Chelator sensing and lipopeptide interplay mediates molecular interspecies interactions between soil bacilli and pseudomonads

Sofija Andric´[1*], Thibault Meyer[1,2*], Augustin Rigolet[1], Anthony Argüelles Arias[1], Sébastien Steels[1], Grégory Hoff[1#], Monica Höfte[3], René De Mot[4], Andrea McCann[5], Edwin De Pauw[5] and Marc Ongena[1]

[1]Microbial Processes and Interactions Laboratory, Terra Teaching and Research Center, Gembloux Agro-Bio Tech, University of Liège, Gembloux, Belgium

[2]UMR Ecologie Microbienne, F-69622, University of Lyon, Université Claude Bernard Lyon 1, CNRS, INRAE, VetAgro Sup, Villeurbanne, France

[3]Laboratory of Phytopathology, Department of Plants and Crops, Faculty of Bioscience engineering, Ghent University, Gent, Belgium

[4] Centre of Microbial and Plant Genetics, Faculty of Bioscience Engineering, University of Leuven, Heverlee, Belgium

[5] Mass Spectrometry Laboratory, , MolSys Research Unit, Department of Chemistry, University of Liège, Belgium

[#]Current address: Ecology and Biodiversity, Department of Biology, Utrecht University, Padualaan 8, 3584 CH, Utrecht, The Netherlands

* Equal contribution

Abstract

Some bacterial species are important members of the rhizosphere microbiome and confer protection to the host plant against pathogens. However, our knowledge is still limited about the multitrophic interactions determining the ecological fitness of these biocontrol bacteria in their highly competitive natural niche. In

this work, we have investigated the molecular mechanisms underlying interactions between *B. velezensis,* considered as model plant-associated and beneficial species in the *Bacillus* genus, and *Pseudomonas* as a rhizosphere-dwelling competitor. Our data show that *B. velezensis* boosts its arsenal of specialized antibacterials upon the perception of the secondary siderophore pyochelin produced by phylogenetically distinct pseudomonads and some other genera. We postulate that *B. velezensis* has developed some chelator sensing systems to learn about the identity of its surrounding competitors. Illustrating the multifaceted molecular response of *Bacillus*, surfactin is another crucial component of the secondary metabolome mobilized in interbacteria competition. Its accumulation not only enhances motility capability but, unexpectedly, this lipopeptide also acts as a chemical trap that reduce the toxicity of other lipopeptides released by *Pseudomonas* challengers. This favors the persistence of *Bacillus* populations upon competitive root colonization. Our work thus highlights new ecological roles for bacterial secondary metabolites acting as key drivers of social interactions.

INTRODUCTION

Soil is one of the richest ecosystems in terms of microbial diversity and abundance[1]. However, the scarcity of resources makes it one of the most privileged environment for competitive interspecies interactions [2,3]. A subset of the bulk soil microbes has evolved to dwell in the rhizosphere compartment surrounding roots due to continued nutrient-enriched exudation from the plant. Compared to bulk soil, microbial warfare in the rhizosphere is presumably even more intense as the habitat is spatially restricted and more densely populated[3]. Besides rivalry for nutrients (exploitative), interference competition is considered as a key factor driving microbial interactions and community assembly. It can involve signal interference or toxins deployed by contact-dependent delivery systems[4,5] but is mainly mediated at distance through the emission of various molecular weapons. The molecular basis of interference interactions and their phenotypic outcomes between diverse soil bacterial species have been amply investigated in the last decade[6,7].

Like some genera of the Proteobacteria, Actinobacteria, and Bacteroidetes taxa, bacilli belonging to the *B. subtilis* complex are ubiquitous members of the rhizosphere microbiome[2,8,9]. Among these species, *B. velezensis* has emerged as a model for plant-associated bacilli and displays strong potential as biocontrol agents reducing diseases caused by phytopathogens[10]. *B. velezensis* distinguish from other species of the *B. subtilis* group regarding their richness in biosynthetic gene clusters (BGCs, representing up to 13% of the

54 whole genome) responsible for the synthesis of bioactive secondary metabolites (BSMs) [11,12]. This
55 chemically-diverse secondary metabolome includes volatiles, terpenes, non-ribosomal (NR) dipeptides,
56 cyclic lipopeptides (CLPs) and polyketides (PKs), but also ribosomally synthesized lantibiotics and larger
57 bacteriocins (RiPP) [13,14]. BSMs are involved in the biocontrol activity via direct inhibition of infectious
58 microbes and/or via stimulation of the plant immune system[15,16]. From an ecological viewpoint, BSMs also
59 contribute to competitiveness in the rhizosphere niche thanks to multiple and complementary functions as
60 drivers of developmental traits, as antimicrobials, or as signals initiating cross-talk with the host plant[17–19 20–
61 26].

62 Mostly guided by practical concerns for use as biocontrol agents, research on BSMs has mainly focused on
63 the characterization of their biological activities. However, the impact of environmental factors that may
64 modulate their expression under natural conditions has been much poorly investigated. It includes
65 interactions with other organisms sharing the niche. Some recent reports illustrate how soil bacilli may
66 adapt their behavior upon sensing bacterial competitors but almost exclusively focusing on developmental
67 traits (sporulation, biofilm formation, or motility)[6]. Unlike other genera such as *Streptomyces*, it remains
68 largely unknown to what extent bacilli in general and *B. velezensis* in particular, may modulate the
69 expression of their secondary metabolome upon interaction with other bacteria[2,8,9,27]. Through this work,
70 we wanted to investigate the molecular outcomes of interspecies interactions that *B. velezensis* may
71 engage. We selected *Pseudomonas* as challenger considering that species of this genus are also highly
72 competitive and commonly encountered in rhizosphere microbiomes[8]. We performed experiments under
73 nutritional conditions mimicking the oligotrophic rhizosphere environment and used contact-independent
74 settings for pairwise interaction which also obviously best reflect the real situation in the soil. Our data
75 revealed that the two bacteria initiate a multifaceted interactions mostly mediated by the non-ribosomally
76 synthesized components of their secondary metabolome. We pointed out unsuspected roles for some of
77 these BSMs in the interaction context. Beyond its role as a metal chelator, the *Pseudomonas* secondary
78 siderophore pyochelin acts as a signal triggering dual production of PKs and RiPP in *Bacillus* while specific
79 lipopeptides modulate the inhibitory interaction between the two species. This resulted in marked
80 phenotypic changes in *B. velezensis* such as higher antibacterial potential, enhanced motility and protective
81 effect via chemical trapping. We also illustrate the relevance of these outcomes in the context of
82 competitive root colonization.

83

84 **RESULTS**

85 ***B. velezensis* modulates its secondary metabolome and boosts antibacterial activity upon sensing**
86 ***Pseudomonas* metabolites**

87 In this study, we used *B. velezensis* strain GA1 as BSM-rich and genetically amenable isolate representative
88 of the species. Genome mining with AntiSMASH 5.0 [28] confirmed the presence of all gene clusters necessary
89 for the biosynthesis of diverse known BSMs typically formed by this bacterium (Supplementary Table 1).
90 Based on the exact mass and absence of the corresponding peaks in deletion mutants, most of the
91 predicted non-ribosomal (NR) secondary metabolites were identified in cell-free crude supernatants via
92 optimized UPLC-MS (Supplementary Fig. 1). It includes the whole set of cyclic lipopeptides (CLPs of the
93 surfactin, fengycin and iturin families) and polyketides (the PKs difficidin, macrolactin and bacillaene) with
94 their multiple co-produced structural variants, as well as the siderophore bacillibactin. We verified that all
95 these compounds are readily formed in the so-called exudate-mimicking (EM) medium reflecting the
96 specific content in major carbon sources typically released by roots of Solanaceae (such as tomato) plants[29].
97 In addition to these NR products, genes encoding RiPPs such as amylocyclicin and amylolysin are also
98 present in GA1 but these compounds could not be reliably detected in culture broths. The NR dipeptide
99 bacilysin was also predicted but not detected. We selected as the main interaction partner the plant-
100 associated *Pseudomonas* sp. strain CMR12a based on its biocontrol potential and its production of multiple
101 secondary metabolites [30–34]. Genome mining confirmed the potential of CMR12a to synthesize a range of
102 BSMs, including antimicrobial phenazines, the siderophores pyoverdin (structure confirmation in
103 Supplementary Fig. 2) and (enantio-)pyochelin as well as two structurally distinct CLPs, sessilin and orfamide
104 (Supplementary Table 1). In contrast to *Bacillus*, the capacity to co-produce two different CLPs is a quite rare
105 trait for non-phytopathogenic pseudomonads and represented an additional criterion for selecting strain
106 CMR12a for this study[30,34–36]. In the case of CMR12a, all these compounds were detected in EM culture
107 broth but most of them are more efficiently produced upon growth in Casamino Acids medium (CAA)
108 commonly used for *Pseudomonas* cultivation (Supplementary Fig. 1).

109 Our prime objective was to evaluate the intrinsic potential of *B. velezensis* to react to the perception of
110 *Pseudomonas* metabolites in an experimental setting avoiding interferences due to diffusion constraints in a
111 semi-solid matrix or due to the formation of impermeable biofilm structures. The first assays were
112 performed by growing GA1 in agitated liquid EM medium supplemented or not with (sterile) BSM-
113 containing spent medium of CAA-grown CMR12a (CFS, cell-free supernatant). At low dose (2% (v/v)), the

114 addition of this CFS extract led to a marked increase in the production of some GA1 NR metabolites.
115 Significantly higher amounts were measured for surfactin, bacillaene and its dehydrated variant, difficidin
116 and its oxidized form, and bacillibactin (Fig. 1a, b) but not for fengycin, iturin nor macrolactin (Fig 1a).

117 Such boost in BSM synthesis triggered by *Pseudomonas* CFS was associated with an increase in the
118 antibacterial activity of the corresponding extracts when tested for growth inhibition of *Xanthomonas*
119 *campestris* and *Clavibacter michiganensis* used respectively as representative of Gram-negative and Gram-
120 positive plant pathogenic bacteria of agronomical importance[37] (Fig. 1c). Since most of the BSMs are not
121 commercially available and our attempt to purify PKs failed due to chemical instability, we could not use
122 individual compounds for their specific involvement in bacterial inhibition. As an alternative, we generated
123 and tested a range of GA1 knockout mutants including the Δ*sfp* derivative specifically repressed in 4'-
124 phosphopantetheinyl transferase essential for the proper functioning of the PK and NRP biosynthesis
125 machinery. Full loss of anti-*Xanthomonas* activity in Δ*sfp* extracts indicated a key role for NR BSMs and ruled
126 out the possible involvement of other chemicals known for their antibacterial activity such as bacilysin or
127 RiPPs (Fig. 1d). Loss of function of mutants specifically repressed in the synthesis of individual compounds
128 pointed out the key role of (oxy)difficidin and to a lower extent of (dihydro)bacillaene in *Xanthomonas*
129 inhibition (Fig. 1d). These two polyketides are also responsible for GA1 inhibitory activity toward other
130 important bacterial phytopathogens such as *Pectobacterium carotovorum*, *Agrobacterium tumefaciens* and
131 *Rhodococcus fasciens* but are not involved in the inhibition of plant pathogenic *Pseudomonas* species for
132 which bacilysin may be the active metabolite (Supplementary Fig. 3). However, as illustrated below in the
133 next sections, *B. velezensis* does not display significant toxicity against CMR12a and other non-pathogenic
134 soil *Pseudomonas* isolates tested here. Stimulation of PKs synthesis upon sensing CMR12a is not specific to
135 GA1 and was also observed in other *B. velezensis* strains such as S499, FZB42 and QST713 with well-known
136 biocontrol potential[38–40] (Supplementary Fig. 4).

137 In contrast to *Xanthomonas*, the enhanced antibiotic activity against *Clavibacter* is not mediated by NR
138 products as shown by the fully conserved activity in the Δ*sfp* mutant (Fig. 1d). We, therefore, suspected
139 from genomic data that some RiPPs such as amylocyclicin could be involved in inhibition. This hypothesis
140 was supported by the 80% reduction in antibiotic potential observed for the Δ*acnA* mutant knocked out for
141 the corresponding biosynthesis gene (Fig. 1d). Besides, RT-qPCR data revealed a highly induced expression
142 of the *acnA* in GA1 cells upon supplementation with CMR12a CFS (Fig. 1e) but we were not able to provide

143 evidence for higher accumulation of the mature peptide in the medium. Enhanced expression of the *acnA*
144 gene in presence of *Pseudomonas* products was also observed for strain S499 (Supplementary Fig. 5).

145 **Pyochelin acts as a signal sensed by *Bacillus* to stimulate polyketide production**

146 We next wanted to identify the signaling molecules secreted by *Pseudomonas* that are sensed by *Bacillus*
147 cells and lead to improved BSMs production. For that purpose, we used dihydrobacillaene (2H-bae) as an
148 indicator of the *Bacillus* response because it represents the most consistent and highly boosted polyketide
149 species. We first compared the triggering potential of CFS obtained from knockout mutants of CMR12a
150 specifically lacking the different metabolites identified (Supplementary Fig. 1). Only extracts from mutants
151 impaired in the production of siderophores and more specifically pyochelin were significantly affected in
152 PKs-inducing potential (Fig. 2a). Possible involvement of this compound was supported by the drastic
153 reduction in the activity of CFS prepared from CMR12a culture in CAA medium supplemented with Fe^{3+}
154 where siderophore expression is repressed (Supplementary Fig. 6). We also performed bioactivity-guided
155 fractionation and data showed that only extracts containing pyoverdine and/or pyochelin displayed
156 consistent PKs-triggering activity (Supplementary Fig. 7). HPLC-purified compounds were also tested
157 independently at a concentration similar to the one used in CFS CAA extract and it revealed a much higher
158 PK-triggering activity for pyochelin compared to the main pyoverdine isoform (Fig. 2b). Dose-dependent
159 assays further indicated that supplementation with pyoverdine, as strong chelator[41], caused some iron
160 limitation in the medium which is sensed by GA1. It is reflected first by the marked increase in production of
161 the siderophore bacillibactin in GA1 wild-type (Fig. 2c) and by the reduced growth of a Δ*dhbC* mutant
162 repressed in bacillibactin synthesis upon pyoverdin addition (Fig. 2d, Supplementary Fig. 8 for statistics).
163 This last result indicates that pyoverdine in its ferric form cannot be taken up by GA1 despite the presence
164 of several transporters for exogenous siderophores in *B. velezensis* similar to those identified in *B.*
165 *subtilis*[42,43] based on genome comparison (Supplementary Table 2). We, therefore, assumed that iron stress
166 mediated by pyoverdine only induces a rather limited boost in PKs production. We validate that such
167 response is not due to iron starvation by supplementing GA1 culture with increasing doses of the 2,2'-
168 dipyridyl chemical chelator that cannot be taken up by Bacillus cells (Fig. 2b). By contrast, the addition of
169 pyochelin with a much lower affinity for iron does not activate bacillibactin synthesis (Fig. 2c) and does not
170 affect Δ*dhbC* growth at the concentrations used (Fig. 2d). We concluded that the activity of this compound
171 referred to as secondary siderophore is not related to iron-stress. If internalized, pyochelin can cause
172 oxidative stress and damage in other bacteria as reported for *E. coli* [44,45] . However, the absence of toxicity

173 toward GA1 indicates that pyochelin is not taken up by *Bacillus* cells and thus clearly acts as a signal
174 molecule perceived at the cell surface. PKS boost also occurred upon addition of CFS obtained from other
175 *Pseudomonas* isolates such as *P. protegens* Pf-5[46] and *P. aeruginosa* PAO1 [47] producing pyochelin-type
176 siderophores. However, PKS stimulation was also observed in response to *P. tolaasi* CH36[48] and
177 *Pseudomonas* sp. A214[49] that do not form pyochelin indicating that some other unidentified BSMs may act
178 as triggers in other strains (Supplementary Figure 9).

179 **Enhanced motility as distance-dependent and surfactin-mediated response of *Bacillus***

180 Surfactin production is also stimulated by CMR12a CFS and by pure pyochelin (Fig. 1a and Supplementary
181 Fig. 10). Based on the mutant analysis, this multifunctional CLP does not contribute to the antibacterial
182 potential of *B. velezensis* (Fig 1d and Supplementary Fig. 3) but is notably involved in developmental
183 processes of multicellular communities such as biofilm formation and motility[50]. We, therefore, wanted to
184 test a possible impact of *Pseudomonas* on the motile phenotype of *B. velezensis* upon co-cultivation on
185 plates. We observed distance-dependent enhanced motility on gelified medium containing high agar
186 concentrations (1.5% m/v) which phenotypically resembles the sliding-type of motility illustrated by typical
187 "van Gogh bundles"[50] (Fig. 3a). This migration pattern is flagellum independent but depends on multiple
188 factors including the synthesis of surfactin which reduces friction at the cell-substrate interface[50]. We thus
189 suspected such enhanced motility to derive from an increased formation of the lipopeptide. This was
190 supported by the almost full loss of migration of the Δ*srfaA* mutant in these interaction conditions (Fig. 3b).
191 Moreover, spatial mapping via Maldi-FT-ICR MS imaging confirmed a higher accumulation of surfactin ions
192 in the interaction zone and around the *Bacillus* colony when it is growing at a short or intermediate distance
193 from the *Pseudomonas* challenger compared to the largest distance where the motile phenotype is much
194 less visible (Fig. 3c). These data indicate that *Bacillus* cells in the microcolony perceive some soluble signal
195 diffusing from the *Pseudomonas* colony to a limited distance.

196 **Interplay between CLPs drives antagonistic interactions**

197 Furthermore, we observed that, besides modulating secondary metabolite synthesis, confrontation with
198 *Pseudomonas* may also lead to some antagonistic outcomes. GA1 growth as planktonic cells is slightly
199 inhibited upon supplementation of the medium with 2% v/v CMR12a CFS but this inhibition is much marked
200 at higher doses (Supplementary Fig. 11). To identify the *Pseudomonas* compound retaining such antibiotic
201 activity, we tested the effect of CFS from various CMR12a mutants impaired in the synthesis of lipopeptides

202 and/or phenazines. Even if some contribution of other compounds cannot be ruled out, it revealed that the
203 CLP sessilin is mainly responsible for toxic activity toward GA1 grown in liquid cultures but also when the
204 two bacteria are grown at close proximity on gelified EM medium (Fig 4a). Nevertheless, we observed that
205 the sessilin-mediated inhibitory effect is markedly reduced by delaying CFS supplementation at 6h of culture
206 instead of adding at the beginning of incubation (Fig 4b). This suggested that some early secreted *Bacillus*
207 compounds may counteract the toxic effect of sessilin. We hypothesized that surfactin can play this role as
208 it is the first detectable BSM to accumulate in significant amounts in the medium at early growth. We tested
209 the surfactin-deficient mutant in the same conditions and observed that its growth is still strongly affected
210 indicating that no other GA1 compound may be involved in toxicity alleviation. Chemical complementation
211 with purified surfactin allowed to restore growth to a large extent, providing further evidence for a
212 protective role of the surfactin lipopeptide (Fig. 4b). Such sessilin-dependent inhibition also occurred when
213 bacteria were confronted on solid CAA medium favoring *Pseudomonas* BSM production (Fig. 4c-I). In these
214 conditions, the formation of a white precipitate in the interaction zone was observed with CMR12a wild
215 type but not when GA1 was confronted with the Δ*sesA* mutant (Fig. 4c). UPLC-MS analysis of ethanol
216 extracts from this white-line area confirmed the presence of sessilin ions but also revealed an accumulation
217 of surfactin from GA1 in the confrontation zone (Fig. 4d). The involvement of surfactin in precipitate
218 formation was confirmed by the absence of this white-line upon testing the Δ*srfaA* mutant of GA1 (Fig. 4c).
219 The loss of surfactin production and white-line formation was associated with a higher sensitivity of the
220 *Bacillus* colony to the toxin secreted by *Pseudomonas*. Altogether, these data indicate that surfactin acts as
221 a chemical trap and inactivates sessilin via co-aggregation into insoluble complexes.

222 A similar CLP-dependent antagonistic interaction and white-line formation were observed upon co-
223 cultivation of GA1 with *P. tolaasii* strain CH36 producing tolaasin (Fig. 4c), a CLP structurally very similar to
224 sessilin (only differing by a single amino acid residue, Supplementary Fig. 12). However, this chemical
225 aggregation is quite specific regarding the type of CLP involved since it was not visible upon the interaction
226 of GA1 with other *Pseudomonas* strains forming different CLP structural groups that are not toxic for
227 *Bacillus* (Fig. 4c, see Supplementary Fig. 12 and 13 for identification and structures). Sessilin/tolaasin-
228 dependent toxicity and white-line formation were also observed when other surfactin-producing *B.*
229 *velezensis* isolates are confronted with CMR12a and CH36 (Supplementary Figs. 14 and 15, respectively).
230 Although the chemical basis and the steochiometry of such molecular interaction remain to be determined,
231 it probably follows the same rules as observed for the association between sessilin/tolaasin and other

232 endogenous *Pseudomonas* CLPs such as WLIP or orfamide[35] or between CLPs and other unknown
233 metabolites[51,52].

234 **BSM-mediated interactions drive competitive root colonization**

235 Our *in vitro* data thus point out how *B. velezensis* may modulate its secondary metabolome when
236 confronted to *Pseudomonas*. To appreciate the relevance of our findings in a more realistic context, we next
237 evaluated whether such BSM interplay may also occurred upon root co-colonization of tomato plantlets and
238 its possible impact on *Bacillus* fitness. When inoculated independently, CMR12a colonized roots more
239 efficiently than GA1 within the first 5 days, most probably due to a higher intrinsic growth rate[53]. Upon co-
240 inoculation, CMR12a colonization rate was not affected but GA1 populations were reduced compared to
241 mono-inoculated plantlets (Fig. 5a). UPLC-MS analysis of methanolic extracts prepared from co-bacterized
242 roots (and surrounding medium) revealed substantial amounts of pyochelin (Supplementary Fig. 16)
243 indicating that the molecule is readily formed under these conditions and could therefore also act as a
244 signal *in planta.* Probably due to the low populations of GA1, we could not detect Bacillus PKs and RiPPs in
245 these extracts. However, a significantly enhanced expression of gene clusters responsible for the synthesis
246 of bacillaene, difficidin and amylocyclicin was observed in GA1 cells confronted to *Pseudomonas* compared
247 to single inoculation (Fig 5b). It indicated that the metabolite response observed in GA1 cell cultures in EM
248 medium may also occur upon competitive colonization where the bacteria feed exclusively on root
249 exudates.

250 Lipopeptides involved in the interference interaction described in the previous section are also readily
251 formed upon single and dual root colonization (Fig. 5d). We hypothesized that the inhibitory effect of
252 sessilin may impact the colonization potential of GA1 in presence of CMR12a which was confirmed by the
253 increase in GA1 populations co-inoculated with the Δ*sesA* mutant (Fig 5c). Moreover, colonization by the
254 Δ*srfaA* mutant is more impacted compared to WT when co-cultivated with CMR12a and a significant gain in
255 root establishment is recovered upon co-colonization with the Δ*sesA* mutant (Fig. 5d). The sessilin-surfactin
256 interplay thus also occurs *in planta.* Sessilin would confer a competitive advantage to CMR12a during
257 colonization by inhibiting GA1 development but efficient surfactin production on roots, however, may
258 provide some protection to *Bacillus* cells.

259

260 **DISCUSSION**

261 Our current knowledge on the molecular interaction between soil bacilli and pseudomonads is rather
262 limited, except the fact that the Type VI secretion system and the antibiotic 2,4-diacetylphloroglucinol may
263 impact key developmental traits in *B. subtilis* such as biofilm formation and/or sporulation rate[54,55]. Here we
264 show that the model species *B. velezensis* can mobilize a substantial part of its secondary metabolome to
265 face *Pseudomonas* competitors. Our data provide the first evidence for the enhanced synthesis of broad-
266 spectrum PKs (bacillaene and difficidin-type acting independently or in synergy) together with stimulation of
267 bacteriocin production in *Bacillus* upon perception of other bacteria both in contact-independent *in vitro*
268 settings and upon competitive root colonization. This correlates with an enhanced antibacterial potential
269 which is of interest for biocontrol but which can also be considered as a defensive strategy to persist in its
270 natural competitive niche. *B. velezensis* calls its antibiotic arsenal upon sensing *Pseudomonas* but we also
271 show that the bacterium also recruits its multifunctional surfactin CLP to improve multicellular mobility. It
272 may represent an escape mechanism enabling *Bacillus* cells to relocate after detecting harmful challengers.
273 Improved motility of *B. subtilis* has been already described upon sensing competitors such as *Streptomyces*
274 *venezuelae*[6,56] but no relationship was established with enhanced production of BSMs potentially involved in
275 the process. We also highlight a new role for surfactin acting as a chemical shield to counteract the toxicity
276 of exogenous CLPs. Intraspecies CLP co-precipitation has been reported[30] but our results make sense of this
277 phenomenon in the context of interference interaction between two different genera. In planta, this new
278 function of surfactin contributes to *Bacillus* competitiveness for root invasion. This has to be added to other
279 previously reported implications of surfactin in *B. subtilis* interspecies interactions such as interfering with
280 the growth of closely related species in synergy with cannibalism toxins [57], inhibiting the development of
281 *Streptomyces* aerial hyphae [58] or participating in the expansion and motility of the interacting species [59].

282 Furthermore, we exemplify that PKs stimulation in *B. velezensis* is mainly mediated by pyochelin (even if
283 other secreted products can also play some role) produced by *Pseudomonas* as secondary siderophore. Our
284 data show that *Bacillus* perceives pyochelin in a way independent of iron stress and piracy indicating that
285 beyond its iron-scavenging function, this siderophore may thus also act as infochemical in interspecies
286 cross-talk. In the pairwise system used here, pyochelin signaling superimposes the possible effect of iron
287 limitation in the external medium which may also result in enhanced production of antibacterial
288 metabolites by *Bacillus* as occasionally reported[60]. That said, due to the limitation in bioavailable iron,
289 almost all known rhizobacterial species have adapted to produce their siderophores to compete for this

290 essential element. Siderophore production is thus probably the most widely conserved trait among soil
291 bacteria. It also means that upon recognition of exogenous siderophores, any isolate may somehow identify
292 surrounding competitors. However, some of these siderophores are structurally very variable and almost
293 strain-specific (such as pyoverdins from fluorescent pseudomonads) while some others are much more
294 widely distributed across species and even genera (enterobactin-like, citrate). In both cases, their
295 recognition would not provide proper information about the producer because too specific or too general
296 respectively. Interestingly, the synthesis of pyochelin and its structurally very close enantio form is
297 conserved in several but not all *Pseudomonas* sp. as well as in a limited number of species belonging to
298 other genera such as *Burkholderia* and *Streptomyces*. We,therefore, hypothesize that *Bacillus* may have
299 evolved some chelator-sensing systems targeting siderophores that are conserved enough to be detected
300 but restricted to specific microbial phylogenetic groups. With this mechanism, soil bacilli would rely on
301 siderophores as public goods to accurately identify competitors and respond in an appropriate way like
302 remodeling its BSM secretome. This novel concept of chelator sensing represents a new facet of the
303 siderophore-mediated social interactions. Whether it is used for other secondary siderophores than
304 pyochelin and if yes whether this adaptative trait can be generalized to other soil-dwelling species remains
305 to be determined.

306 In summary, we provide the first evidence that the model *B. velezensis* species can mobilize a substantial
307 part of his secondary metabolome upon a perception of *Pseudomonas* competitors. Beyond the notion of
308 specialized metabolites, we point out unsuspected additional functions for some of these small molecules in
309 the context of interactions between bacterial clades that are important members of the plant-associated
310 microbiome. This metabolite response is viewed as a way to mount a multi-faceted defensive strategy to
311 gain fitness and persistence in its natural competitive niche.

312

313 **MATERIAL AND METHODS**

314 **Bacterial strains and growth conditions**

315 Strains and plasmids used in this study are listed in Supplementary Table S1. *B. velezensis* strains were
316 grown on recomposed exudate (EM) solid medium [29] or with shaking (160 rpm), at 30°C in EM liquid
317 medium. Deletion mutants of *B. velezensis* were selected on chloramphenicol (5 µg/ml) and/or phleomycin

318 (4 µg/ml) on Lysogeny broth (LB) (10 g l^{-1} NaCl, 5 g l^{-1} yeast extract and 10 g l^{-1} tryptone). *Pseudomonas* sp.
319 strains were grown on King B (20 g l^{-1} of bacteriological peptone, 10 g l^{-1} of glycerol and 1.5 g l^{-1} of K_2HPO_4,
320 1.5 g l^{-1} of $MgSO_4.7H_2O$, pH=7) and casamino acid (CAA) solid and liquid medium (10 g l^{-1} Casamino acid, 0.3
321 g l^{-1} K_2HPO_4, 0.5 g l^{-1} $MgSO_4$ and pH=7) with shaking (120 rpm), at 30°C. The phytopathogenic bacterial
322 strains were grown on LB and EM solid and liquid media and with shaking (150 rpm), at 30°C.

323 **Construction of deletion mutants of *B. velezensis* GA1**

324 All deletion mutants were constructed by marker replacement. Briefly, 1 kb of the upstream region of the
325 targeted gene, an antibiotic marker (chloramphenicol or phleomycine cassette) and downstream region of
326 the targeted gene was PCR amplified with specific primers (listed in Supplementary Table S2). The three
327 DNA fragments were linked by overlap PCR to obtain a DNA fragment containing the antibiotic marker
328 flanked by the two homologous recombination regions. This latter fragment was introduced into *B.*
329 *velezensis* GA1 by natural competence induced by nitrogen limitation[61]. Homologous recombination event
330 was selected by chloramphenicol resistance (or phleomycin resistance for double mutants) on LB medium.
331 All gene deletions were confirmed by PCR analysis with the corresponding UpF and DwR specific primers
332 and by the loss of the corresponding BSMs production.

333 Transformation of the *B. velezensis* GA1 strain was performed following the protocol described by Jarmer et
334 al., [61] with certain modifications. One fresh GA1 colony was inoculated into LB liquid medium at 37°C (160
335 rpm) until reaching an OD_{600nm} of 1.0. Afterwards, cells were washed one time with peptone water and one
336 time with a modified Spizizen minimal salt medium called MMG liquid medium (19 g l^{-1} K_2HPO_4 anhydrous; 6
337 g l^{-1} KH_2PO_4; 1 g l^{-1} Na_3 citrate anhydrous; 0.2 g l^{-1} $MgSO_4$ $7H_2O$; 2 g l^{-1} Na_2SO_4; 50 µM $FeCl_3$ (sterilized by
338 filtration at 0.22 µm); 2µM $MnSO_4$; 8 g l^{-1} glucose; 2 g l^{-1} L-glutamic acid; pH 7.0), 1µg of DNA recombinant
339 fragment was added to the GA1 cells suspension adjusted to an OD_{600nm} of 0.01 into MMG liquid medium.
340 One day after incubation at 37°C with shaking at 165 rpm, bacteria were spread on LB plates supplemented
341 with the appropriated antibiotic to select positive colonies.

342 **Construction of deletion mutants of *Pseudomonas* sp. CMR12a**

343 Pyochelin and pyoverdine mutants of *Pseudomonas* sp. CMR12a were constructed using the I-SceI system
344 and the pEMG suicid vector [62,63]. Briefly, the upstream and downstream region flanking the *pchA*
345 (*C4K39_5481*) or the *pvdI* (*C4K39_6027*) genes were PCR amplified (with primers listed in the

346 Supplementary Table S2), linked via overlap PCR and inserted into the pEMG vector. The resulting plasmid
347 (listed in Supplementary Table S1) was integrated by conjugation into the *Pseudomonas* sp. CMR12a
348 chromosome via homologous recombination. Kanamycin (25µg/mL) resistant cells were selected on King B
349 agar plates and transformed by electroporation with the pSW-2 plasmid (harboring I-SceI system).
350 Gentamycin (20µg/ml) resistant colonies on agar plates were transferred to King B medium with and
351 without kanamycine to verify the loss of the antibiotic (kanamycin) resistance. *Pseudomonas* mutants were
352 identified by PCR with the corresponding UpF and DwR specific primers and via the loss of pyochelin or/and
353 pyoverdin production (see section Secondary metabolites analysis).

354 ***Pseudomonas* sp. cell-free supernatant**

355 *Pseudomonas sp.* strains were grown overnight on LB solid medium, at 30°C. Cells were adjusted to OD_{600nm}
356 of 0.05 by resuspension with 100 ml of CAA and when appropriate supplemented with 20 µg/l of $FeCl_3.6H_2O$
357 (iron supplementation). Cultures were shaken at 120 rpm at 30°C for 48 h and then centrifuged at 5000 rpm
358 at room temperature (22°C) with a Universal 320 centrifuge (Hettich) for 20 min. The supernatant was
359 sterilized with a 0.22 µm pore size Sterile PES Syringe (ROCC S.A.) filters and stored at -20°C until needed.

360 **Dual interaction and time course experiment**

361 *B. velezensis* strains were grown overnight on LB solid medium, at 30°C. Cells were resuspended in 2 ml of
362 $EM_{1/2}$ liquid medium to a final OD_{600nm} of 0.1 in which 1, 2, 4, or 8% v/v (depending on the experiment) of
363 *Pseudomonas* CFS were added while the control remained without the addition of CFS. *B. velezensis* liquid
364 cultures were shaken in an incubator at 300 rpm at 30°C for 24 h. During the culture time course
365 experiment, at 8, 24, and 36 h, the OD_{600nm} was measured with VWR, V-1200 Spectrophotometer to
366 correlate it with *Pseudomonas* CFS effect on *Bacillus* growth and BSMs production. In addition, 2 ml of the
367 (co-)culture supernatants were sampled, centrifugated at 5000 rpm at room temperature (approx. 22°C) for
368 10 min to extract supernatants and collect the cells. Cell-free (co-)culture supernatants were obtained by
369 sterilization with a 0.22µm pore size Sterile CA Syringe filters and thus used for analytical analysis of
370 secondary metabolites (as mentioned above) and antibacterial assays. For some experiments using 2H-bae
371 as a marker, the CFS obtained from the double mutant sessilin and orfamide (Δ*sesA-ofaBC*) was used as a
372 control instead of CFS from CMR12a wild-type because it yielded a higher response and lower inhibition
373 interferences by CLPs. The remaining cells after supernatant collection were stored at -80°C to avoid RNA
374 degradation, until performing RT-qPCR analysis.

375 **Antimicrobial activity assays**

376 Antibacterial activity of the *B. velezensis* supernatant generated after dual interaction with *Pseudomonas*
377 CFS was tested against *X. campestris* pv. *campestris* and *C. michiganensis* subsp. *michiganensis.* The activity
378 of co-culture supernatants was quantified in microtiter plates (96-well) filled with 250 µl of LB liquid
379 medium, inoculated at OD_{600nm}= 0.1 with *X. campestris* pv. *campestris* and *C. michiganensis* subsp.
380 *michiganensis* and supplemented with 2% or 6% v/v of *B. velezensis* supernatants, respectively. The activity
381 of (co-)culture supernatants was estimated by measuring the pathogen OD_{600nm} every 30min during 24h
382 with a Spectramax® (Molecular Devices, Wokingham, UK), continuously shaken, at 30°C. For estimating the
383 activity of co-culture supernatants on solid medium, 5 µl supernatantwas applied to a sterile paper disk (5
384 mm diameter). After drying, diskswere placed on LBAsquare plates previously inoculated with a confluent
385 layer of *X. campestris* pv. *campestris* or *C. michiganensis* subsp. *michiganensis*. LB liquid medium was used
386 as negative control. Plates were incubated at 25°C for 48 h. Three repetitions were done and the inhibition
387 zones from the edge of the paper discs to the edge of the zone were measured.

388 **RNA isolation and RT-qPCR.**

389 RNA extraction and Dnase treatment were carried out using the NucleoSpin RNA Kit (Macherey Nagel,
390 Germany), following the Gram + manufacturer's protocol. RNA quality and quantity were performed with
391 Thermo scientific NanoDrop 2000 UV-vis Spectrophotometer. The gyrase gene was chosen as a
392 housekeeping gene to normalize mRNA levels between different samples. The target gene in this study was
393 *acnA*. Primer 3 program available online was used for primer design and primers were synthesized by
394 Eurogentec. The primer efficiency was evaluated and primer pairs showing an efficiency between 90 and
395 110% in qPCR analysis were selected. Reverse transcriptase and RT-qPCR reactions were conducted using
396 the Luna® Universal One-Step RT-qPCR Kit (New England Biolabs, Ipswich, MA, United States). The reaction
397 was performed with 50 ng of total RNA in a total volume of 20 µL: 10 µL of luna universal reaction mix, 0.8
398 µL of each primer (10 µM), 5 µL of cDNA (50ng), 1µL of RT Enzyme MIX, 2.4 µl of Nuclease-free water. The
399 thermal cycling program applied on the ABI StepOne was: 55°c for 10 min, 95C °C for 1 min, 40 cycles of 95
400 °C for 10 s and 60 °C for 1 min, followed by a melting curve analysis performed using the default program of
401 the ABI StepOne qPCR machine (Applied Biosystems). The real-time PCR amplification was run on the ABI
402 step-one qPCR instrument (Applied Biosystems) with software version 2.3. Analysis was conducted on gene
403 expression level and has been quantified using the $2^{-\Delta\Delta CT}$ method[64].

404 **Secondary metabolite analysis**

405 For detection of BSMs, *B. velezensis* and *Pseudomonas* sp. were cultured in EM and CAA as described above.
406 After an incubation period of 24 h for *B. velezensis*, if not differentially indicated, and 48 h for *Pseudomonas*
407 sp., supernatants of the bacteria were collected and analyzed by UPLC MS/MS and UPLC qTOF MS/MS.
408 Analysis of *Bacillus* metabolites was performed by UPLC MS/MS using an Acquity UPLC® BEH C 18 column
409 (L=50mm, D= 2.1mm, Particles diameter= 1.7µm) (Waters, milford, MA, USA). The volume injected was 10
410 µl. Mass spectrometer (Acquity UPLC ® Class H SQD (Water, milford, MA, USA)) was set in negative mode
411 ESI- and positive more ES+ (cone voltage: 60V) with a mass range from m/z 300.00 to 2048.00. Analysis of
412 *Pseudomonas* metabolites was performed by Q-TOF (tandem mass spectrometry, quadrupole and Time of
413 flight detector combined) (Agilent, Waldbronn, Germany) and a diode array detector (DAD) 190 to 601 nm
414 (steps: 1nm). The volume injected was 10µl. The mass analysis was performed in positive mode ES+ (Dual
415 AJS ESI) (Vcap= 3500V, Nozzle Voltage= 1000V), with a mass range from m/z 180 to 1700.

416 **Bioguided fractionation**

417 *Pseudomonas* CFS were concentrated with a C18 cartridge 'Chromafix, small' (Macherey-Nagel, Düren,
418 Germany). The column was conditioned with 10 ml of MeOH followed by 10ml of milliQ water. Then, 20 ml
419 of supernatant flowed through the column. The metabolites were eluted with 1 ml of a solution of
420 increasing acetonitrile/water ratio from 5:95 to 100:0 (v/v). The triggering effect of these fractions on
421 *Bacillus* 2H-bae production was tested in 48 wells microplate containing 1 ml of EM medium inoculated
422 with *B. velezensis* GA1 (OD_{600nm} = 0.1) and 4% v/v of aforementioned *Pseudomonas* fractions, growing for
423 24h, with shaking at 300 rpm and 30°C. Afterward, the production of 2H-bae was quantified compared to
424 controls and crude supernatant.

425 **Purification of pyochelin and pyoverdine**

426 Pyoverdine and pyochelin were purified in two steps. Firstly, *Pseudomonas* CFS were concentrated with a
427 C18 cartridge (as indicated in section Bioguided fractionation) and eluted with 2 times 2ml of a solution of
428 water and ACN (15 and 30% of ACN (v/v)). Secondly, the fractions were injected on HPLC for purification
429 performed on an Eclipse+ C18 column (L=150mm, D= 3.0mm, Particles diameter 5µm) (Agilent, Waldbronn,
430 Germany). The volume injected was 100 µl. The UV-Vis absorbance was measured with a VWD Agilent
431 technologies 1100 series (G1314A) detector (Agilent, Waldbronn, Germany). The lamp used was a

432 Deuterium lamp G1314 Var Wavelength Det. (Agilent, Waldbronn, Germany). Two wavelengths were
433 selected: 320 nm, used for the detection of pyochelin, and 380 nm, used for the detection of pyoverdine.
434 The fractions containing the pyoverdine and pyochelin were collected directly at the detector output.
435 Further, the purity of the samples was verified by two detectors, a diode array detector (DAD) 190 to 601
436 nm (steps: 1nm) and a Q-TOF (tandem mass spectrometry, quadrupole and Time of flight detector
437 combined) (Agilent, Waldbronn, Germany). Electrospray ionization was performed in positive mode (ESI+)
438 (Dual AJS ESI) (Vcap= 3500V, Nozzle Voltage= 1000V), with mass range from m/z 200 to 1500. Finally, the
439 concentration of pyoverdine and pyochelin were estimated by utilization of Beer Lambert law formula,
440 $A=\varepsilon lc$ (A: absorbance; Ɛ: molar attenuation coefficient or absorptivity of the attenuating species; l: optical
441 path length and c: concentration of molecule). l value for pyochelin and pyoverdine is 1 cm while Ɛ is 4000
442 $L.mol^{-1}.cm^{-1}$ or 16000 $L.mol^{-1}.cm^{-1}$, respectively [65]. The absorbance was measured with VWR, V-1200
443 Spectrophotometer, at 320nm (pH=8) for pyochelin and 380nm (pH=5) for pyoverdine[65]. Further, the
444 absorbance value was used for calculating the final concentration. The fragmentation pattern of
445 *Pseudomonas* sp. CMR12a pyoverdine was obtained by UPLC MS/MS analysis of m/z = 1288.5913 ion in
446 positive mode with a fragmentation energy at 75V and compared to the one described in *P. protegens* Pf-5
447 [1].

448 **Confrontation, white line formation and motility test**

449 For confrontation assays on agar plates*, Bacillus* and *Pseudomonas.* strains were grown overnight in $EM_{1/2}$
450 and CAA liquid mediums, respectively. After bacterial washing in peptone water and adjustment of OD_{600nm}
451 to 0.1, 5 µl of bacterial suspension was spotted at 1 mm, 5 mm and 7.5 mm distance onto an $EM_{1/2}$ agar
452 plate. For the white line formation experiments, *B. velezensis* line was applied with a cotton stick and 5 µl of
453 *Psuedomonas* sp. cell suspensions were spotted at a 5 mm distance onto CAA agar plates. Plates were
454 incubated at 30 °C and images taken after 24 h. Photographs were captured using CoolPix camera (NiiKKOR
455 60x WIDE OPTICAL ZOOM EDVR 4.3-258 mm 1:33-6.5), photos were thus proceeded in Adobe Photoshop CC
456 2019 or by direct edition.

457 **Maldi-FT-ICR MS imaging**

458 Mass spectrometry images were obtained as recently described (Kune et al 2019) using a FT-ICR mass
459 spectrometer (SolariX XR 9.4T, (Bruker Daltonics, Bremen, Germany)) mass calibrated from 200 m/z to
460 2,300 m/z to reach a mass accuracy of 0.5 ppm. Region of interest from agar microbial colonies were

461 directly collected from the Petri dish and transferred onto an ITO Glass slide (Bruker, Bremen, Germany),
462 previously covered with double sided conductive carbon tape. The samples were dried under vacuum and
463 covered with an α-cyano-4-hydroxycinnamic acid (HCCA) matrix solution at 5mg/mL (70:30
464 acetonitrile:water v/v). In total, 60 layers of HCCA matrix were sprayed using the SunCollect instrument
465 (SunChrom, Friedrichsdorf, Germany). FlexImaging 5.0 (Bruker Daltonics, Bremen, Germany) software was
466 used for MALDI MS imaging acquisition, with a pixel step size for the surface raster set to 100 µm.

467 ***In planta* competition**

468 For *in planta* studies, tomato seeds (*Solanum lycopersicum* var. Moneymaker) were sterilized in 75%
469 ethanol with shaking for 2 min. Subsequently, ethanol was removed and seeds were added to the 50 ml
470 sterilization solution (8.5 ml of 15% bleach, 0.01 g of Tween 80 and 41.5 ml of sterile ultra-pure water) and
471 shaken for 10 min. Seeds were thereafter washed five times with water to eliminate stock solution residues.
472 Further, seeds were placed on square Petri dishes (5 seeds/plate) containing Hoagland solid medium (14 g/l
473 agar, 5 ml stock 1 (EDTA 5,20 mg/l; $FeSO_4x7H_2O$ 3,90 mg/l; H_3B0_3 1,40 mg/l; $MgSO_4x7H_2O$ 513 mg/l;
474 $MnCl_2x4H_2O$ 0,90 mg/l, $ZnSO_4x7H_2O$ 0,10 mg/l; $CuSO_4x5H_2O$ 0,05 mg/l; 1 ml in 50 ml stock 1, $NaMo0_4x2H_2O$
475 0,02 mg/l 1 ml in 50 ml stock 1), 5 ml stock 2 (KH_2PO_4 170 mg/l; 5 ml stock 3 : $KN0_3$ 316 mg/l, $Ca(NO_3)_2$ $4H_2O$
476 825 mg/l), pH=6,5) and placed in the dark for three days. Afterwards, 6 seeds were inoculated with 2 µl of
477 overnight culture (OD_{600}=0.1) of the appropriate strains (control) or with a mix of *Bacillus* and *Pseudomonas*
478 cells (95:5 ration) (interaction), and grown at 22°C under a 16h/8h night/day cycle with constant light for
479 three days. After the incubation period, to determine bacterial colonization levels, bacteria from roots of six
480 plants per condition were detached from roots by vortexing for 1 min in peptone water solution
481 supplemented with 0.1% of Tween. Serial dilutions were prepared and 200 µl of each were plated onto LBA
482 medium using plating beads. After 24 h of incubation at 30 °C for *Pseudomonas* and at 42°C for *Bacillus*,
483 colonies were counted. Colonization results (six plants *per* strain) were log-transformed and statistically
484 analyzed. Three independent assays were performed with six plants each for *in planta* competition assays.
485 To measure bacterial BSMs production *in planta*, a rectangle part (1 x 2.5 cm) of medium close to the
486 tomato roots was sampled. BSMs were extracted for 15 min, with an acetonitrile (85%)/agar solution (1:1).
487 After centrifugation for 5 min at 4000 rpm, the supernatant was recovered for the UPLC analysis (section
488 Secondary metabolites analysis).

489 **Statistical analysis**

490 Statistical analyses were performed using GraphPad PRISM version 6 software (GraphPad) by using a paired
491 T-test or Mann-Whitney test. For multiple comparisons, one-way ANOVA and Tukey test were used in
492 RStudio 1.1.423 statistical software environment (R Core Team, 2020). Data represent mean ± s.d. of three
493 independent experiments unless stated otherwise. $P < 0.05$ was considered statistically significant.

494

495

496 **Figure legends**

497

498 **Figure 1. Enhanced production of BSMs and related anti-*bacterial* activities of *B. velezensis* GA1 as a**
499 **response to interaction with *Pseudomonas* sp. CMR12a secreted metabolites. a.** LCMS extracted ions
500 chromatograms (EIC) illustrating relative abundance of ions corresponding to dihydrobacillaene (**I**),
501 bacillaene (**II**), bacillibactin (**III**), oxydifficidin (**IV**), difficidin (**V**), surfactins (**VI**), macrolactins (**VII**), iturins (**VIII**)
502 and fengycins (**IX**) produced by *B. velezensis* GA1, after 24h growth in CFS-supplemented (2 % v/v) EM
503 medium (blue) compared to un-supplemented control (red). **b**. Production of *B. velezensis* GA1 BSMs co-
504 cultivated with 2 % (v/v) of *Pseudomonas* sp. CMR12a CFS. Fold change equals to 1 is represented as a red
505 line and corresponds to the production of metabolites in *B. velezensis* GA1 unsupplemented. Statistical
506 significance of each condition (n=9 within three biological repetitions and three technical replicates) was
507 calculated as a comparison of BSMs production by *B. velezensis* GA1 in treated cultures with the production
508 in related monoculture, by using Mann–Whitney test where ''****'' represents significant difference
509 (P<0.0001) **c.** Enhanced Anti-*Xanthomonas campestris* (**I** and **II**) and anti-*Clavibacter michiganensis* (**III** and
510 **IV**) activities of *B. velezensis* GA1 after growth in CFS-supplemented (4 %) medium (GA1+CFS) compared to
511 control (GA1) assessed by inhibition zone (**I** and **III**) and liquid pathogens culture growth kinetic (**II** and **IV**) **d.**
512 Anti-*Xanthomonas campestris* (I) and Anti- *Clavibacter michiganensis* (II) activities of *B. velezensis* GA1 (GA1
513 wt) BSMs and its derived mutants impaired in production of different BSMs. Metabolites not produced by
514 the different mutants are illustrated with red boxes in the table. Error bars indicate standard error (n=6
515 within two biological repetitions and three technical replicates). Different letters indicate groups of
516 statistically different conditions (one-way ANOVA and Tukey test; P<0.05). **e.** Differential expression of
517 *acnA*, encoding the amylocylicin precusor, upon supplementation with CMR12a CFS compared to control
518 Error bars indicate standard error (n=6 within two biological repetitions and three technical replicates). The
519 statistical difference, in *acnA* gene expression, between the two conditions was calculated by using Mann–
520 Whitney test where ''**'' represents a statistically significant difference between the two conditions
521 (0.001>P<0.05).

522

523 **Figure 2: Enantio-pyochelin as the main *Pseudomonas* sp. CMR12a triggers of the anti-*bacterial* activity of**
524 ***B. velezensis* GA1. a,** Effect of 2% v/v *Pseudomonas* sp. CMR12a mutants CFS on dihydrobacillaene (2H-bae)

525 production by *B. velezensis* GA1. Metabolites not produced by the different mutants are illustrated by red
526 boxes in the table below. Fold change equals to 1 is represented as a red line and corresponds to the
527 production of 2H-bae in *B. velezensis* GA1 unsupplemented culture. Error bars indicate standard error (n= 9
528 with three biological repetitions and three technical replicates). Different letters indicate groups of
529 statistically different conditions (one-way ANOVA and Tukey test; P<0.05). **b,** Differential production of 2H-
530 bae after addition of 0.35µM pure pyoverdine (PVD), 1.4µM pure enantio-pyochelin (PCH), 4% v/v
531 *Pseudomonas* sp. CMR12a cell-free supernatant (CFS CAA) , CMR12a cell-free supernatant from iron
532 supplemented culture (CFS CAA+Fe) and different concentration of iron-chelating agent 2,2'-dipyridyl (DIP).
533 Fold change equals to 1 is represented as a red line and corresponds to the production of 2H-bae in *B.*
534 *velezensis* GA1 unsupplemented culture. Error bars indicate standard error (n=6 within two biological
535 repetitions and three technical replicates). Different letters indicate groups of statistically different
536 conditions (one-way ANOVA and Tukey test; P-value < 0.05). **c,** Effect of pure PVD and PCH on bacillibactin
537 and 2H-bae production by *B. velezensis* GA1. *B. velezensis* GA1 culture was supplemented with different
538 concentration of pure PVD or pure PCH. Fold change equals to 1 is represented as a red line and
539 corresponds to the production of 2H-bae in *B. velezensis* GA1 unsupplemented culture. Error bars indicate
540 standard error (n=6 within two biological repetitions and three technical replicates). Different letters
541 indicate groups of statistically different conditions (one-way ANOVA and Tukey test; P<0.05). **d.** Growth
542 curve of GA1 WT and bacillibactin KO mutant (ΔdhbC) upon supplementation with pyoverdine (pvd) and
543 pyochelin (pch) at concentration corresponding to the ones in co-culture experiment (4% (v/v))
544 pseudomonas CFS . Error bars indicate standard error (n=6 with two biological repetitions and three
545 technical replicates).

546

547 **Figure 3: Enhanced motility of *Bacillus* is driven by distance and surfactin production . a,** *B. velezensis* GA1
548 and P. sp. CMR12a phenotypes when cultured alone (left panel) or in confrontation at short distance
549 (1cm)(right panel). **b,** Phenotype and motile of GA1 in confrontation with CMR12a at short distance (1cm)
550 compared to his surfactin defficient mutant (Δ*srfaA*) **c,** Maldi FT-ICR MSI (Mass spectrometry Imaging)
551 heatmaps showing spatial localisation and relative abundance of C14-Surfactin ($[M+Na]^+$) when *B.*
552 *velezensis* GA1 is in confrontation with CMR12a at different distances.

553

554 **Figure 4: Overview of the unsuspected roles of bacterial CLPs in *Bacillus-Pseudomonas* interaction**: **a,**
555 Illustration of *B. velezensis* GA1 growth inhibition cultured in gelosed media in confrontation (**I**) or in liquid
556 with 6%v/v (**II**) with *Pseudomonas* sp. CMR12a wild-type (CFS) or mutants devoid of production of
557 orfamides and phenazines (Δ*ofaBC-phz*), sessilins (Δ*sesA*), sessilins and orfamides (Δ*sesA-ofaBC*), sessilins
558 and phenazines (Δ*sesA-phz*), or sessilins, orfamides and phenazines (Δ*sesA-ofaBC-phz*). Error bars indicate
559 standard error (n=6 within two biological repetitions and three technical replicates). Different letters
560 indicate groups of statistically different conditions (one-way ANOVA and Tukey test; P<0.05). **b, Left** :
561 Optical density of B. velezensis GA1 WT (GA1) after 7h culture supplemented with CMR12a WT CFS
562 (CMR12a) or sessilin KO mutant (Δ*sesA*).**Right :** Optical density of B. velezensis GA1 surfactin KO mutant
563 (Δ*srfaA*) after 7h culture supplemented with CMR12a WT CFS (CMR12a) and pure surfactin (CMR12a +
564 surfactin). Growth inhibition of CMR12 sessilin mutant CFS (Δ*sesA*). Unsupplemented culture of both B.
565 velezensis strains are mentioned as control culture**. c-** White line formation obtained by confrontation with
566 surfactin and sessilin/tolaasin producing strains (*B. velezensis* GA1 confronted to *Pseudomonas* sp. CMR12a
567 (**I**) or *P.* tolaasii CH36 (**II**). CLPs impaired mutants (Δ*srfaA,* and Δ*sesA* or Δ*tolA*) do not show any white-line.
568 Other *Pseudomonas* CLPs producers do not cause white-line formation or impact the *B. velezensis* GA1
569 growth (WCU-84, SS101, BW11M1, RW10S2).. CLPs produced by the aforementioned *Pseudomonas* strains
570 are listed in the table below. **d.** 3D LC-MS chromatogram after solid-liquid extraction of white-line showing
571 the presence of sessilin and surfactin.

572

573 **Figure 5: *Bacillus-Pseudomonas in planta* competition and CLPs role**. **a.** *Bacillus* (GA1) or *Pseudomonas*
574 (CMR12a) population recovered at 3 days post inoculation (dpi) when inoculated alone (GA1, CMR12a) or
575 coinoculated (co-inoculation) . Error bars indicate standard error (n=16) of 3 independent experiments
576 including at least 4 plants per condition. Statistical difference between the conditions was calculated by
577 using Mann–Whitney test where ''****'' and ''***'' represent significant differences (P<0.0001 and
578 0.0001>P<0.001, respectively). **b.** Relative gene expression of *srfaA, dfnA, baeJ* and *acnA*a fter 3 days of co-
579 inoculation on tomato plant roots. Error bars indicate standard error (n=3, each dot represents the mean of
580 three technical repetitions). Fold change equals to 1 is represented as a red line and corresponds to the
581 gene expressions in *B. velezensis* GA1 monoculture. Statistical significance of each group was calculated as a
582 comparison of genes expressions by *B. velezensis* GA1 in treated cultures with the one in monocultures, by
583 using T-test where ''*'', ''***'' and ''****'' represent significant difference (0.01>P<0.05, 0.0001>P<0.001

584 and P<0.0001, respectively). **c.** Recoverd population at 3dpi of *B. velezensis* GA1 WT (GA1) or his surfactin
585 impaired mutant (Δ*srfA*) co-inoculatied with *P. sp.* CMR12a (CMR12a) or his sessilin KO mutant (Δ*sesA*) .
586 Error bars represent standard deviations (n=16 to 18) of 3 independent experiments including at least 4
587 plants per condition. Statistical difference between two conditions was calculated by using Mann–Whitney
588 test where ''**'' indicates a significant difference (0.001>P<0.05). **d.** LC-MS EIC illustrating relative *in planta*
589 production of sessilin and surfactin by monocultures of *B. velezensis* GA1 (GA1) and co-cultures of wild
590 types (GA1+CMR12a) and *B. velezensis* GA1 and *Pseudomonas* sp. CMR12a impaired in sessilin production
591 (GA1+Δsessilin).

592

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608

609

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756

Figure 1

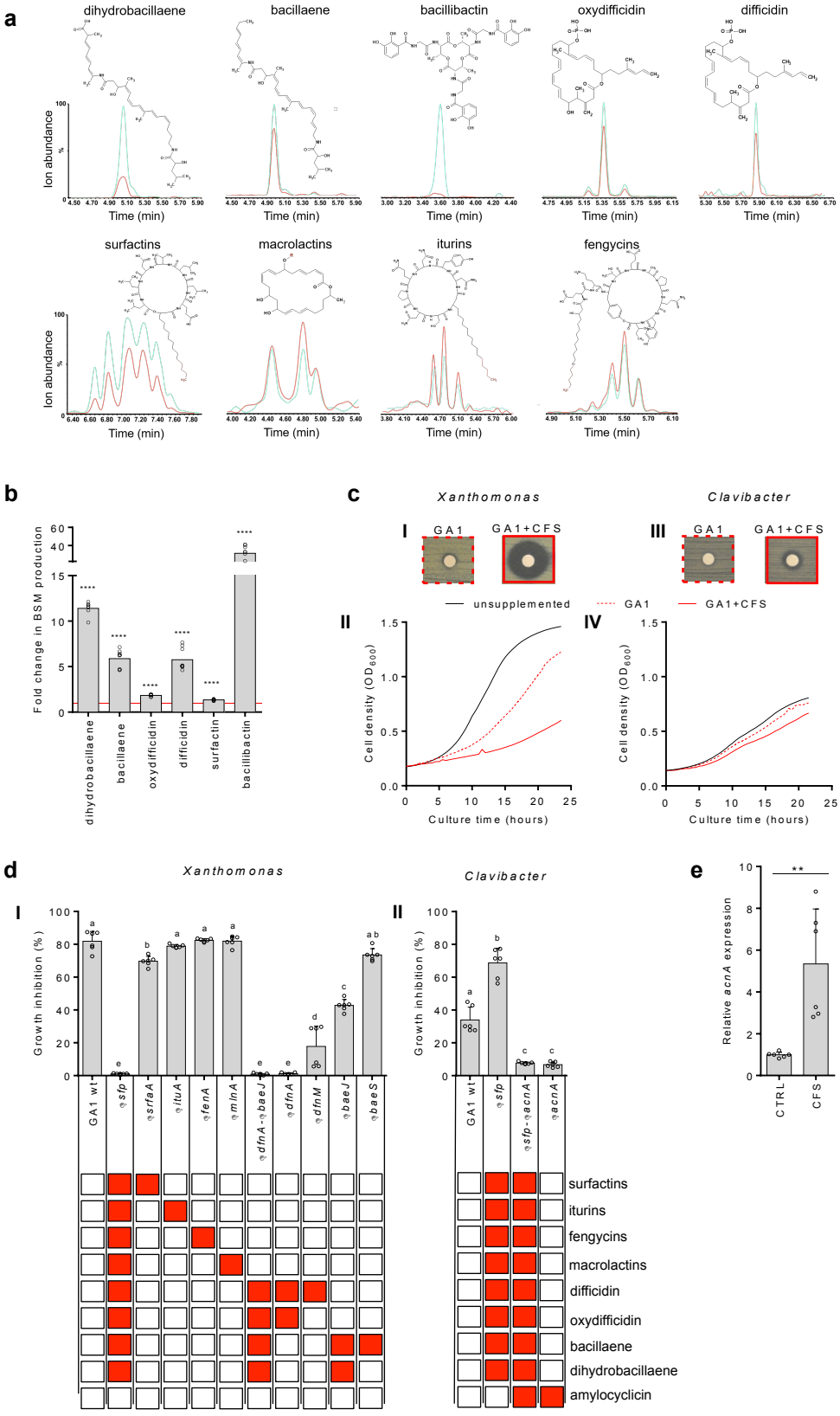

a
dihydrobacillaene
bacillaene
bacillibactin
oxydifficidin
difficidin
surfactins
macrolactins
iturins
fengycins
Ion abundance
Time (min)
b
Fold change in BSM production
dihydrobacillaene
bacillaene
oxydifficidin
difficidin
surfactin
bacillibactin
c
Xanthomonas
Clavibacter
I
GA1
GA1+CFS
III
unsupplemented
GA1
GA1+CFS
II
IV
Cell density (OD600)
Culture time (hours)
d
Xanthomonas
Clavibacter
I
II
Growth inhibition (%)
GA1 wt
Δsfp
ΔsrfaA
ΔituA
ΔfenA
ΔmlnA
ΔdfnA-ΔbaeJ
ΔdfnA
ΔdfnM
ΔbaeJ
ΔbaeS
Δsfp-ΔacnA
ΔacnA
surfactins
iturins
fengycins
macrolactins
difficidin
oxydifficidin
bacillaene
dihydrobacillaene
amylocyclicin
e
Relative acnA expression
CTRL
CFS

Figure 2

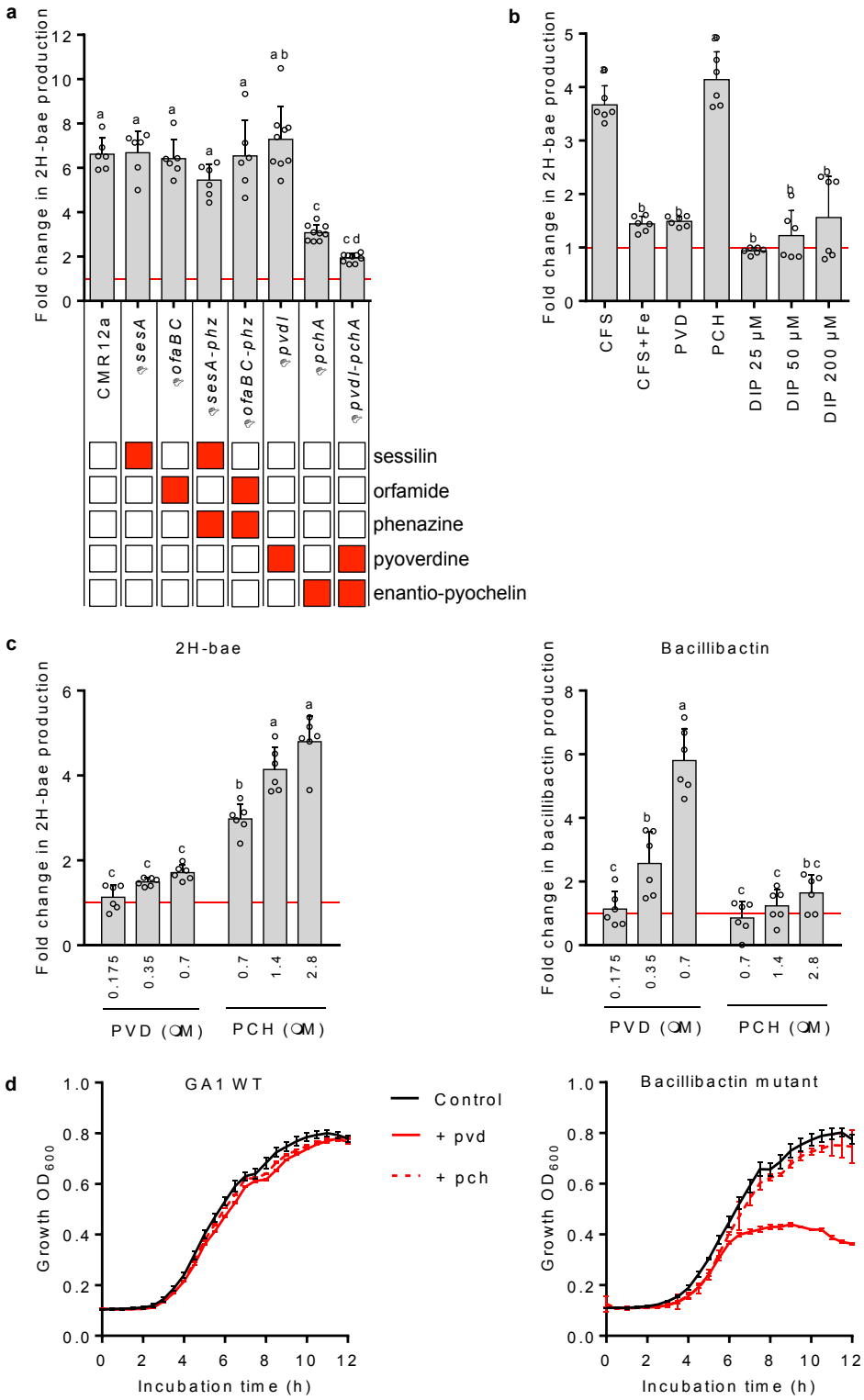

a
Fold change in 2H-bae production
CMR12a
sesA
ofaBC
sesA-phz
ofaBC-phz
pvdI
pchA
pvdI-pchA
sessilin
orfamide
phenazine
pyoverdine
enantio-pyochelin
b
Fold change in 2H-bae production
CFS
CFS+Fe
PVD
PCH
DIP 25 µM
DIP 50 µM
DIP 200 µM
c
2H-bae
Fold change in 2H-bae production
PVD (µM)
PCH (µM)
Bacillibactin
Fold change in bacillibactin production
d
GA1 WT
Growth OD600
Incubation time (h)
Control
+ pvd
+ pch
Bacillibactin mutant

Figure 3

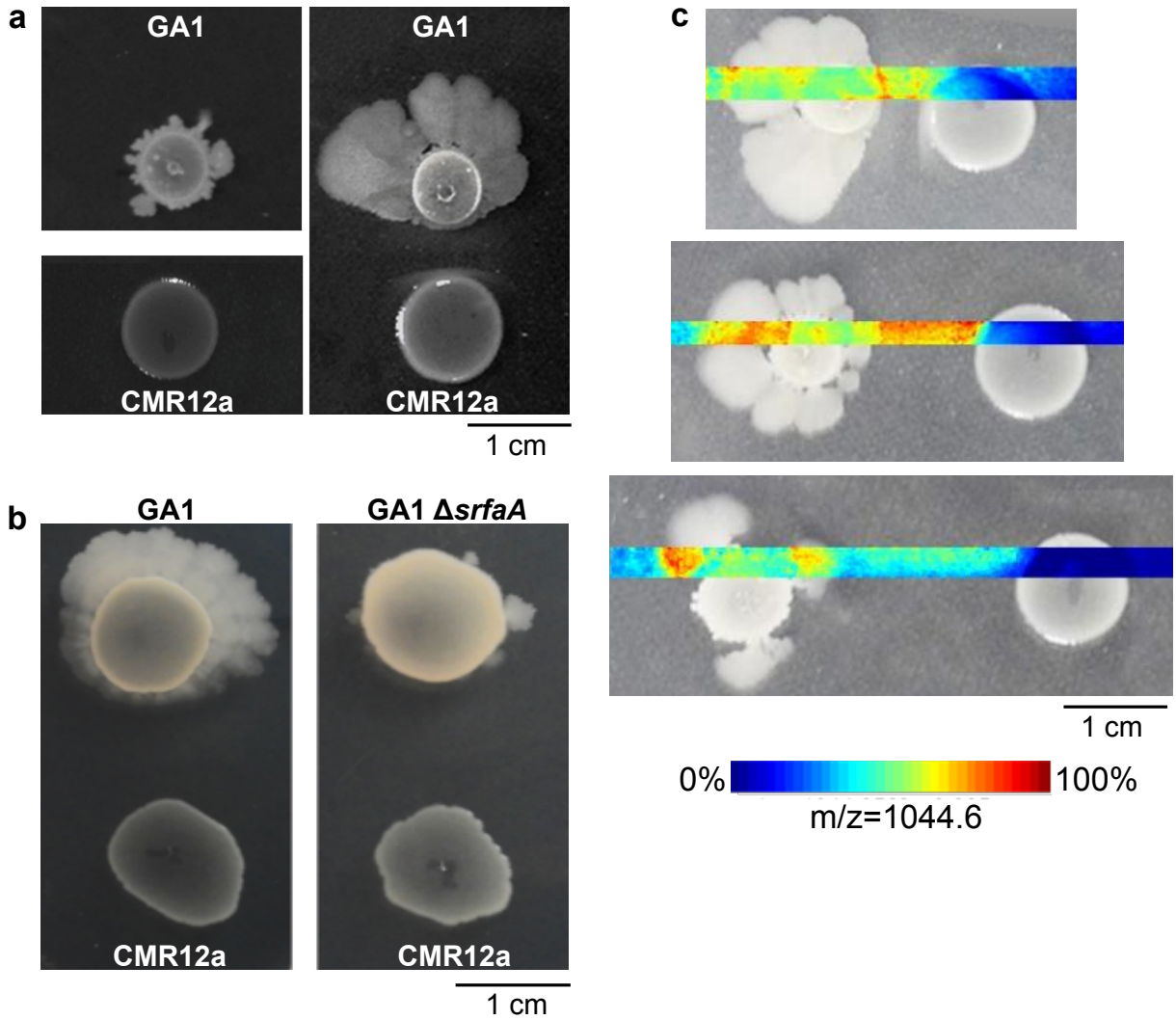

a
GA1
GA1
CMR12a
CMR12a
1 cm
b
GA1
GA1 Δ*srfaA*
CMR12a
CMR12a
1 cm
c
1 cm
0%
100%
m/z=1044.6

Figure 4

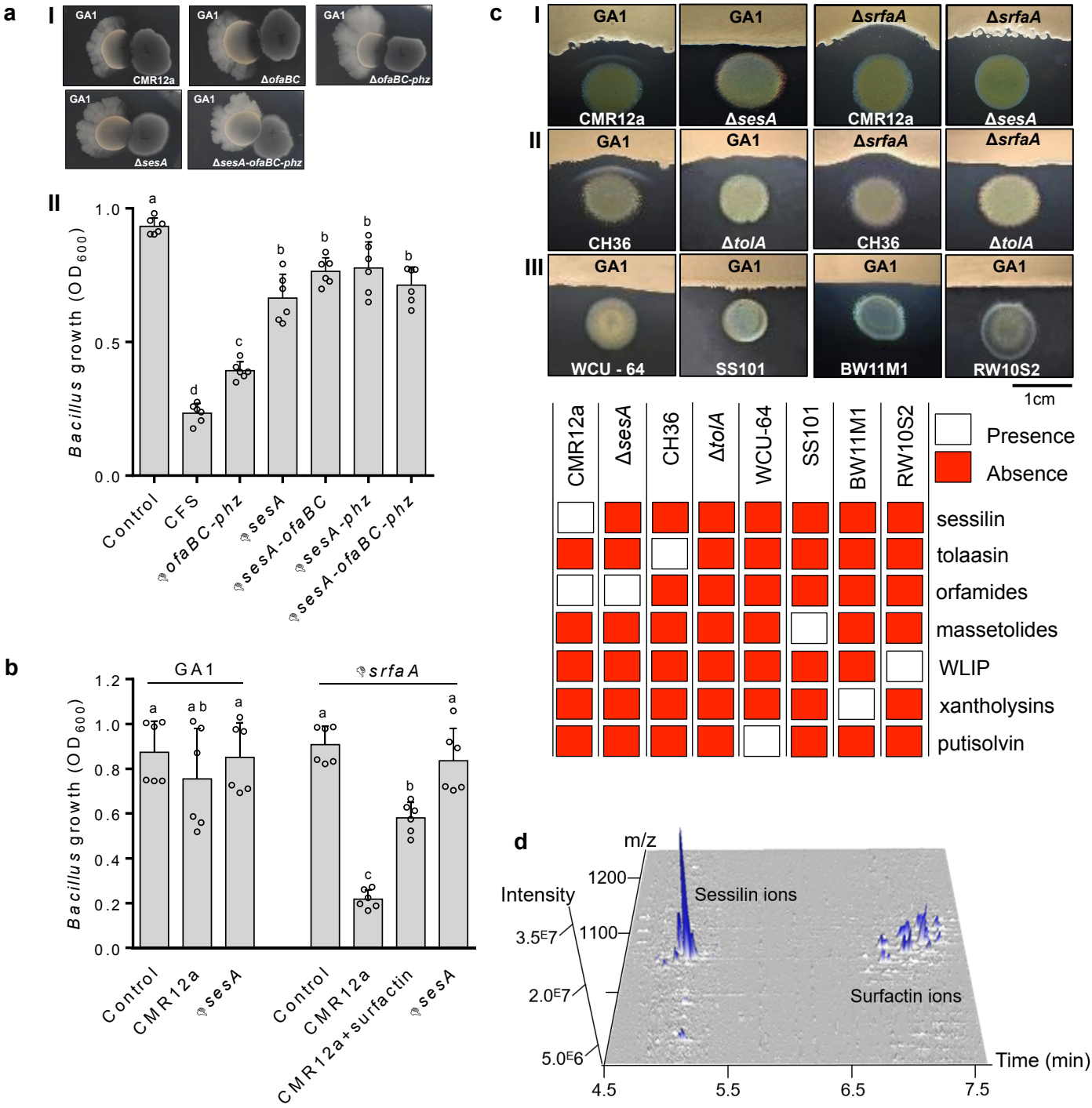
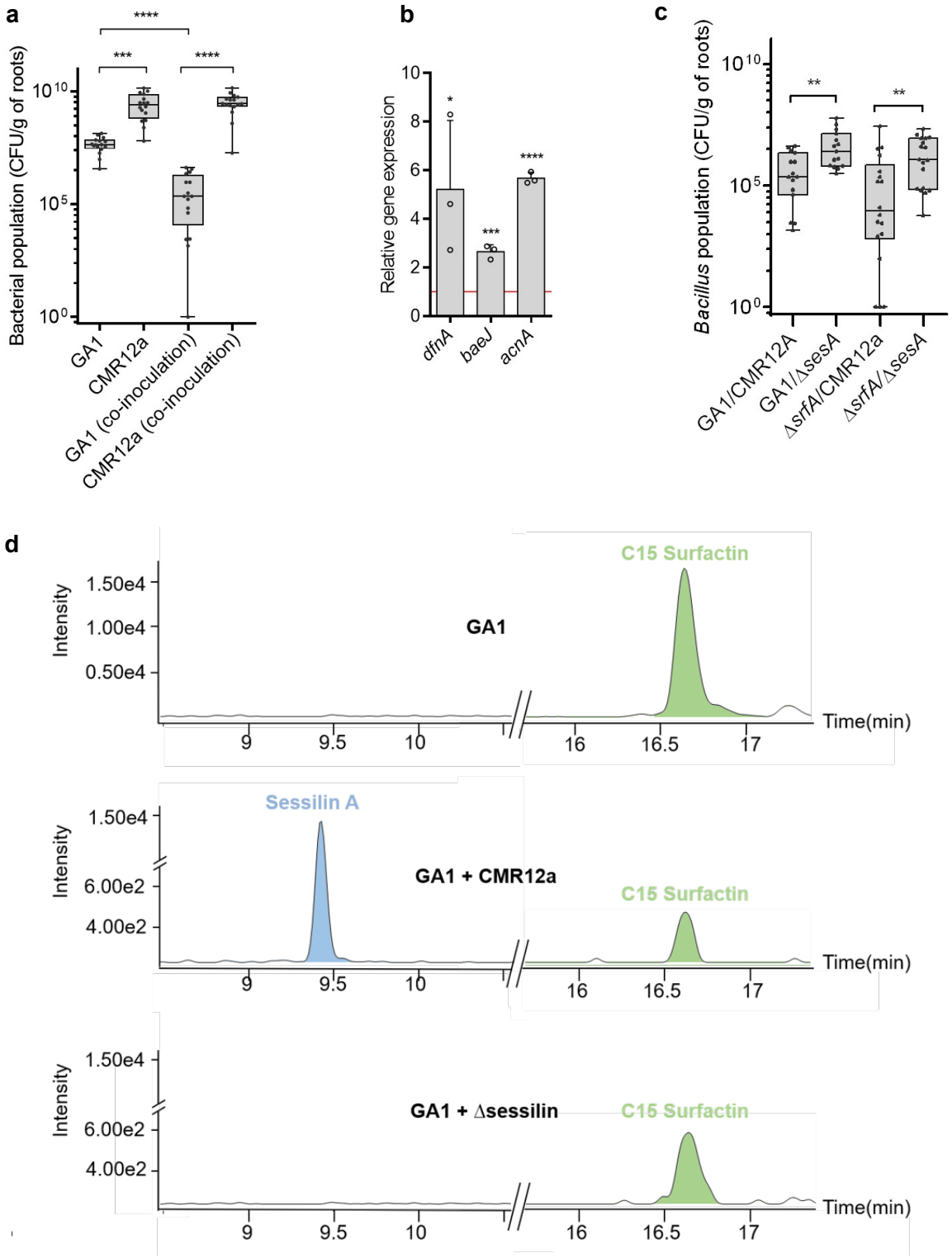

a
I
GA1
CMR12a
ΔofaBC
ΔofaBC-phz
ΔsesA
ΔsesA-ofaBC-phz
II
Bacillus growth (OD600)
1.0
0.5
0.0
Control
CFS
ofaBC-phz
sesA
sesA-ofaBC
sesA-phz
sesA-ofaBC-phz
b
GA1
srfaA
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Control
CMR12a
sesA
CMR12a+surfactin
c
I
GA1
ΔsrfaA
CMR12a
ΔsesA
II
CH36
ΔtolA
III
WCU - 64
SS101
BW11M1
RW10S2
1cm
Presence
Absence
sessilin
tolaasin
orfamides
massetolides
WLIP
xantholysins
putisolvin
d
m/z
Intensity
1200
1100
3.5E7
2.0E7
5.0E6
Sessilin ions
Surfactin ions
Time (min)
4.5
5.5
6.5
7.5

Figure 5


a
Bacterial population (CFU/g of roots)
GA1
CMR12a
GA1 (co-inoculation)
CMR12a (co-inoculation)
b
Relative gene expression
dfnA
baeJ
acnA
c
Bacillus population (CFU/g of roots)
GA1/CMR12A
GA1/ΔsesA
ΔsrfA/CMR12a
ΔsrfA/ΔsesA
d
Intensity
GA1
C15 Surfactin
Time(min)
GA1 + CMR12a
Sessilin A
GA1 + Δsessilin