Observations of Stably Stratified Flow through a Microscale Gap

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Published in:
Journal of the Atmospheric Sciences

Link to article, DOI:
[10.1175/JAS-D-20-0087.1](https://doi.org/10.1175/JAS-D-20-0087.1)

Publication date:
2021

Document Version
Publisher's PDF, also known as Version of record

[Link back to DTU Orbit](#)

Citation (APA):
Vassallo, D., Krishnamurthy, R., Menke, R., & Fernando, H. J. S. (2021). Observations of Stably Stratified Flow through a Microscale Gap. *Journal of the Atmospheric Sciences*, 189-208. <https://doi.org/10.1175/JAS-D-20-0087.1>

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1 Development of a whole cell biosensor for detection of 2, 4-
2 diacetylphloroglucinol (DAPG) producing bacteria from grassland soil

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13 Keywords: Secondary metabolites, *Pseudomonas*, DAPG, Synthetic biology, biocontrol

14 **ABSTRACT**

15 Fluorescent *Pseudomonas* spp. producing the antibiotic 2,4-diacetylphloroglucinol (DAPG)
16 are ecologically important in the rhizosphere as they can control phytopathogens and contribute
17 to disease suppressiveness. DAPG can also trigger a systemic resistance response in plants and
18 stimulate root exudation and branching as well as induce plant-beneficial activities in other
19 rhizobacteria. While studies of DAPG-producing *Pseudomonas* have predominantly focused
20 on rhizosphere niches, the ecological role of DAPG as well as the distribution and dynamics
21 of DAPG-producing bacteria remains less well understood for other environments such as bulk
22 soil and grassland, where the level of DAPG producers are predicted to be low. Here, we
23 construct a whole cell biosensor for detection of DAPG and DAPG-producing bacteria from
24 environmental samples.

25 The constructed biosensor contains a *phlF* response module and either *lacZ* or *lux* genes as
26 output modules assembled on a pSEVA plasmid backbone for easy transfer to different host
27 species and to enable easy future genetic modifications. We show that the sensor is highly
28 specific towards DAPG, with a sensitivity in the low nanomolar range (>20 nM). This
29 sensitivity is comparable to the DAPG levels identified in rhizosphere samples by chemical
30 analysis. The biosensor enables guided isolation of DAPG-producing *Pseudomonas*. Using the
31 biosensor, we probed the same grassland soil sampling site to isolate genetically related DAPG-
32 producing *Pseudomonas kilonensis* strains over a period of 12 months. Next, we used the
33 biosensor to determine the frequency of DAPG-producing Pseudomonads within three
34 different grassland soil sites and show that DAPG producers can constitute part of the
35 *Pseudomonas* population in the range of 0.35-17% at these sites. Finally, we show that the
36 biosensor enables detection of DAPG produced by non-*Pseudomonas* species.

37 Our studies show that a whole-cell biosensor for DAPG detection can facilitate isolation of
38 bacteria that produce this important secondary metabolite and provide insight into the
39 population dynamics of DAPG producers in natural grassland soil.

40

41 **IMPORTANCE**

42 The interest has grown for bacterial biocontrol agents as biosustainable alternatives to
43 pesticides to increase crop yields. To date, we have a broad knowledge of antimicrobial
44 compounds, such as DAPG, produced by bacteria growing in the rhizosphere surrounding plant
45 roots. However, compared to the rhizosphere niches, the ecological role of DAPG as well as
46 the distribution and dynamics of DAPG-producing bacteria remains less well understood for
47 other environments such as bulk and grassland soil. Currently, we are restricted to chemical
48 methods with detection limits and time-consuming PCR-based and probe-hybridization
49 approaches to detect DAPG and its respective producer. In this study, we have developed a
50 whole-cell biosensor, which can circumvent the labor-intensive screening process, as well as
51 increase the sensitivity at which DAPG can be detected. This enables quantification of relative
52 amounts of DAPG-producers, which in turn increases our understanding of the dynamics and
53 ecology of these producers in natural soil environments.

54 **INTRODUCTION**

55 Secondary metabolites are well-known for their potential as drugs in the medical industry. They
56 were initially defined as dispensable, non-vital compounds to their respective producers.
57 However, recent advances in genome mining and microbial ecology are beginning to shed light
58 on the prevalence, role and importance of these metabolites in natural environments. In soil
59 ecology, species of fluorescent *Pseudomonas* isolated from naturally suppressive soils have
60 received a great deal of attention due to their production of antimicrobial secondary
61 metabolites, such as 2,4-diacetylphloroglucinol (DAPG). Suppression of wheat take-all disease
62 caused by *Gaeumannomyces graminis* var. *tritici* was shown to be induced by years of crop
63 monoculture and was associated with the root colonization of DAPG-producing fluorescent
64 *Pseudomonas* (1). Moreover, production of DAPG was shown to be involved in disease control
65 against the causative agent of tobacco black root rot, *Thielaviopsis basicola* (2). It has also been
66 demonstrated that DAPG has antibacterial properties against the pathogen *Erwinia*
67 *carotovorum* subsp. *atroseptica* causing soft rot of potatoes (3).

68 The biosynthetic gene cluster related to DAPG production in *Pseudomonas* comprises eight
69 genes, *phlACBDEFGH* (4, 5). Proteins encoded by the operon of *phlACBD* are responsible for
70 the synthesis of DAPG (4). The type III polyketide synthase, PhlD, initially condenses three
71 malonyl coenzyme A molecules into phloroglucinol (PG), which is further acetylated by the
72 enzyme complex of PhlACB into monoacetylphloroglucinol (MAPG) and DAPG (6). PhlE
73 was identified as a putative membrane transporter with similarities to a known efflux pump in
74 *Staphylococcus aureus* (4). The protein product of *phlG* has been described as a hydrolase that
75 catalyses the degradation of DAPG to MAPG, thus controlling the intracellular levels of DAPG
76 (5, 7). Both *phlF* and *phlH* encode *tetR*-like repressors that inhibit transcription of *phlACBD*
77 and *phlG*, respectively (5, 8). PhlF and PhlH bind to operator sites located in promoters
78 upstream of the genes they regulate, thereby sterically blocking transcription. DAPG serves as

79 the ligand for both repressors. Thus, in the presence of DAPG repression is relieved, leading
80 to expression of *phlACBD* and *phlG*, which in turn leads to induced DAPG biosynthesis and
81 post-translational regulation (5, 8).

82 The complex of species belonging to the group, *Pseudomonas fluorescens*, has been well
83 characterized over the past three decades, due to the potential of several species to act as
84 biocontrol agents in agriculture. A recent study surveyed the phylogenetic relationship between
85 166 type strains of *Pseudomonas* (among which 66 belonged to the *P. fluorescens* group) based
86 on amino acid sequences of 100 gene orthologues, which further proposed the existence of 10
87 subgroups within the *P. fluorescens* clade (9). However, despite the vast diversity among *P.*
88 *fluorescens*, only few species belonging to two subgroups, *P. protegens* and *P. corrugata*, are
89 known to produce DAPG (1, 8). With the advances in genome mining and the increased
90 availability of complete genomes, the biosynthetic gene cluster *phlACBDE* was recently
91 identified in *Pseudomonas* species outside of the *P. fluorescens* group, as well as in two genera
92 of β -proteobacteria (10). However, in these cases production of DAPG has not yet been
93 demonstrated.

94 Identification and enumeration of DAPG-producing microorganisms has, to our knowledge,
95 exclusively relied on DNA probe hybridization and PCR-based techniques. One of the most
96 commonly employed techniques uses colony hybridization combined with a confirmatory PCR
97 to verify the presence of *phlD* (11). A more recent method involves culture-independent real-
98 time PCR to quantify populations of DAPG-producing *Pseudomonas* in the plant rhizosphere
99 (12). A different approach is to quantify the amount of DAPG produced *in situ* by chemical
100 analysis. Bonsall et al. demonstrated that an optimized extraction protocol enabled
101 quantification of DAPG isolated directly from the plant rhizosphere (13). While these
102 techniques have clear advantages, there are several drawbacks that also exist. PCR-based
103 methods are limited by DNA binding of specific primers and measures have to be taken to

104 address the quantity of diverse genotypes of DAPG-producers. Chemical identification, on the
105 other hand, is restricted by detection limits, which is directly correlated to the size of the
106 bacterial population.

107 In recent years, the synthetic biology toolbox has expanded rapidly and the use of genetically
108 engineered molecular circuits to sense molecules and conditions of interest has gained
109 increased attention. These developments have given rise to whole-cell biosensors that utilize
110 natural regulatory systems engineered to detect metabolites and small molecules (14, 15).
111 Whole-cell biosensors rely on molecule recognition to either activate transcription or lift
112 repression of a reporter gene and are thus often highly sensitive with detection limits in the
113 nano- to micromolar range (16–18). Furthermore, whole-cell biosensors are tunable by addition
114 or alteration of genetic parts, which allows for higher sensitivity and increased specificity (15,
115 19). Lastly, biosensors may also be implemented as biological detectors for uncovering
116 metabolic activities *in situ* (20, 21).

117 Studies of DAPG-producing *Pseudomonas* species have predominantly focused on rhizosphere
118 niches, whereas the ecological role of DAPG as well as the distribution and dynamics of
119 DAPG-producing bacteria is not well understood for other environments such as bulk soil and
120 grassland. Here, we construct a whole cell biosensor as an alternative and efficient approach
121 for detection of DAPG and directed isolation of DAPG-producing bacteria from environmental
122 samples.

123 RESULTS

124 Construction of whole-cell DAPG biosensors with high sensitivity and specificity

125 Two whole-cell biosensors were constructed to enable specific detection of DAPG and
126 identification of DAPG-producing bacteria. Both sensors contain an identical module for
127 DAPG sensing in combination with either the *lux* operon or the *lacZ* gene as reporters (Figure
128 1A). The biosensor plasmids were constructed as repressor-mediated modules in an *E. coli* K12
129 $\Delta lacIZYA$ host. In the absence of DAPG, the TetR-like repressor protein, PhlF, binds as a dimer
130 to the *phlO* operator site (8) in the promoter upstream of the reporter gene. As bioavailable
131 DAPG diffuses into the cytoplasm and binds to PhlF, the repression on the target P_{phlF} promoter
132 is relieved. Two reporter modules were chosen. The *lux* operon was used as the output reporter
133 to obtain a highly sensitive response measured in bioluminescence units. To enable agar plate
134 screenings for investigating the distribution and dynamics of DAPG-producing bacteria in
135 natural microbial communities, the *lacZ* gene was used as the second output reporter. To ensure
136 stable inhibition of the P_{phlF} promoter under non-induced conditions, the *phlF* gene is
137 constitutively expressed from the P_{lacIQ} promoter. Both genetic circuits were introduced into a
138 pSEVA plasmid background (Figure 1B) to allow for rapid and simple cloning, as well as
139 efficient mobilization into distinct hosts by triparental mating.

140 To address the sensitivity and specificity of the whole-cell biosensors, microtiter bioassays
141 were conducted. For characterization of the *lux* version (Figure 1C), the *E. coli* host with
142 pSEVA226-DAPG_{lux} was grown in LB broth containing varying concentrations of PG, MAPG
143 or DAPG with continuous measurements of luminescence and cell density. PG and MAPG are
144 both precursors of DAPG and thus similar compounds, allowing determination of biosensor
145 specificity towards DAPG. For each concentration of inducer, the average luminescence was
146 normalized to cell density over a period of 35 minutes (8 data points, $t = 172-207$ min). This
147 corresponds to late exponential growth phase (Supplementary-S1). The whole-cell biosensor

148 exhibited excellent sensitivity towards DAPG with a response to 20 nM being statistically
149 significantly higher than the negative control without added DAPG (Student's t-test, $p = 0.01$).
150 The biosensor did not respond to the concentrations of PG tested, but a minor response to
151 MAPG at $>1.25 \mu\text{M}$ was observed. For characterization of the *lacZ* version (Figure 1D), the *E.*
152 *coli* host with pSEVA225::DAPG_{*lacZ*} was grown in LB broth containing varying concentrations
153 of either PG, MAPG or DAPG to allow for enzymatic expression. Subsequently, the biosensor
154 response was determined in a β -galactosidase microtiter assay. Enzymatic activity of
155 transcribed *lacZ* was estimated by continuously measuring the increase in o-nitrophenol
156 concentration over time (Supplementary-S2). The output is displayed in Miller Units (MU =
157 $(5000 \cdot \text{OD}_{420}/\text{min}) / \text{OD}_{600}$). Exposing the biosensor to $0.625 \mu\text{M}$ of DAPG yielded a
158 statistically significantly higher response compared to the negative control without added
159 DAPG (Student's t-test, $p = 0.0032$).

160

161 **Detection of DAPG production from bacterial colonies during growth on agar surfaces**

162 After addressing the sensitivity and specificity of the biosensor, we proceeded to utilize it in
163 the identification of DAPG-producing bacteria. We investigated the response of the whole-cell
164 biosensor when co-inoculated with known DAPG-producing bacteria commonly found in soil
165 (Figure 2). The biosensor harbouring pSEVA225::DAPG_{*lacZ*} was grown as a lawn on KBmalt
166 agar (31) supplemented with X-gal. Inoculation of DAPG-producing cultures of *P. protegens*
167 CHA0 and *P. protegens* DTU9.1 on these agar plates resulted in induction of the biosensor and
168 production of clear blue halos surrounding the colonies after 24 hours (Figure 2A). We also
169 constructed a $\Delta\text{phlACBD}$ mutant strain of *P. protegens* DTU9.1 by allelic replacement, in
170 which the DAPG biosynthesis genes were deleted (Materials and Methods). As expected, the
171 mutant strain did not elicit a response from the biosensor (Figure 2A) and did not produce
172 DAPG detectable by LCMS analysis (Figure 2B). We note that DAPG production in

173 *Pseudomonas* species has been shown to be high in growth conditions containing maltose, such
174 as the conditions used here (8, 32). In concordance with these findings, we did not observe
175 clear blue halos when LB agar was used (data not shown). We also tested *P. putida* KT2440,
176 which does not contain the DAPG biosynthetic gene cluster. Similar to the *P. protegens*
177 DTU9.1 $\Delta phlACBD$ mutant, *P. putida* did not elicit a response in the biosensor after 24 hours
178 of growth. However, after prolonged incubation (>72 hours) of *P. putida* KT2440, a slight blue
179 colouring was detected surrounding the bacterial colony (Supplementary-S3).

180 Finally, we also explored if our setup can be used to identify DAPG production in species other
181 than *Pseudomonas*. Recently, the genome of *C. vaccinii* MWU328 was shown to contain genes
182 with high similarity to the essential genes required for DAPG biosynthesis (*phlACBDE*) (10).
183 We found that *C. vaccinii* MWU328 produced molecules that induced a response in the
184 biosensor, resulting in a blue halo around the colony. A small amount of DAPG was
185 subsequently confirmed by LCMS (Supplementary-S4).

186

187 **Biosensor-guided identification of DAPG-producing *Pseudomonas***

188 Next, we used our biosensor to guide the identification of DAPG-producing *Pseudomonas*
189 from environmental samples. To this end, we collected soil samples from the same grassland
190 soil site (labelled “P5”) in both August 2018 and August 2019 and randomly isolated 30
191 fluorescent *Pseudomonas* strains at both time points. This site is located in Dyrehaven, which
192 is a Danish natural reserve, thus representing a relatively unaffected, natural soil niche. Using
193 the approach described above, all 60 isolates were screened on KBmalt agar plates
194 supplemented with X-gal and a lawn of the biosensor harbouring pSEVA225::DAPG_{lacZ}. A
195 blue halo indicative of DAPG production was observed for one isolate from 2018 (isolate
196 P5.21) and two isolates from 2019 (isolates P5.52 and P5.53) (Figure 3A). Subsequent LCMS
197 analysis confirmed DAPG production in all three isolates (Supplementary-S5). For taxonomic
198 identification of the 60 isolates, part of the housekeeping gene, *rpoD*, was sequenced for each
199 isolate. The *rpoD* sequences were aligned to a database of 165 *Pseudomonas* type strains (9).
200 Species identification of each isolate was determined based on the highest match to the type
201 strains using nucleotide BLAST on NCBI. The diversity of cultivable fluorescent
202 *Pseudomonas* remained similar, as species of *P. jessenii*, *P. koreensis* and *P. corrugata*
203 subgroups (as well as species of *P. putida*) were identified in the two samplings (Figure 3B).
204 However, isolates belonging to *P. fluorescens* subgroup were only found in 2018.

205
206 The three DAPG-producing *Pseudomonas* were identified as *P. kilonensis*. This species is part
207 of the *P. corrugata* subgroup of fluorescent *Pseudomonas* (Figure 3C, green box). Members
208 of this subgroup are known to harbour the biosynthetic gene cluster required for DAPG
209 production (10). To determine the phylogenetic relationship of the three *P. kilonensis* isolates
210 compared to the *P. corrugata* subgroup, we constructed an *rpoD*-based bootstrap consensus
211 tree (500 replicates) with the Neighbor-Joining method (Figure 3C). As expected, the three *P.*

212 *kilonensis* isolates cluster together with the members of the *P. corrugata* subgroup known to
213 produce DAPG (marked with a blue box). Taken together, these results show that genetically
214 highly related DAPG-producing *Pseudomonas* can be isolated from the same grassland soil
215 site over a 12 month period.

216

217 **Measuring the frequency of DAPG-producing Pseudomonads in grassland soils**

218 To further explore the populations and frequencies of DAPG-producing Pseudomonads in
219 grassland soils, we sampled soil from three additional grassland sites (an area close to P5
220 labelled P5', P8 and P9) (Materials and methods). In total, we isolated 288 *Pseudomonas*
221 strains as libraries in 96-well microplates from each of the three sites (n = 864). The three
222 libraries were then screened for potential DAPG-producers by replica-plating them onto a
223 KBmalt agar supplemented with X-gal and a lawn of the whole-cell biosensor harbouring
224 pSEVA225::DAPG_{lacZ}. Simultaneously, the libraries were screened on separate plates for
225 production of natural β -galactosidases, and isolates displaying a response were discarded from
226 further analyses. Note that in this experimental setup (using replica-plating), the development
227 of blue halos around DAPG-producing colonies took longer time than when larger aliquots of
228 cultures were spotted on the agar plates. After 48 hours of incubation the biosensor elicited a
229 response to six isolates from P5', five isolates from P8 and 49 isolates from P9 (Table 1).
230 Subsequently, we analysed the colonies displaying a blue halo for the presence of *phlD*, as well
231 as their taxonomy by sequencing part of *rpoD* and aligning it to the database of *Pseudomonas*
232 type strains (9). For P5' and P8, one isolate from each site was confirmed to encode the
233 polyketide synthase responsible for DAPG biosynthesis (Table 1) and both isolates were
234 identified as *P. protegens* (Figure 4). For P9, the 49 isolates with a surrounding blue halo were
235 confirmed to have *phlD*, where 38 of those isolates belonged to *P. kilonensis*, while the
236 remaining 11 were identified as *P. protegens* (Figure 4). The false positives from P5' and P8

237 were subsequently analysed with LC-MS (see Materials and Methods) to confirm the absence
238 of DAPG production. DAPG was not detected in any of the nine isolates.

239

240

241

242 **DISCUSSION**

243 In this study we constructed a highly sensitive whole-cell biosensor for detection and guided
244 isolation of DAPG-producing microorganisms. Utilization of genetic circuits to detect and
245 report on the presence of small molecules is associated with advantages and disadvantages.
246 Two apparent caveats associated with the *in vitro* agar-based biosensor-guided identification
247 used in this study are that it is only viable for cultivable organisms and it requires conditions
248 that allow co-cultivation of the isolate of interest with the biosensor. One advantage of the
249 biosensor is that it is not restricted to a narrow range of genotypes of DAPG-producers, which
250 is a limiting factor of PCR-based approaches. Secondly, with a detection limit of >20 nM *in*
251 *vitro* (Figure 1C) the biosensor may have potential for use *in situ* to identify DAPG production
252 in specific soil niches and thus serve as a promising alternative to chemical identification of
253 DAPG, where the detection limit is in the low micromolar range (13).
254 We show that the biosensor is specific towards detection of DAPG. The biosensor did not
255 respond to PG and elicited a minor response towards MAPG. This minor response was absent
256 from its *lacZ* counterpart, which further demonstrates the sensitivity of the *lux* variant. We
257 realize that we have only used two molecules (PG and MAPG) to represent natural DAPG
258 analogues in our specificity assessment. It remains a possibility that other molecules can elicit
259 a biosensor response. In a study by Yan et al. on the PhlH transcriptional regulator, it was
260 demonstrated that multiple molecules with structural similarities to DAPG could bind to PhlH
261 and induce a response, albeit significantly lower than the response induced by DAPG (5).
262 Likewise, it was found that MAPG induced a minor response in the same study (5).
263 In order to demonstrate the biosensor response to bioavailable DAPG on agar surfaces, we
264 inoculated DAPG producers and non-producers on top of the whole-cell biosensor on agar
265 plates. As expected, a blue halo was observed around the DAPG producers. Additionally, a
266 blue colouring was absent around the non-producers after 24 hours. These findings also

267 correlated with the LCMS analysis. However, a slight blue halo was observed surrounding *P.*
268 *putida* after prolonged incubation (>72 hours), suggesting that one or more compounds are
269 being secreted by this strain during late stationary phase, which interacts with PhlF, thus
270 relieving repression of the reporter gene.

271 Subsequently, the biosensor was utilized for guided identification of DAPG-producing
272 fluorescent *Pseudomonas*. The isolates were selected on $\frac{1}{4}$ KB⁺⁺⁺, which has been shown to be
273 optimal for isolation of fluorescent *Pseudomonas*, including DAPG producers (27). We
274 screened 30 randomly isolated *Pseudomonas* from the P5 site in both 2018 and 2019. DAPG
275 producers were detected in both samplings based on a clear blue halo surrounding their colonies
276 and were identified as *P. kilonensis* species, which are known to produce DAPG (10). Part of
277 the *rpoD* gene was sequenced for all isolates and aligned to a database of *Pseudomonas* type
278 strains (9), which revealed a remarkably similar diversity over a 12 month period (i.e. the same
279 *Pseudomonas* subgroups were sampled at both time points). However, despite the low
280 sampling depth, the species abundance appears to shift from *P. jessenii* to *P. koreensis*. From
281 an ecological point of view, it is of interest to note that the DAPG producers seem to persist
282 over time in relatively similar quantities.

283 Lastly, we sought to optimize the screening assay to a high-throughput 96-well microplate
284 format. We isolated 288 *Pseudomonas* species from each of three soil sites (P5', P8 and P9).
285 The sites are located in a Danish natural reserve (Dyrehaven), thus we argued that they
286 represent pristine grassland soil niches. It is worth noting that bulk soil is an extremely harsh
287 environment with low nutrient availability, which might explain the low amount of CFU g⁻¹ of
288 *Pseudomonas* compared to rhizosphere environments (11–13). We identified 6 and 5 isolates
289 with blue halos around their colonies in P5' and P8, respectively. Yet, only one isolate from
290 each site was confirmed to encode the polyketide synthase responsible for DAPG production.
291 Picard et al. isolated 156 *Pseudomonas* from bulk soil, but no DAPG producers were identified,

292 although DAPG producers were isolated at a later stage from roots of maize plants grown in
293 the same soil (33). It was speculated that DAPG-producing *Pseudomonas* are present in bulk
294 soil in quantities $<2.6 \cdot 10^2$ CFU g⁻¹, which is comparable to the findings obtained in our study
295 of grassland soil at P5' (6×10^1 g⁻¹) and P8 (2×10^1 g⁻¹) (Table 1). In P9, on the other hand,
296 17% of the isolates displayed a blue halo around their colonies and the presence of *phlD* was
297 confirmed for all isolates. However, during the course of our study it became apparent that a
298 deer-feeding site was located near the sampling site, with wheat being the main feed. This could
299 potentially explain the high frequency of DAPG producers in P9, as DAPG-producing
300 *Pseudomonas*, which are known to be associated with the roots of wheat (1), might have
301 translocated into the soil surrounding the feeding site due to animal activities.

302 The false positive isolates from the high-throughput screening (i.e. isolates that resulted in a
303 biosensor response without the presence of DAPG biosynthesis genes) could potentially
304 produce compounds similar to those made by *P. putida* (as described above), which interfere
305 with PhlF. The *phlF* gene encoded by the biosensor was cloned from *P. protegens* CHA0 (22),
306 where it naturally functions as a transcriptional repressor of *phlACBD* (8). The false positives
307 identified in our screen may secrete yet unknown secondary metabolites that interact with the
308 PhlF repressor, thus inducing biosynthesis of DAPG. This finding highlights the possibility for
309 microbe-microbe interactions *in situ* leading to induced DAPG production by adjacent non-
310 producers. Interestingly, we found two isolates belonging to the *P. putida* group in the high-
311 throughput screening, which may indicate that certain species of this group produce molecules
312 that can induce expression of DAPG. Surprisingly, we also identified isolates of *P. koreensis*
313 and *P. mandelii* that elicit a response from the biosensor, which further enhances the potential
314 of yet unexplored microbe-microbe interactions that could be addressed in future studies.

315 In conclusion, this study demonstrates the use of an engineered whole-cell biosensor for guided
316 identification of DAPG-producing microorganisms. This approach surpasses the limits of

317 previous PCR-based and chemical identification methods, although future optimization to
318 further increase sensitivity and reduce unexpected response to false positives might be required.

319

320

321 MATERIALS AND METHODS

322 Strains, media and growth conditions

323 Plasmid cloning and genetic circuit characterization were performed in *Escherichia coli* K12
324 $\Delta lacIZYA$ or *E. coli* CC118- λpir . Cells were cultured in Luria-Bertani (LB) broth (Lennox,
325 Merck, St. Louis, MO, USA) with appropriate antibiotics. The antibiotic concentration used
326 was 25 $\mu\text{g ml}^{-1}$ for kanamycin, 10 $\mu\text{g ml}^{-1}$ for chloramphenicol and 8 $\mu\text{g ml}^{-1}$ for tetracycline.
327 The engineered whole-cell biosensor was cultured by inoculating a single colony in 5 ml LB
328 broth supplemented with kanamycin and incubating overnight at 37°C with shaking (200 rpm).
329 For characterization of the biosensor response to DAPG-producers, control strains were
330 routinely cultured by inoculating a single colony in 5 ml LB broth and incubating overnight at
331 30°C with shaking (200 rpm). Control strains include: *Pseudomonas putida* KT2440,
332 *Pseudomonas protegens* DTU9.1 (previously isolated by our group), *Pseudomonas protegens*
333 DTU9.1 $\Delta phlACBD$ (see below) and *Chromobacterium vaccinii* MWU328.

334

335 Plasmid circuit construction

336 Plasmid construction and DNA manipulation was performed following standard molecular
337 biology techniques. The strain *E. coli* K12 $\Delta lacIZYA$ was transformed with all plasmid
338 constructs by chemical transformation. The plasmid pAJM847 (Accession: MH101727.1),
339 comprising the P_{lacIQ} promoter, *phlF*, the induction operon terminator (IOT) and the P_{phlF}
340 promoter, was a kind gift from Christopher Voigt (22). The genetic circuit from pAJM847 was
341 re-organized into pSEVA225T (Accession: KC847299.1) (23) to obtain the DAPG biosensor.
342 To this end, the fragment containing the P_{lacIQ} promoter, *phlF* and the induction operon
343 terminator was PCR amplified with AvrII and EcoRI overhangs ($P_{lacI_AvrII_fw}$: 5'-
344 TAAGCACCTAGGCGTTTTGGTCCAATGG, Term847_EcoRI_rev: 5'-

345 TGCTTAGAATTCAGCGAGGAAGCACC). Purified PCR product was digested with
346 appropriate restriction enzymes and inserted in pSEVA225T to yield pSEVA225::P_{lacIQ}-*phlF*.
347 Subsequently, the fragment containing the P_{phlF} promoter was PCR amplified with EcoRI and
348 HindIII overhangs (PphlF_EcoRI_fw: 5'-TAAGCAGAATTCCGACGTACGGTGG,
349 PphlF_HindIII_rev: 5'-TGCTTAAAGCTTTATTTCCCCTCTTTCTCTAG). Purified PCR
350 product was restriction-digested and inserted in pSEVA225::P_{lacIQ}-*phlF* to yield the *lacZ*
351 version of the DAPG biosensor (pSEVA225::DAPG_{lacZ}). The *lux* operon (*luxCDABE* from *P.*
352 *luminescens*) was PCR amplified from pUC18-mini-TN7T-Gm-*lux* with HindIII and SpeI
353 overhangs (Lux_HindIII_fw: 5'-GAATTCAAGCTTATGACTAAAAAATTTTCATTC,
354 Lux_SpeI_rev: 5'-GAATTCAGTAGGATATCAACTATCAAAC). Purified PCR
355 product was restriction-digested and inserted in pSEVA225::DAPG_{lacZ} to yield the *lux* version
356 (pSEVA226::DAPG_{lux}).

357

358 **Deletion of *phlACBD* by allelic replacement**

359 To abolish DAPG production in *P. protegens* DTU9.1, the biosynthesis genes, *phlACBD*, were
360 deleted by allelic replacement according to Hmelo et al. (24). In short, DNA fragments directly
361 upstream of *phlA* (Up_F: 5'-ATCCCGTCTAGACAGAGATTTTCGCAGTAAAAAG, Up_R:
362 5'-GGCGGGACAGGCACAGGCAGTCACATTTCTCTTGATTCCATTCTTTTC) and
363 directly downstream of *phlD* (Down_F: 5'-TGTGACTGCCTGTGCCTG, Down_R: 5'-
364 ATCCGGGAGCTCTTGAAAGCCGCCAGCC) were PCR amplified. The fragments were
365 joined by splicing-by-overlap extension PCR with XbaI and SacI overhangs using primers
366 Up_F and Down_R. The purified PCR product was restriction-digested and inserted in pNJ1
367 (25). The resulting plasmid was mobilized into *P. protegens* DTU9.1 via triparental mating
368 with *E. coli* HB101 harbouring the helper plasmid pRK600. Merodiploid transconjugants were

369 initially selected on *Pseudomonas* Isolation Agar (PIA, Merck, St. Louis, MO, USA)
370 supplemented with 50 $\mu\text{g ml}^{-1}$ tetracycline. A second selection was performed on NSLB agar
371 (10 g l^{-1} tryptone, 5 g l^{-1} yeast extract, 15 g l^{-1} Bacto agar) with 15% sucrose. Candidates for
372 successful deletion were confirmed by PCR and verified by Sanger sequencing at Eurofins
373 Genomics.

374

375 **Luminescence dose/response microplate assay**

376 Overnight cultures of the whole-cell biosensor harbouring pSEVA226-DAPG_{lux} were prepared
377 in six biological replicates as described above. 96-well black, clear bottom microplates (In
378 Vitro, Denmark) were prepared with LB broth supplemented with kanamycin and varying
379 concentrations of PG, MAPG or DAPG (0, 0.005, 0.01, 0.02, 0.039, 0.078, 0.156, 0.3125,
380 0.625, 1.25, 2.5, 5, 7.5, 10 and 15 μM). The overnight cultures were diluted to inoculate the
381 microplates to an initial $\text{OD}_{600} = 0.01$. The plates were sealed with a semipermeable membrane
382 (Breathe-Easy, Merck, St. Louis, MO, USA) and incubated in a Cytation5 microplate reader
383 for 5 hours at 37°C with shaking (600 rpm) with continuous measurements of luminescence
384 and absorbance at OD_{600} . This data and any other microplate assay data was collected with the
385 Gen5 2.07 software and exported to Excel 2016 and GraphPad for data analysis.

386

387 **β -galactosidase dose/response microplate assay**

388 Overnight cultures of the whole-cell biosensor harbouring pSEVA225-DAPG_{lacZ} were
389 prepared in triplicate as described above. Transparent 96-well microplates (TPP, Merck, St.
390 Louis, MO, USA) were prepared with LB broth supplemented with kanamycin and varying
391 concentrations of PG, MAPG or DAPG (0, 0.625, 1.25, 2.5, 5, 7.5, 10 and 15 μM). The
392 overnight cultures were diluted to inoculate the microplates to an initial $\text{OD}_{600} = 0.01$. Cultures

were grown for 3 hours at 37°C with shaking (600 rpm), followed by measuring the end-point absorbance at OD₆₀₀. Subsequently, 20 µl from each well was transferred to new transparent 96-well microplates and mixed with 80 µl permeabilization buffer (0.1 M Na₂HPO₄, 0.02 M KCl, 0.002 M MgSO₄, 0.8 mg ml⁻¹ hexadecyltrimethylammonium bromide, 0.4 mg ml⁻¹ sodium deoxycholate, 5.4 µl ml⁻¹ β-mercaptoethanol) (26). The plates were incubated at 30°C with shaking (600 rpm) for 30 minutes to facilitate cell lysis. Next, 28 µl lysed cell culture from each well was transferred to 96-well black microplates and mixed with 172 µl substrate solution (0.06 M Na₂HPO₄, 0.04 M NaH₂PO₄, 1 mg ml⁻¹ o-nitro-phenyl-β-D-galactopyranoside, 2.7 µl ml⁻¹ β-mercaptoethanol) (26). The plates were sealed with a semipermeable membrane (Breathe-Easy, Merck, St. Louis, MO, USA) and incubated in a Cytation5 microplate reader for 16 hours at 37°C with shaking (600 rpm) with continuous measurements of absorbance at OD₄₂₀. Data was collected and analysed, as mentioned above.

Detecting DAPG from bacterial cultures grown on agar surfaces

Overnight cultures of the whole-cell biosensor harbouring pSEVA225-DAPG_{lacZ} and the control strains *P. putida* KT2440, *P. protegens* DTU9.1, *P. protegens* DTU9.1 Δ*phlACBD* and *C. vaccinii* MWU328 were prepared as described above. The biosensor was normalized to OD₆₀₀ = 1.0 and spread on King's agar B supplemented with malt extract (KBmalt) (20 g l⁻¹ Proteose peptone No. 3, 1.5 g l⁻¹ K₂HPO₄, 1.5 g l⁻¹ MgSO₄, 7.5 g l⁻¹ malt extract, 10 ml l⁻¹ glycerol and 20 g l⁻¹ Bacto agar) supplemented with 25 µg ml⁻¹ 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal, Thermo Fisher Scientific). Overnight cultures of the control strains were normalized to OD₆₀₀ = 1.0 and inoculated as 20 µl spots on the agar plates containing the biosensor. Agar plates were incubated at 30°C for 24-96 hours. Plates were inspected for blue halos surrounding the bacterial spots every 24 hours.

Detection of DAPG by LCMS

Overnight cultures of the control strains *P. putida* KT2440, *P. protegens* DTU9.1, *P. protegens* DTU9.1 Δ *phlACBD* and *C. vaccinii* MWU328 were prepared as described above. The cultures were normalized to OD₆₀₀ = 1.0, inoculated as 20 μ l spots on KBmalt and malt agar plates and incubated at 30°C for 24 hours. An agar plug (6 mm diameter) of the bacterial culture was transferred to a vial and extracted with 1 ml of isopropanol:ethyl acetate (1:3, v/v), containing 1% formic acid, under ultrasonication for 60 min. The extracts were then transferred to new vials, evaporated under N₂, and re-dissolved in 200 μ l of methanol for further sonication over 15 min. After centrifugation at 13400 rpm for 3 min, the supernatants were transferred to HPLC vials and subjected to ultrahigh-performance liquid chromatography-high resolution electrospray ionization mass spectrometry (UHPLC-HRESIMS) analysis. UHPLC-HRESIMS was performed on an Agilent Infinity 1290 UHPLC system equipped with a diode array (DAD) detector. UV-visible spectra were recorded from 190 to 640 nm. Liquid chromatography of 1 μ l extract was carried out using an Agilent Poroshell 120 phenyl-hexyl column (2.1 $\times$ 150 mm, 1.9 μ m) at 60°C using acetonitrile and H₂O, both containing 0.02 M formic acid, as mobile phases. Initially, a linear gradient of 10% acetonitrile/H₂O to 100% acetonitrile over 10 minutes was employed, followed by isocratic wash of 100% acetonitrile for 2 minutes. The gradient was returned to 10% acetonitrile/H₂O in 0.1 minute and finally isocratic condition of 10% acetonitrile/H₂O for 1.9 minutes, all at a flow rate of 0.35 ml min⁻¹. Mass-spectrometry detection was performed in positive ionization on an Agilent 6545 QTOF MS equipped with an Agilent Dual Jet Stream electrospray ion source with a drying gas temperature of 250°C, drying gas flow of 8 l min⁻¹, sheath gas temperature of 300°C and sheath gas flow of 12 L min⁻¹. Capillary voltage was set to 4000 V and nozzle voltage to 500 V. Mass-spec data analysis and processing were performed using Agilent MassHunter Qualitative Analysis B.07.00.

442 **Isolation of fluorescent *Pseudomonas* from grassland soil**

443 Three sites of undisturbed grassland were chosen (P5, 55°78'88"N, 12°55'83"E; P8,
444 55°79'52"N, 12°58'06"E; P9, 55°79'12"N, 12°57'51"E). Soil was collected approximately 10
445 centimetres below the grass surface. Five grams of soil were suspended in 30 ml of sterile water
446 and shaken vigorously for 1 min on a Vortex mixer. In order to isolate fluorescent
447 *Pseudomonas* the samples were serially diluted and plated onto ¼ KB⁺⁺⁺ (7.5 g l⁻¹ King's agar
448 B, 10 ml l⁻¹ glycerol, 7.5 g l⁻¹ Bacto agar) supplemented with 100 µg ml⁻¹ cycloheximide, 13
449 µg ml⁻¹ chloramphenicol and 40 µg ml⁻¹ ampicillin, as previously described (27). Agar plates
450 were incubated at 30°C for 48 hours. Fluorescent colonies were identified under UV light and
451 re-streaked on LB agar plates. Species identification of the soil isolates was performed by PCR,
452 amplifying part of the *rpoD* gene with primers (PsEG30F: 5'-ATYGAAATCGCCAARCG,
453 PsEG790R: 5'-CGGTTGATKTCCTTGA) (28). PCR products were purified, sequenced and
454 aligned to a database of 166 known type strains of *Pseudomonas* (9).

456 **Biosensor-guided identification of DAPG-producers from grassland soil**

457 Thirty fluorescent *Pseudomonas* were randomly selected from sample site P5 both in 2018 and
458 2019 as described above. Isolates were cultured overnight in LB broth at 30°C with shaking
459 (200 rpm). An overnight culture of the biosensor harbouring pSEVA225-DAPG_{lacZ} was
460 normalized to OD₆₀₀ = 1.0 and spread on KBmalt plates supplemented with 25 µg ml⁻¹ X-gal.
461 Overnight cultures of the *Pseudomonas* isolates were inoculated on the agar plates as 20 µl
462 spots. Plates were incubated at 30°C for 24-48 hours. Plates were inspected for blue halos
463 surrounding the bacterial spots every 24 hours. The DAPG-producing isolates were identified
464 by PCR-based species identification, as described above. The phylogenetic relationship
465 between the DAPG-producing isolates was determined by analysing a phylogenetic tree with
466 representatives of each *P. fluorescens* subgroup (9). In short, the PCR amplified part of the

467 *rpoD* genes were Sanger sequenced and aligned using the MUSCLE algorithm, followed by
468 construction of a bootstrap consensus tree (500 replicates) by the neighbour-joining method in
469 MEGA X (29).

470

471 **High-throughput screening for DAPG-producers in grassland soil**

472 In 2019, 288 fluorescent *Pseudomonas* were randomly selected from three sample sites (P5',
473 55°78'78"N, 12°56'07"E; P8 and P9 – see coordinates above). Fluorescent colonies were
474 streaked on LB agar OmniTray™ (Nunc™, Nalge Nunc International, Rochester, NY, USA)
475 and incubated for 24 hours at 30°C. Isolates were cultured in transparent 96-well microplates
476 in terrific broth (TB; 12 g l⁻¹ tryptone, 24 g l⁻¹ yeast extract, 0.17 M KH₂PO₄, 0.72 M K₂HPO₄
477 and 5 ml l⁻¹ glycerol). An overnight culture of the biosensor harbouring pSEVA225-DAPG_{lacZ}
478 was normalized to OD₆₀₀ = 0.5 and spread on KBmalt OmniTrays supplemented with 50 µg
479 ml⁻¹ X-gal. The *Pseudomonas* isolates were inoculated on the OmniTrays with sterile
480 replicators. The OmniTrays were incubated at 30°C for 48 hours and inspected for blue halos
481 surrounding the *Pseudomonas* colonies. Candidate isolates exhibiting a blue halo were
482 screened with PCR for the presence of *phlD* with primers (B2BF: 5'-
483 ACCCACC GCAGCATCGTTTATGAGC, BPR4: 5'-
484 CCGCCGGTATGGAAGATGAAAAAGTC) (30). Moreover, *rpoD* was amplified from
485 candidate colonies for species identification with primers PsEG30F and PsEG790R.

486

487 **Data availability**

488 All data are included in the article and the supplemental material. Plasmid constructs and
489 bacterial strains, including *Pseudomonas* isolates, will be made available by the corresponding
490 author upon request.

491 **Acknowledgements**

492 We would like to thank Prof. Victor de Lorenzo for providing the pSEVA plasmid used as
493 vector background for the biosensor. Secondly, we thank Prof. Christopher Voigt for providing
494 the plasmid pAJM847. Moreover, we want to thank Pavelas Sazinas for advice on phylogenetic
495 analysis, as well as Susanne Koefoed for technical assistance. We thank the members of the
496 Centre for Microbial Secondary Metabolites (CeMiSt) for discussions.

497

498

499 **Funding**

500 This study was funded by the Danish National Research Foundation (DNRF137) for the
501 Centre for Microbial Secondary Metabolites.

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- 608
- 609

610 **Figure 1. Design of highly specific and sensitive whole-cell biosensors for DAPG detection.**

611 **A)** Schematic illustration of the genetic circuit representing the DAPG biosensors with varying
612 reporter modules. **B)** Map of the biosensor present on a pSEVA plasmid background with an
613 RK2 replicon (orange), origin of transfer (dark grey) and a kanamycin resistance gene (light
614 green). Terminators (T0, T1 and IOT – dark red) **C)** The response of the whole-cell biosensor
615 harbouring pSEVA226-DAPG_{lux} to DAPG and similar molecules (PG and MAPG) measured
616 in luminescence per OD₆₀₀. **D)** The response of the whole-cell biosensor harbouring
617 pSEVA225-DAPG_{lacZ} to DAPG and similar molecules (PG and MAPG) measured in Miller
618 Units.

619
620 **Figure 2. Detection of DAPG produced by bacterial colonies grown on agar surfaces. A)**

621 Four fluorescent *Pseudomonas* were grown on a lawn of the biosensor with
622 pSEVA225::DAPG_{lacZ} on KBmalt media supplemented with X-gal. Wildtype *P. protegens*
623 CHA0 and DTU9.1 known to produce DAPG elicited a biosensor response after 24 hours,
624 whereas the negative controls *P. putida* KT2440 and a Δ *phlACBD* mutant of *P. protegens*
625 DTU9.1 did not. **B)** Extracted ion chromatograms (EIC) for DAPG (m/z 211.0601 $\pm$ 5 ppm) of
626 the four *Pseudomonas* extracts confirm the production of DAPG after 24 hours by *P. protegens*
627 CHA0 and DTU9.1.

628
629 **Figure 3. Examination of fluorescent *Pseudomonas* from grassland soil for DAPG**

630 **production. A)** 30 fluorescent *Pseudomonas* isolated from grassland soil in 2018/2019 were
631 grown on a lawn of the biosensor with pSEVA225::DAPG_{lacZ} on KBmalt media supplemented
632 with X-gal. The biosensor responded to one isolate from 2018 and two from 2019. **B)** Part of
633 the *rpoD* gene was sequenced and aligned to a database of 165 type strains of *Pseudomonas*.
634 The three isolates eliciting a response from the biosensor were identified as *P. kilonensis*, which

635 is part of the *P. corrugata* subgroup (9). C) An *rpoD*-based Neighbor-Joining tree representing
636 the phylogenetic relationship of the three *P. kilonensis* isolates to the *P. corrugata* subgroup.
637 *P. protegens* CHA0 was included as outlier. A bootstrap consensus tree (500 replicates) of the
638 *rpoD* PCR products was constructed via the Neighbor-Joining method. The bootstrap
639 percentage values are depicted next to each branching point. The *P. corrugata* subgroup is
640 depicted in a green box, whereas the species of this subgroup known to produce DAPG are
641 placed in a blue box, along with the three soil isolates.

642

643 **Figure 4. Taxonomic identification of isolates with a surrounding blue halo in the high-**
644 **throughput screening assay.** For each isolate, part of the *rpoD* gene was sequenced and
645 aligned to a database of 165 type strains of *Pseudomonas*. In both P5' and P8, one isolate was
646 identified as *P. protegens*, whereas the remaining isolates belonged to either the *P. putida* group
647 or *P. koreensis* and *P. mandelii* subgroups of *P. fluorescens*, according to Hesse et al. (9). In
648 P9, the *rpoD* gene of 49 isolates displaying a blue halo was sequenced. Only species of *P.*
649 *kilonensis* and *P. protegens* were identified.

650

TABLE 1. Frequencies of DAPG producers in natural soil microbiomes

	Total CFU/g soil ^a	Blue halo		<i>phlD</i> PCR verified	
		Count	Percentage ^b	Count	CFU/g soil ^c
P5'	$1.7 \cdot 10^4$	6/288	2.08%	1/6	60
P8	$5.7 \cdot 10^3$	5/288	1.74%	1/5	20
P9	$6.9 \cdot 10^3$	49/288	17.01%	49/49	$1.2 \cdot 10^3$

651

a: Total CFU of bacteria cultivable on ¼ KB⁺⁺⁺ media, which is

652

predominantly *Pseudomonas*.

653

b: Percentage refers to the number of isolates displaying a blue halo in

654

relation to the 288 colonies tested.

655

c: CFU/g soil of potential DAPG producers were calculated using the ratio of PCR-

656

confirmed isolates compared to the total number of tested isolates times CFU/g soil

657

of cultivable *Pseudomonas*.

■ *P. protegens*
■ *P. corrugata*
■ *P. putida*
■ *P. koreensis*
■ *P. mandelii*