages for data
803 analysis and visualizations: Tidyverse⁷¹, Broom⁷², DECIPHER⁷³, DESeq2⁶⁸, emmeans⁷⁴,
804 ggthemes⁷⁵, pheatmap⁷⁶, multcomp⁷⁷, phyloseq⁷⁸, phytools⁷⁹, vegan⁸⁰ in combination
805 with custom functions.

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807

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