Fungal-associated molecules induce key genes involved in the biosynthesis of the antifungal secondary metabolites nunamycin and nunapeptin in the biocontrol strain *Pseudomonas fluorescens* In5

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1 Fungal-associated molecules induce key genes involved in the biosynthesis of the antifungal
2 secondary metabolites nunamycin and nunapeptin in the biocontrol strain *Pseudomonas*
3 *fluorescens* In5

4

5 **Running Title: Regulation of cyclic lipopeptides in *P. fluorescens* In5**

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16

17 **Abstract:**

18 *Pseudomonas fluorescens* In5 synthesizes the antifungal cyclic lipopeptides (CLPs) nunamycin and
19 nunapeptin, which are similar in structure and genetic organization to the pseudomonas-derived
20 phytotoxins syringomycin and syringopeptin. Regulation of syringomycin and syringopeptin is
21 dependent on the two-component global regulatory system GacS/GacA, and the SalA, SyrF, and
22 SyrG transcription factors, which activate syringomycin synthesis in response to plant signalling
23 molecules. Previously, we demonstrated that a specific transcription factor, NunF, positively
24 regulates the synthesis of nunamycin and nunapeptin in *P. fluorescens* In5 and that the *nunF* gene is
25 upregulated by fungal-associated molecules. This study focusses on further unravelling the complex
26 regulation governing CLP synthesis in *P. fluorescens* In5. Promoter fusions were used to show that
27 the specific activator NunF is dependent on the global regulator of secondary metabolism GacA and
28 is regulated by fungal-associated molecules and low temperatures. In contrast, GacA is stimulated
29 by plant signalling molecules leading to the hypothesis that *P. fluorescens* is a hyphosphere-
30 associated bacterium encoding transcription factor genes that respond to signals indicating the
31 presence of fungi and oomycetes. Based on these findings, we present a model for how synthesis of
32 nunamycin and nunapeptin is regulated by fungal- and oomycete-associated molecules.

33
34 **Importance:**

35 Cyclic lipopeptide (CLP) synthesis gene clusters in pseudomonads display a high degree of synteny
36 and the structure of the peptides synthesized is very similar. Accordingly, the genomic island
37 encoding the synthesis of syringomycin and syringopeptin in *P. syringae* pv. *syringae* closely
38 resembles that of *P. fluorescens* In5, which code for synthesis of the antifungal and anti-oomycete
39 peptides nunamycin and nunapeptin, respectively. However, the regulation of syringomycin and
40 syringopeptin synthesis is different from that of nunamycin and nunapeptin synthesis. While CLP
41 synthesis in the plant pathogenic *P. syringae* pv. *syringae* is induced by plant signalling molecules,
42 such compounds do not significantly influence synthesis of nunamycin and nunapeptin in *P.*

43 *fluorescens* In5. Instead, fungal-associated molecules positively regulate anti-fungal peptide
44 synthesis in *P. fluorescens* In5 while the synthesis of the global regulator GacA in *P. fluorescens*
45 In5 is positively regulated by plant signal molecules but not fungal-associated molecules.

46

47 Introduction

48 Members of the genus *Pseudomonas* are ubiquitous inhabiting many environments including
49 agricultural soils where they may promote plant growth and health, and suppress plant pathogens
50 (1-4). One of the major mechanisms by which pseudomonads suppress plant pathogens such as
51 fungi and oomycetes is through the production of a variety of secondary metabolites capable of
52 inhibiting fungal growth. A very large number of publications describe bioactive compounds
53 produced by pseudomonads most notably cyclic lipopeptides (CLPs), 2,4-diacetylphloroglucinol
54 (DAPG), phenazines (PHZs), pyrrolnitrin (PRN), pyoluteorin (PLT), 2,5-dialkylresorcinol,
55 quinolones, gluconic acids, rhamnolipids, siderophores, and hydrogen cyanide (4-6). Some groups
56 produce several of the bioactive compounds whereas others only synthesize a selection of the
57 compounds. Zhao and coworkers performed an *in silico* analysis of genome sequences representing
58 a variety of pseudomonads (7). They found that strains of *Pseudomonas protegens* produced CLPs,
59 siderophores, DAPG, PRN, PLT and other bioactive compounds, some strains of *P.*
60 *brassicacearum*, *P. fluorescens*, and *P. thivervalensis* synthesized siderophores, and DAPG but not
61 CLPs, PRN, or PLT, whereas strains affiliated to *P. syringae*, *P. brassicacearum*, *P. fluorescens*, *P.*
62 *corrugata*, and *P. poae* only produced CLPs and siderophores but not DAPG, PRN, or PLT. The
63 CLPs produced by this last group comprise syringomycin/syringopeptin (from *P. syringae* pv.
64 *syringae*) (8), brasmycin/braspeptin (from *Pseudomonas* sp. 11K1) (7), nunamycin/nunapeptin
65 (from *P. fluorescens* In5) (9), cormycin/corpeptin (from *P. corrugata*) (10), and
66 thanamycin/thanapeptin (from *Pseudomonas* sp. SH-C52) (11) (Fig. S1). The peptide encoding
67 genes from these pseudomonads show a high degree of synteny (Fig. S1) and thus, the peptides
68 synthesized are very similar: all the -mycin peptides are cyclic and composed of nine amino acids
69 and the -peptins are 22 amino acid cyclic peptides (7). In addition to genes involved in the synthesis
70 of thanamycin and thanapeptin in *Pseudomonas* sp. SH-C52, the large genomic island also
71 contained genetic information for synthesis of antimicrobial dipeptides, brabantamide A, B, and C
72 (11-12). Mining the genome sequences of pseudomonads similar to *Pseudomonas* sp. SH-C52

revealed similar brabantamides-like synthesis genes (Fig. S1) and in this paper, we also show that *P. fluorescens* In5 is capable of synthesizing brabantamide-like compounds. Synthesis of secondary metabolites in pseudomonads is complex and regulated by a plethora of molecules and systems. The two component global regulatory system GacS/GacA has been shown to activate a number of cellular processes in *Pseudomonas* species (13-15) including synthesis of lipopeptides and other virulence factors (16-19). The GacS/GacA system often works together with the Rsm system (repressor proteins RsmA and RsmE in addition to small RNAs *rsmY* and *rsmZ*) (20, 21) and *N*-acylhomoserine lactone (*N*-AHL)-mediated quorum sensing systems (22, 23). In addition to the overarching GacS/GacA system, other genes may regulate secondary metabolite production, for example special sigma factors (24), D-3-phosphoglycerate dehydrogenase, an Hsp70 protein family chaperone encoded by *dnaK*, and PrtR, an anti-sigma factor and transmembrane activator (25). Finally, genes that encode specific transcriptional regulators are often found in the large genomic islands that comprise all genes necessary for the synthesis of secondary metabolites. In *P. syringae* pv. *syringae* the regulatory genes *sala*, *syrF*, and *syrG* involved in synthesis of syringomycin and syringopeptin are located next to the syringomycin encoding gene, *syrE* (26), and in *P. fluorescens* In5 the *nunF* gene homologous to *syrF* in *P. syringae* pv. *syringae* is situated next to the nunamycin genes *nunD* and *nunE* (Fig. S1) (27). In *P. syringae* pv. *syringae*, the GacS/GacA system positively regulates the expression of the *sala* gene (18) and the *sala* gene product positively regulates the *syrF* and *syrG* genes which in turn positively regulate syringomycin synthesis genes (29). In an early paper from 1991, Mo and Gross showed that one of the genes, *syrB*, involved in syringomycin synthesis was induced by plant signal molecules in accordance with the fact that *P. syringae* pv. *syringae* is a plant pathogen (29). This observation was confirmed in a later publication in which Wang et al. showed by microarray and promotor fusion analyses that the whole *syr-syp* region was upregulated by the plant phenolic glucosides arbutin, salicin and phenyl- β -D-glucopyranoside (30).

We have previously shown that in *P. fluorescens* In5, NunF, a homolog to SyrF, positively regulates synthesis of both nunamycin and nunapeptin and that *nunF* expression was strongly upregulated in response to both carbon sources indicating the presence of a fungus (27) and during interaction with the fungus *Fusarium graminearum* (31). In this study, we investigate the induction pattern of In5 *nunF* and *gacA* in relation to fungal and plant signal molecules, and we show that In5 *gacA* is slightly upregulated by arbutin but not by fungal molecules whereas *nunF* is upregulated only by fungal signaling molecules but not by plant phenolics. Finally, we summarize our results in a model for how nunamycin/nunapeptin is regulated when the bacterium comes into contact with a fungus.

Results and Discussion

GacA and NunF are positive regulators of nunamycin and nunapeptin

Comparison of genomic islands encoding cyclic peptides in different *Pseudomonas* strains reveals a large degree of synteny between the regions that encode the synthesis of e.g. nunamycin/-peptin, brasmycin/-peptin, syringomycin/-peptin, thanamycin/-peptin, and cormycin/-peptin (Supplementary Fig. S1). Not only nonribosomal peptide synthetase genes show synteny, regulatory genes that typically are located adjacent to the synthetase genes also display synteny (7, 9, 11). In *P. syringae* pv. *syringae* B301D, production of syringomycin and syringopeptin was shown to be controlled by the LuxR-type regulatory genes *salA*, *syrF*, and *syrG* (20) and in *P. fluorescens* In5, *nunF*, a LuxR homolog to *syrF*, was shown to be one of the key regulators of nunamycin and nunapeptin synthesis (27).

Here, we show that synthesis of nunamycin, nunapeptin, and brabantamides is regulated by the Gac-system. Fig 1A shows that nunamycin, nunapeptin, and brabantamide-like compounds are produced in a *P. fluorescens* In5 wild type strain but not in a *gacA* knock-out mutant, resulting in

124 inability of the mutant to inhibit the growth of both fungi and oomycetes (Fig. 1B). In a previous
125 publication, we showed that synthesis of nunamycin and nunapectin was also dependent on the
126 presence of an intact *nunF* gene, and we subsequently demonstrated that *nunF*-promotor::*mCherry*
127 fusions can be used to monitor the expression of the gene encoding the activator NunF (27). We
128 have therefore used the *nunF*::*mCherry* and *gacA*::*mCherry* fusions to examine the transcriptional
129 activity from the *nunF* and *gacA* promoters in relation to the genetic background and supplemented
130 carbon source. Fig. 1C shows that the *nunF* promotor was switched on after ca. 13-14 hours after
131 inoculation and induction in the wild type strain but it was totally inactive in a *gacA* mutant.
132 Activity of the *nunF* promotor was lowered to ca. 14% in a *nunF* knock out mutant indicating that
133 NunF was positively autoregulating its own expression. Expression from the *gacA* promotor was
134 analyzed similarly. In wild type cells, the *gacA* promotor was gradually activated ca. 9 hours after
135 inoculation and induction and transcription continued for ca. 9-10 hours in contrast to transcription
136 from the *nunF* promotor that only lasted ca. 3 hours. The *gacA* promotor showed no dependency on
137 *nunF* since the level of mCherry fluorescence in a *nunF* knock out mutant was similar to that in
138 wild type cells. However, the *gacA* promotor was upregulated ca. 100% in a *gacA* mutant indicating
139 that synthesis of GacA was also autoregulated but in a negative way with GacA acting as a
140 repressor of its own expression (Fig. 1C).

141

142 *Regulation of gacA and nunF promoters by plant and fungal associated molecules*

143 Mo and Gross (1991) showed that plant signal molecules activated the *sydB* gene, which is required
144 for syringomycin production by *P. syringae* pv. *syringae*, and we demonstrated that in *P.*
145 *fluorescens* In5 the *nunF* gene, which is an activator for synthesis of nunamycin and nunapectin,
146 may be regulated by fungal-associated molecules (27, 29). Here, we analyze the activity of the *gacA*
147 and *nunF* promoters from strain In5 in relation to fungal (trehalose, glycerol) and plant (arbutin,
148 salicin, phenyl- β -D-glucopyranoside) associated molecules. Fig. 2A shows that the *gacA* promotor
149 from *P. fluorescens* In5 responded to the plant signal molecules, arbutin and phenyl- β -D-

150 glucopyranoside, whereas the fungal associated molecule trehalose showed no induction as the
151 expression pattern was similar to that of water or DMSO addition. The *nunF* promotor on the other
152 hand showed no induction with the plant signal molecules, arbutin, salicin, or phenyl- β -D-
153 glucopyranoside but was induced by trehalose (Fig. 2B) and glycerol (Fig. 5). Helicin and esculin
154 that showed no activity on the *syrB* promotor in the Mo and Gross study (29) were also tested in
155 this study but no induction of *nunF* was observed (data not shown).

156
157 *Crude fungal extracts upregulate nunF but not gacA promoters in P. fluorescens In5.*

158 Since fungal but not plant-associated molecules induced the *nunF* promotor, we tested if crude
159 fungal extracts could also activate the promotor. Fig. 3A shows that the *gacA* promotor was not
160 induced by extracts derived from the plant pathogen *Fusarium culmorum* or the fungal biocontrol
161 agents *Clonostachys rosea* or *Trichoderma harzianum*. However, the *nunF* promotor was
162 significantly upregulated by extracts from all three fungi, irrespectively if they were pathogenic or
163 non-pathogenic. Heat inactivation of the extracts lowered the level of induction to ca. 60-70% of the
164 untreated extracts, but the level was still ca. 100% higher than activation with trehalose (Fig. 3B).
165 Similar results were obtained when testing extracts obtained from fungal phytopathogen
166 *Rhizoctonia solani* and the plant pathogenic oomycete *Pythium aphanidermatum* (Fig. S2)

167
168 *nunF and syrF promoters respond differently to fungal extracts and molecules.*

169 NunF and SyrF have been shown to activate the synthesis of nunamycin/-peptin and syringomycin/-
170 peptin, respectively in *P. fluorescens* In5 and *P. syringae* pv. *syringae* (26-28). We cloned the *syrF*
171 promotor from *P. syringae* pv. *syringae* B728a in a promotor::*mCherry* fusion vector similar to the
172 vector with *nunF* promotor::*mCherry* fusion and transformed the *syrF* and *nunF* promotor fusions
173 into *P. syringae* and *P. fluorescens* In5, respectively in order to compare the induction of *syrF*
174 compared to that of *nunF*. As observed previously, the *nunF* promotor was induced by a *Fusarium*-
175 derived extract and by trehalose (Figure 4A). The *syrF* promotor in *P. syringae* displayed a lower

176 induction by the fungal extract and hardly any induction with the fungal associated molecule
177 trehalose.

178

179 *nunF* promotor expression and production of nunamycin, nunapectin, and brabantamides is
180 temperature dependent.

181 All the above experiments were conducted at 28°C , the optimal growth temperature for *P.*
182 *fluorescens* In5. However, previous experiments have shown that inhibition of fungi and oomycetes
183 is more efficient at 10-15°C compared to 20°C (32). As a result, we also investigated the effect of
184 temperature on the expression of the *nunF* promotor again using promotor::*mCherry* fusions, and
185 quantified the production of nunamycin, nunapectin, and brabantamide-like compounds using LC-
186 MS. Four experiments were run in parallel at 10°C, 15°C, 20°C, and 28°C and promotor activities
187 were followed over ca. 47 hours and at the end, secondary metabolites were measured by LC-MS.
188 The *nunF* promotor fusions showed maximal induction at 20°C, ca. 75% at 15°C and ca. 20-25%
189 activity was measured at 28°C (Fig. 5). At 10°C, the cells grew very slowly and no induction was
190 observed after 47 hours. Similarly, expression from the *gacA* promotor was measured at the four
191 temperatures. No expression from the *gacA* promotor could be monitored at 10°C, 15°C, or 20°C
192 whereas at 28°C, *gacA* expression was observed as previously described (Figs. 1, 2, 3). The fact that
193 only the *nunF* promotor was induced at 10°C, 15°C, or 20°C, temperatures normally found in
194 Greenlandic soils, could indicate that NunF is the real activator of production of nunamycin,
195 nunapectin, and brabantamides in *P. fluorescens* In5. GacA, although essential for peptide
196 production (Fig. 1), may serve other global functions. Also shown in Fig. 5 is induction with the
197 fungal associated molecules, trehalose and glycerol, and with glucose. The *nunF* promotor fusion
198 was upregulated with all three compounds whereas the *gacA* promotor was not induced. Glucose
199 showed the highest induction of *nunF* at 15°C and at 20°C, whereas glycerol was the best inducer at
200 28°C. The reason for this difference could be due to different rates of uptake of glucose, glycerol,
201 and trehalose and/or different metabolic efficiencies.

202

203 In a parallel experiment, cells were cultivated at 10°C, 15°C, 20°C, and 28°C and supplemented
204 with either glucose, trehalose, or glycerol. Supernatants were extracted and the presence of
205 nunamycin, nunapeptin, and brabantamide-like compounds was measured by LC-MS. Fig. 6 shows
206 that all three secondary metabolites were produced with glucose, glycerol, and trehalose as
207 inducers. The optimal temperature for production was at 15°C followed by 10°C and 20°C. No or
208 almost no synthesis of nunamycin, nunapeptin or brabantamide-like compounds was observed at
209 28°C. This result supports the previous observation that maximal inhibition of fungal and oomycete
210 growth was at 10-15°C but is in contradiction with the promotor fusion results above. However, the
211 promotor fusion experiments measure transcription, whereas LC-MS and inhibition experiments
212 also monitor post-translational processes notably ribosomal translation of the large non-ribosomal
213 peptide synthetase complexes, non-ribosomal peptide synthesis, and transport of the peptides.

214

215 *Model for synthesis of secondary metabolites in P. fluorescens In5 interacting with fungi.*

216 *P. syringae* pv. *syringae* is a plant pathogen and thus, synthesis of the phytotoxins syringomycin
217 and syringopeptin is induced by plant signalling molecules. In contrast, *P. fluorescens* In5, a fungal
218 pathogen, does not respond to plant-associated molecules but rather to molecules indicating the
219 presence of fungi and/or oomycetes. The model below (Fig. 7) explains how synthesis of
220 nunamycin and nunapeptin is regulated as a consequence of interaction between the bacterium and
221 its host fungus.

222 *Step 1: Bacterium and fungus are far away from each other.* Neither glycerol or trehalose (or
223 glucose) are common in soil since they are readily metabolized by bacteria.

224 In bacterium: NunF and GacA regulators and nunamycin and nunapeptin are expressed
225 constitutively at very low levels.

226 In fungus: The main carbon storage is fat. Glucose, glycerol, and trehalose concentrations
227 are relatively low. Turgor is high.

228 *Step 2: Bacterium and fungus approach each other and come close.*

229 In bacterium: GacA is expressed at low, constitutive level; NunF is induced by the leaking
230 glucose, glycerol, and trehalose from fungal hyphae. Nunamycin and nunapectin still
231 expressed constitutively at very low level.

232 In fungus: The membrane starts to leak. Fat is converted to glycerol to try to restore turgor
233 pressure and trehalose is synthesized to protect membranes and proteins from apparent
234 drying. Glycerol and trehalose (and glucose) leaks out of the fungus and is taken up by the
235 bacterium.

236 *Step 3: Bacterium and fungus have been in close contact for some time.*

237 In bacterium: GacA is still expressed at low, constitutive level; NunF is expressed at high
238 level due to the leaking glucose, glycerol, and trehalose and induces production at high
239 levels and export of nunamycin and nunapectin.

240 In fungus: The membrane leaks more. The fungus tries to restore turgor pressure as before
241 but the now more leaking membrane makes turgor pressure drop. The fungus starts to die
242 while the bacterium growth rate increases.

243

244 *Summary*

245 In this study, promoters from *gacA* and *nunF* genes that regulate CLP synthesis in *P. fluorescens*
246 In5 were fused to a reporter gene encoding the red fluorescent protein mCherry. *P. fluorescens* In5
247 cells harboring the promotor fusions were cultivated in the presence of different carbohydrates and
248 fungal extracts and the expression from the promoters was studied by measuring the fluorescence
249 generated. The promotor expressing the specific activator, NunF, was shown to be dependent on the
250 global regulator of secondary metabolism, GacA, and it was regulated by fungal-associated
251 molecules and low temperatures. In contrast, GacA was stimulated by plant signalling molecules. In
252 a parallel experiment with the In5 *nunF* promotor and the homologous *syrF* promotor from the
253 closely related *P. syringae* pv. *syringae*, it was shown that the *syrF* promotor responded much less

254 to fungal extracts and not at all to trehalose, one of the molecules indicating the presence of fungi.
255 This let us to hypothesize that *P. fluorescens* In5 is a hyphosphere-associated bacterium encoding
256 transcription factor genes that respond to signals indicating the presence of fungi and oomycetes,
257 whereas *P. syringae* pv. *syringae* as previously shown is a plant pathogen.

258 **Materials and Methods**

259 *Bacterial and fungal strains*

260 Strains used in this study are listed in Table 1. Bacterial strains were routinely cultured in liquid or
261 solid (1.5% agar) Lysogeny Broth (LB) medium at 28°C if no other specific temperature is
262 mentioned. Fungal strains were routinely cultured on potato dextrose agar (PDA) at 25°C.

264 *Insertional mutagenesis of gacA by homologous recombination*

265 Gene knockout by homologous recombination of *gacA* was carried out as previously described²⁷.
266 Primers used for insertional mutagenesis of *gacA* were used to amplify the *gacA* gene from *P.*
267 *fluorescens* In5 and gentamicin resistance gene from pSEVA641. Primers used were block 1: 5'-
268 CCATGATTACGCCAAGCTCTAGGGATAACAGGGTAATCCCGACCTGTTATGGCTGGAT
269 GAAGC-3' and
270 5'-ATCGTAATTATTGGGGACCCGAATGGCCTGGACCATTTCG-3', block 2: 5'-
271 GGAAATGGTCCAGGCCATTTCGGGTCCCCAATAATTACGATTAAATTTGACATAAG-3'
272 and 5'-TTGCCC GGCAAATACCAGGCGGACCGTTGTCCAATTTACCGAAC-3', block 3: 5'-
273 GGTA AATTGGACAACGGTCCGCCTGGTATTTGCCGGGC-3'
274 and 5'-
275 GACGGCCAGTGCCAAGCTCATTACCCTGTTATCCCTACCCCTACCAGCCCGACGCAC-3'.
276 The blunt-end-amplified fragments were subsequently ligated using Gibson assembly ® (NEB) into
277 *Bam*HI-digested pEX100T and subsequently transformed into electrocompetent *P. fluorescens* In5
278 cells as previously described²⁷.
279

280 *Construction of mCherry based reporter of gacA gene expression*

281 A reporter strain of *P. fluorescens* In5 harbouring the *gacA* promoter region fused to mCherry was
282 constructed as previously described³³. The primers used were 5'-
283 GACGCAAATCAGCGCGTTGTGGCCGGATCCTCTAGAGCACGTAACCGAAGCATTTCAG
284 -3' and 5'-
285 GCTCACCATATGTTTTTCCTCCTAAGCTTGCATGCTCGTGTAATGCCTGTACGAACG-3'.
286 The resultant construct was then transformed into *P. fluorescens* WT and *gacA* mutant as previously
287 described³³.

288
289 *Preparation of plant- and fungal-associated molecules*

290 The plant phenolic β -glucosides arbutin, sialicin, phenyl- β -D- glucopyranoside, esculin and helicin
291 were purchased from Sigma-Aldrich. All compounds were dissolved in DMSO except for arbutin
292 which was dissolved in sterile water. Fungal extracts from four different fungi (*Rhizoctonia solani*,
293 *Fusarium culmorum*, *Clonostachys rosea*, *Trichoderma harzianum*) and the oomycete *Pythium*
294 *aphanidermatum* were prepared as follows. Fungi were cultured on PDA plates and incubated for 5-
295 7 days at 25°C. Fungal mycelia were then scraped off each plate and resuspended in 500 μ l sterile
296 water. Cell lysis was performed in a FastPrep-24TM 5G bead beater (MP Biomedicals) using glass
297 beads size 212–300 μ m/425–600 μ m in the ratio 1:1 (Sigma-Aldrich). After cell disruption, the
298 lysate was transferred to sterile 1.5 ml sterile tube and centrifuged 5 mins at 13, 000 g and the
299 supernatant subsequently used in bioreporter assays. Heat inactivation of fungal extracts was
300 performed by heat treatment at 95°C for 10 minutes with a subsequent centrifugation step at 13,000
301 g for 5 minutes.

302
303 *Biomonitoring of gacA and nunF*

304 A microtiter plate reader assay was used to monitor expression of the *gacA* and *nunF* genes.
305 Reporter strains carrying either *gacA* or *nunF* on the dual-fluorescence reporter plasmid

pSEVA237RB were assayed as follows. Bioreporter strains were grown in 10 ml Defined Fusarium Medium (DFM)²⁷ supplemented with 0.5% cellobiose shaking at 200 rpm 28°C overnight. Cells were washed twice with 0.9% wv⁻¹ NaCl and resuspended in 0.9% wv⁻¹ NaCl to a final OD_{600nm} of 0.1. For testing plant phenolic compounds the following was added to each well; 178 µl DFM supplemented with 0.05% cellobiose and 2 µl of plant phenolic compounds (100 µM final concentration) or 2 µl of either trehalose (100 µM final concentration), DMSO (control) or water (control), and 20 µl cells or 0.9% wv⁻¹ NaCl (control) with an OD_{600nm} of 0.1 (final OD_{600nm}= 0.01). For testing fungal extracts compounds the following was added to each well: 173 µl DFM supplemented with 0.05% cellobiose and 7µl of fungal extract or water (control) and 20 µl cells or 0.9% wv⁻¹ NaCl (control) with an OD_{600nm} of 0.1. Growth and fluorescence were measured every hour for 48 hours at 28°C in FLUOstar Omega Microplate Reader (BMG LABTECH). Analysis of *gacA* and *nunF* gene expression for all experiments were performed using biological triplicates. The average and the standard error of the mean (SE) was calculated and the graphs show the average and the average plus or minus SE.

Culturing experiment with different carbon sources at 10°C, 15°C, 20°C and 28°C

To investigate the effect of different carbon sources on secondary metabolite production, *P. fluorescens* In5 was grown overnight in DFM supplemented with 0.5% wv⁻¹ cellobiose, shaking 200 rpm at 28°C. Cells were washed twice with 0.9% wv⁻¹ NaCl and resuspended to an OD_{600nm}=1.0 and 2 ml added to 100 ml Erlenmeyer flasks containing either 0.5% wv⁻¹ trehalose, glycerol, cellobiose, and glucose. Flasks were incubated 48 hours shaking 200 rpm at either 10°C, 15°C, 20°C, or 28°C. Analysis of secondary metabolite production was performed using biological triplicates. Peptide extraction and Liquid Chromatography – High Resolution Mass Spectrometry (LC-HRMS) Metabolite extraction and LC-HRMS analysis was conducted as described previously²⁷. Relative abundance represent the peak area in the chromatogram of the extracted ion per total phospholipid

331 (per cell). Total phospholipids is an accepted measure of living biomass since this it is proportional
332 to total membrane area.

333

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341 **Conflict of interest**

342 The authors declare no conflict of interest.

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- 452

453 **Legends to Figures:**

454 **Fig. 1. Expression from the *nunF* promotor, synthesis of nunamycin, nunapeptin, and**
455 **brabantamides, and antifungal activity is dependent on the GacA product. A,** Production of
456 nunapeptin, nunamycin, and brabantamide-like compounds in wild type *P. fluorescens* In5 (1) and
457 in *gacA* knock out mutant (2). Y-axis shows ion counts, *i.e.* the peak area of extracted ion
458 chromatograms for each of the specific analytes. Triplicate cultures were extracted and the
459 concentration of nunapeptin, nunamycin, and brabantamides was measured. **B,** Inhibition of
460 *Rhizoctonia solani* hyphal growth in the presence of wild type (WT) *P. fluorescens* In5 cells and
461 *gacA* knock out mutant ($\Delta gacA$) cells. The growth of *R. solani* hyphae in the presence and in the
462 absence of *P. fluorescens* In5 was measured and the percentage inhibition of radial growth (PIRG)
463 was calculated. **C,** Analysis of *nunF* (P_{nunF}) and *gacA* (P_{gacA}) promotors in *P. fluorescens* In5
464 wild type (WT) and *gacA* knock out mutant ($\Delta gacA$) cells using promotor::*mCherry* promotor
465 fusions. Cells were grown at 28°C in DFM medium supplemented with glucose (0.04% w/v) in a 96
466 well microtiter format with shaking. Growth and mCherry fluorescence was measured every hour
467 for 47 hours in a FLUOstar Omega Microplate Reader. Lines represent average of three biological
468 repetitions. Solid lines show the averages of fluorescence per cell and the dotted lines in the same
469 color represent the average plus/minus the standard error of the mean.

470

471 **Fig. 2. Response of *gacA* and *nunF* promotors to fungal and plant associated molecules. *gacA***
472 **(A) and *nunF* (B) promotors fused to *mCherry* and analyzed in *P. fluorescens* In5 cultivated in 96-**
473 **well microtiter plates in DFM supplemented with plant phenolics (arbutin, salicin, or phenyl- β -D-**
474 **glucopyranoside (“phenol” in the legend next to the curves)), fungal associated trehalose, or**
475 **glucose. Negative controls were blank, water (arbutin is dissolved in water), and DMSO (salicin**
476 **and phenyl- β -D-glucopyranoside are dissolved in DMSO). Growth and fluorescence were measured**
477 **every hour for 47 hours at 28°C in a FLUOstar Omega Microplate Reader. Lines represent average**

478 of three biological repetitions. Solid lines show the averages of fluorescence per cell and the dotted
479 lines in the same color represent the average plus/minus the standard error of the mean.

480

481

482 **Fig. 3. Response of *gacA* and *nunF* promoters to fungal extracts.** *gacA* (A) and *nunF* (B)
483 promoters fused to *mCherry* and analyzed in *P. fluorescens* In5 cultivated in 96-well microtiter
484 plates in DFM supplemented with extracts from *Fusarium culmorum* (F.c.), *Trichoderma*
485 *harzianum* (T.h.), and *Clonostachys rosea* (C.r.). Positive (trehalose) and negative (water, blank)
486 controls were included. T.h. inact. and C.r. inact. indicate fungal extracts inactivated by heating to
487 95°C for 10 minutes. Growth and fluorescence were measured every hour for 47 hours at 28°C in a
488 FLUOstar Omega Microplate Reader. Lines represent average of three biological repetitions. Solid
489 lines show the averages of fluorescence per cell and the dotted lines in the same color represent the
490 average plus/minus the standard error of the mean.

491 .

492

493 **Fig. 4. Response of *P. fluorescens* In5 *nunF* and *P. syringae* pv. *syringae* *syrF* promoters to a**
494 **fungal extract, plant phenolics, trehalose, and glucose.** *nunF* (A) and *syrF* (B) promoters fused
495 to *mCherry* and analyzed in *P. fluorescens* In5 and *P. syringae*, respectively. Cells were cultivated
496 in 96-well microtiter plates in DFM supplemented with plant phenolics (arbutin or phenyl-β-D-
497 glucopyranoside (“phenol” in the legend next to the curves)), fungal associated trehalose, or
498 glucose. Negative controls were blank and water. Growth and fluorescence were measured every
499 hour for 47 hours at 28°C in a FLUOstar Omega Microplate Reader. Lines represent average of
500 three biological repetitions. Solid lines show the averages of fluorescence per cell and the dotted
501 lines in the same color represent the average plus/minus the standard error of the mean.

502

503

504 **Fig. 5. Influence of temperature on *P. fluorescens* In5 *gacA* and *nunF* promoters.** *nunF* (A, C,
505 E, G) and *gacA* (B, D, F, H) promoters fused to *mCherry* and analyzed in *P. fluorescens* In5
506 cultivated in 96-well microtiter plates in DFM supplemented with 0.5% wv-1 trehalose, glycerol, or
507 glucose. Cells were cultivated at 10°C (A, B), 15°C (C, D), 20°C (E, F), and 28°C (G, H). Growth
508 and fluorescence were measured every hour for 47 hours in a FLUOstar Omega Microplate Reader.
509 X axis, hours; Y axis, mCherry/cell density. Lines represent average of three biological repetitions.
510 Solid lines show the averages of fluorescence per cell and the dotted lines in the same color
511 represent the average plus/minus the standard error of the mean.

512

513 **Fig. 6. Effect of carbon source and temperature on secondary metabolite production by *P.***
514 ***fluorescens* In5.** *P. fluorescens* In5 was grown overnight in DFM supplemented with 0.5% wv-1
515 cellobiose, shaking 200 rpm at 28°C. Cells were washed twice with 0.9% wv-1 NaCl and
516 resuspended to an OD_{600nm}=1.0 and 2 mls added to 100 ml Erlenmeyer flasks containing either
517 0.5% wv-1 trehalose, glycerol, or glucose. Flasks were incubated 48 hours shaking 200 rpm at
518 either 10°C, 15°C, 20°C, or 28°C. Production of nunapeptin (A), nunamycin (B) and brabantamides
519 (C) was performed using biological triplicates. Bars represent the average of the triplicates,
520 triangles are average plus standard error of the mean (SE), and squares are average minus SE.
521 Peptide extraction and Liquid Chromatography – High Resolution Mass Spectrometry (LC-HRMS)
522 analysis was performed as described previously (27). Relative abundance represent the peak area in
523 the chromatogram of the extracted ion per total phospholipid.

524

525 **Fig. 7. Model for how synthesis of nunamycin and nunapeptin is regulated.** In step 1 (1), the
526 fungus and *P. fluorescens* In5 (In5) are far away from each other. *P. fluorescens* In5 synthesizes
527 constitutively small amounts of GacA, NunF, nunamycin and nunapeptin. The main carbon storage

528 in the fungus is fat. Concentration of glucose (Glu), trehalose (Tre) and glycerol (Gly) is low and
529 turgor is high. The bacterium and fungus have come closer in step 2 (2) where nunamycin and
530 nunapeptin are still produced constitutively at low levels. Nunamycin and nunapeptin permeabilize
531 the fungal cell membrane and the fungus starts to leak cellular constituents. NunF is induced by
532 leaking glucose, glycerol, and trehalose from the fungal hyphae whereas GacA is expressed at low,
533 constitutive level. The fungus tries to restore turgor pressure by converting fat to glycerol. At the
534 same time, trehalose is synthesized in order to protect membranes and proteins from drying.
535 Glycerol, trehalose and glucose leak out of the cell and are being taken up by the bacterium. The
536 uptake of compounds from step 2 triggers upregulation of nunamycin and nunapeptin synthesis and
537 possibly also export of the peptides in step 3 (3). GacA is still expressed at low, constitutive level,
538 but leaking glucose, glycerol, and trehalose induce NunF expression resulting in increased amounts
539 of nunamycin and nunapeptin leading to further permeabilization of the membrane and leakage of
540 more intracellular components. The fungus tries to stabilize turgor pressure as in step 2, but as the
541 membrane is compromised by increased amounts of nunamycin and nunapeptin, the turgor pressure
542 drops and the fungus starts to die while the bacterial growth rate increases.

543

Table 1 Strains and plasmids used in this study

Strains	Relevant Characteristics	Reference
Plasmid		
pUX-BF13	RK6 replicon-based helper plasmid; Ap ^r	34
pEX100T	<i>oriT bla sacB</i>	9
pEX100T:: <i>gacA</i> ::Gm ^r	<i>oriT bla sacB gacA</i> ; Gm ^r Amp ^r	This study
pSEVA237RB	Kan ^r ; mCherry and EBF2 genes	33
pSEVA237RB:: <i>P_{gyrB}</i>	Kan ^r ; mCherry gene, <i>gyrB</i> promoter (<i>P_{gyrB}</i>) fused to EBF2	33
pSEVA237R:: <i>P_{gacA}</i> B:: <i>P_{gyrB}</i>	Kan ^r ; <i>gacA</i> promoter (<i>P_{gacA}</i>) fused to mCherry gene, <i>P_{gyrB}::EBF2</i>	This study
pSEVA237R:: <i>P_{nunF}</i> B:: <i>P_{gyrB}</i>	Kan ^r ; <i>nunF</i> promoter (<i>P_{nunF}</i>) fused to mCherry gene, <i>P_{gyrB>::EBF2}</i>	33
Bacterial		
<i>Escherichia coli</i> DH5	<i>endA1 hsdR17 supE44 thi-1 recA1 U169 deoR</i>	NEB, UK
<i>Pseudomonas fluorescens</i> In5	Wild type	9
<i>P. fluorescens</i> In5	Δ <i>nunF</i>	31
<i>P. fluorescens</i> In5	Δ <i>gacA</i>	This study
Fungal		
<i>Rhizoctonia solani</i>	Wild type	32
<i>Pythium aphanidermatum</i>	Wild type	32
<i>Fusarium culmorum</i>	Wild type	35
<i>Trichoderma harzianum</i>	Wild type	37
<i>Clonostachys rosea</i>	Wild type	36

A **B**