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5 **A synthetic system that senses *Candida albicans* and inhibits virulence**

6 **factors**

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19 **Abstract**

20 Due to a limited set of antifungals available and problems in early diagnosis invasive fungal

21 infections caused by *Candida* species are among the most common hospital-acquired

22 infections with staggering mortality rates. Here, we describe an engineered system able to

23 sense and respond to the fungal pathogen *Candida albicans*, the most common cause of

24 candidemia. In doing so, we identified hydroxyphenylacetic acid (HPA) as a novel molecule

25 secreted by *C. albicans*. Furthermore, we engineered *E. coli* to be able to sense HPA

26 produced by *C. albicans*. Finally, we constructed a sense-and-respond system by coupling

27 the *C. albicans* sensor to the production of an inhibitor of hypha formation thereby reducing

28 filamentation, virulence factor expression and fungal-induced epithelial damage. This system

29 could be used as a basis for the development of novel prophylactic approaches to prevent

30 fungal infections.

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33 **Introduction**

34 Fungal pathogens cause diverse types of infections ranging from superficial to systemic and

35 claim about 1.5 million lives per year[1]. *Candida* species are among the most common

36 opportunistic fungal pathogens and represent the fourth-leading cause of hospital-acquired

37 bloodstream infections with mortality rates of more than 40% in immunocompromised

38 patients[1–4].

39   *Candida albicans* represents the main cause of candidemia, being responsible for

40 more than 40% of all cases worldwide[2]. *C. albicans* is a commensal colonizing the

41 gastrointestinal and genitourinary tracts of healthy individuals[5]. However, under

42 immunocompromised conditions, *C. albicans* can penetrate epithelia and cause severe

43 systemic infections[6]. A key virulence factor of *C. albicans* is its morphologic plasticity; it can

44 reversibly switch between a yeast and a filamentous or true hyphal morphology depending on

45 the environmental conditions. On epithelial surfaces, initial adhesion and subsequent

46 filamentation are required for efficient epithelial penetration[7]. In addition, several virulence

47 factors such as Candidalysin, a peptide that damages epithelial cells, are produced

48 exclusively by the hyphal form[8].

49   Difficulties associated with rapid and reliable diagnosis as well as lack of specific

50 disease manifestations contribute to the high mortality rates of systemic fungal infections[9].

51 Antifungal prophylaxis is currently applied to high risk individuals. While effective in preventing

52   *Candida* infections[10,11], prolonged prophylaxis can lead to the emergence of resistant strains

53   or to an increase of species, that are intrinsically resistant to the limited set of antifungals

54   available[10,12]. Development of novel antifungals with fungistatic or fungicidal activity is limited

55   by the relative dearth of specific targets in these eukaryotic pathogens. Thus, more recently

56   approaches targeting specific virulence factors have been gaining interest. This strategy can

57   increase the number of potential targets, that are specific to the pathogen, and will exert less

58   evolutionary pressure for the development of resistance compared to growth inhibition. In the

59   case of *C. albicans*, inhibition of filamentation represents an important target because it could

60   prevent physical penetration of epithelia as well as expression of hypha-specific virulence

61   genes[7].

62   A number of studies have shown that members of the human microbiota can inhibit

63   hypha formation by *C. albicans*[13–16]. However, the inhibitory substance is usually produced

64   constitutively and does not represent a specific response to the presence of the fungus. One

65   example of a bacterial secreted molecule that efficiently blocks hypha formation by *C.*

66   *albicans*, is cis-2-dodecenoic acid (BDSF), a diffusible signal factor that is produced by

67   *Burkholderia cenocepacia*[13].

68   Synthetic biology approaches using genetically engineered bacteria have

69   demonstrated great potential in the prophylaxis or treatment of cancer, metabolic disorders

70   and infectious diseases[17–20]. Using engineered microbes offers several advantages over

71   traditional therapeutics including lower cost and higher specificity due to more targeted

72   treatment[17]. A number of approaches using engineered bacteria to counteract or prevent

73   bacterial and viral infections have been reported[21–26]. However, there have been no reports

74   describing the use of reprogrammed microorganisms for treatment of or prophylaxis against

75   fungal infections.

76   In this study, we developed an engineered system able to sense a fungal pathogen

77   and respond by inhibition of fungal virulence factors. We discovered hydroxyphenylacetic acid

78   (HPA) as a novel molecule secreted by *C. albicans* and used engineered *E. coli* cells carrying

79   a HPA sensor to detect the presence of the fungus. Furthermore, we developed a sense-and-

80   respond system by coupling the sensor to the production of BDSF to inhibit filamentation and

81   virulence gene expression in *C. albicans* thereby protecting epithelial cells from fungal-

82   mediated damage.

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85   **Results**

86   **System design**

87   The overall goal of this work was to construct and characterize an engineered, commensal *E.*

88   *coli* strain capable of first, detecting the presence of *C. albicans* and second, producing a

89   hypha inhibitor in response to detection thereby preventing filamentation (**Figure 1**). The

90   sensor should be able to sense a substance secreted by the fungus and control expression of

91   a hypha inhibition module, which will lead to the production of an inhibitor of hypha formation.

92   Thus, bacteria carrying this system would be able to protect an epithelium from being

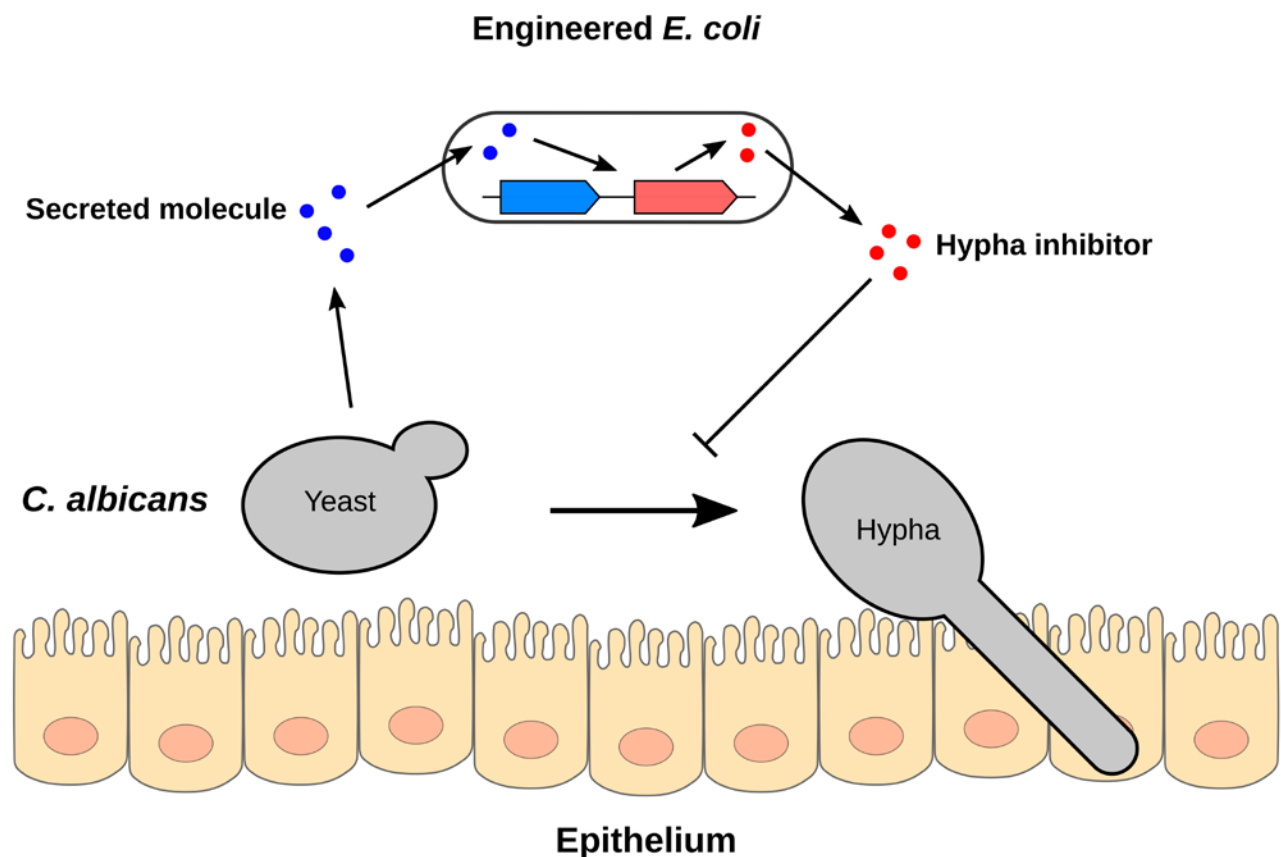93   damaged by *C. albicans*.

94

95   **Figure 1. Design of a *C. albicans* sensing and hypha inhibition system.** *C. albicans* can penetrate

96   host epithelia by switching from a yeast to a hyphal morphology. Engineered *E. coli* cells can sense *C.*

97   *albicans* secreted molecules and respond by production of an inhibitor of hypha formation thereby

98   preventing epithelial damage and penetration.

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Engineered *E. coli* can sense a molecule produced by *C. albicans*

To engineer *E. coli* to sense C*. albicans*, we first focused our efforts on the known fungal quorum sensing molecule tyrosol (4-hydroxyphenylethanol), which is constitutively produced under various conditions including growth within a patient[27]. *E. coli* W strains are able to launch a transcriptional response to the structurally related substance 4-hydroxyphenylacetic acid (4-HPA)[28]. Therefore, we reasoned that the structural similarity between tyrosol and 4-HPA could serve as a starting point for the construction of a tyrosol sensor.

We first tested for tyrosol production by *C. albicans*. We harvested *C. albicans* culture supernatants and confirmed tyrosol production by HPLC-MS, which increased upon supplementation of the medium with its precursor tyrosine (**Figure S1a**). As a control we also determined the levels of HPA in fungal culture supernatants. Surprisingly, we detected HPA production by *C. albicans* and the levels increased upon tyrosine supplementation (**Figure 2a**). Furthermore, we could detect HPA in yeast and hyphal supernatants (**Figure S1b**). Based on its retention time we were able to identify the secreted molecule as being either 3-HPA or 4-HPA. Thus, *C. albicans* is able to produce HPA presumably via a pathway similar to tyrosol.

E. coli has the ability to respond to 4-HPA and can be engineered to record its production by *C. albicans*. The *E. coli* 4-HPA sensor found in W strains consists of a transporter (HpaX) and a transcription factor (HpaA)[28,29]. Upon import by HpaX and binding to HpaA, 4-HPA can activate transcription from the downstream P_{BC} promoter[28].To test this system, we constructed a sensor plasmid carrying the *hpaX* and *hpaA* genes and the P_{BC} promoter upstream of a *gfp* encoding gene (**Figure 2b**). Cells carrying the sensor plasmid (PAS691) showed increased GFP expression upon induction with 4-HPA (**Figure S1c**). Furthermore, the absence of the HpaX transporter (PAS692) decreased the sensitivity of the sensor. Tyrosol did not lead to efficient induction even in the presence of HpaX. To determine if the 4-HPA sensor can detect *C. albicans*, we incubated *E. coli* PAS691 with different dilutions of fungal supernatants and measured GFP levels after 24 hours (**Figure 2b**). *C. albicans* culture supernatants were able to strongly induce the 4-HPA sensor and tyrosine supplementation of the fungal culture further increased the induction level.
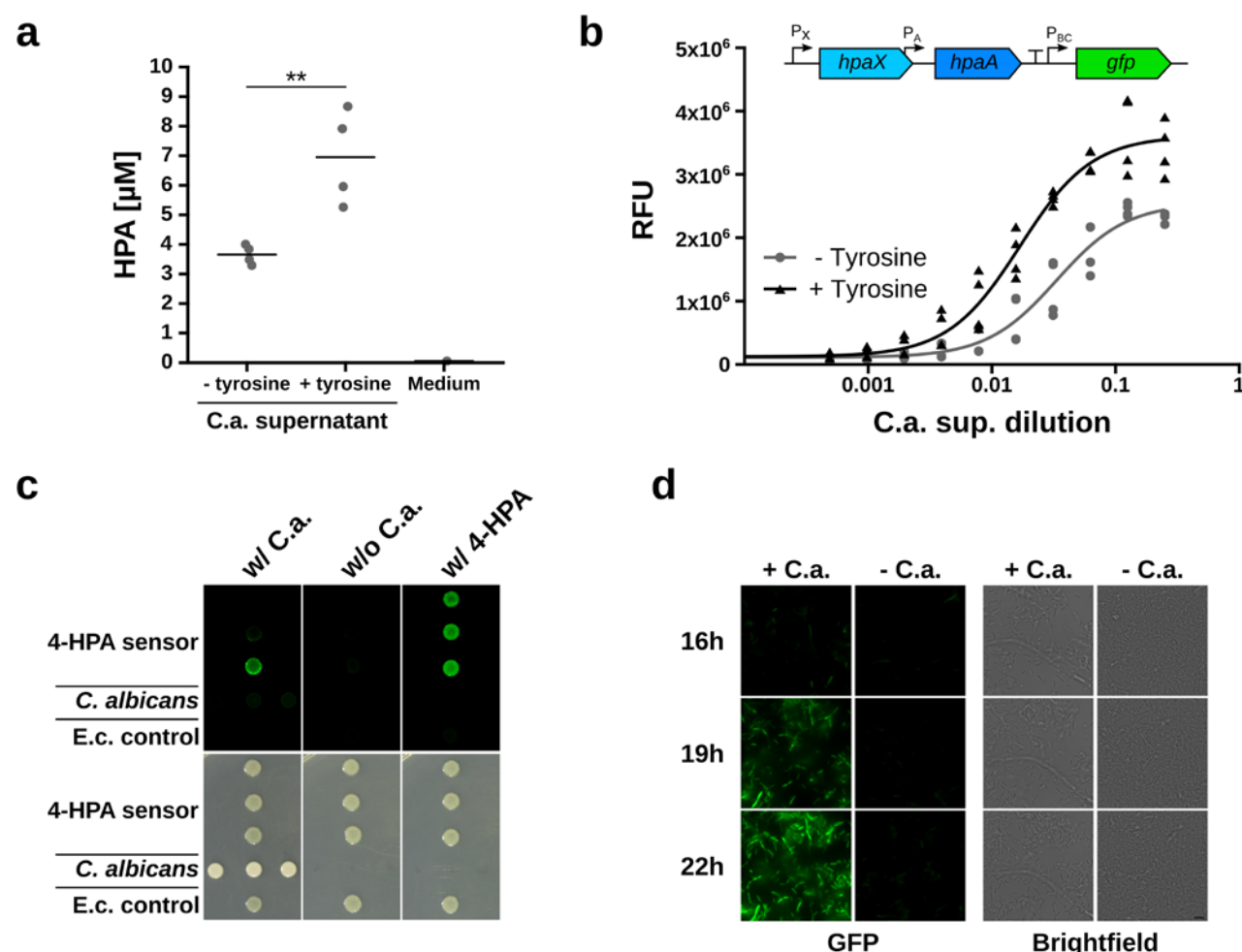
Figure 2. Construction of a *C. albicans* sensor. (a) *C. albicans* produces hydroxyphenylacetic acid (HPA). HPA levels in culture supernatants were determined by HPLC-MS. Addition of tyrosine increases HPA production. Means of biological replicates are indicated. One-tailed Student's t-test: **P<0.01; (b) Dose-response of *E. coli* PAS691 to *C. albicans* culture supernatant. GFP was placed under the control of the 4-HPA inducible P_{BC} promoter. HpaX imports 4-HPA into the cell and HpaA activates the P_{BC} promoter upon binding to 4-HPA. A four-parameter log-logistic dose-response model was fitted to the data. (c) *E. coli* 4-HPA sensor (PAS691) senses *C. albicans*. *E. coli* and *C. albicans* were cocultured on LB agar plates supplemented with glycerol at 37°C. Sensor induction was determined after 24 hours of coculture by imaging using GFP excitation/emission filters (upper panel) and brightfield (lower panel). E. c. control cells carried the empty plasmid (PAS697). 4-HPA was added to the medium as indicated. (d) Time-lapse microscopy of *E. coli* PAS691 and *C. albicans* coculture in liquid minimum medium at 37°C. Images were taken at the indicated time points. Scale bar represents 5 μm.

147

148   The sensor *E. coli* can directly detect the presence of *C. albicans*. We spotted *C.*

149 *albicans* on agar plates and incubated them for 24 hours to allow for the production of HPA.

150 Then, PAS691 sensor cells were spotted in proximity onto the same agar plate and incubated

151 for additional 24 hours prior to imaging. Only bacterial cells in close proximity to *C. albicans*

152 showed an elevated GFP signal, which declined with increasing distance from the fungal cells

153 consistent with induction by a diffusible compound produced by *C. albicans* (**Figure 2c**).

154 Importantly, we observed no significant induction in the absence of *C. albicans* and increased

155 fluorescence upon addition of 4-HPA to the medium. We observed variation in the GFP

156 intensity within PAS691 sensor spots, which might be due to copy number variation of the

157 sensor-encoding plasmid. *C. albicans* spots showed low levels of autofluorescence.

158 Furthermore, we also incubated *E. coli* PAS691 in liquid medium in the absence or presence

159 of *C. albicans* and observed GFP induction only when the fungus was present (**Figure 2d**).

160 Thus, our *E. coli* 4-HPA sensor can be used to detect *C. albicans*.

161

162 **Engineered *E. coli* can inhibit filamentation**

163 *E. coli* can be engineered to induce production of BDSF, an inhibitor of *C. albicans* hypha

164 formation that is produced naturally by *B. cenocepacia*[13]. The enzyme responsible for BDSF

165 production has been identified as *B. cenocepacia* RpfF and expression in *E. coli* leads to

166 production and secretion of BDSF[30]. To enable inducible RpfF expression in *E. coli* we placed

167 the *rpfF* gene downstream of an anhydrotetracyline-(aTC-)inducible promoter (**Figure 3a**).

168 RpfF has dehydratase and thioesterase activities and is able to produce BDSF from the acyl

169 carrier protein (ACP) thioester of 3-hydroxydodecanoic acid, a fatty acid synthesis

170 intermediate[30].

171   First, we confirmed that *C. albicans* hypha formation can be inhibited by BDSF (**Figure**

172 **3b**). To test our BDSF producing cells, we induced bacterial cells with aTC, harvested culture

173 supernatants at different time points and used them for hypha inhibition assays. Starting at 8

174 hours post induction *E. coli* supernatants were able to inhibit *C. albicans* filamentation (**Figure**

175 **3c**). Thus, *E. coli* cells carrying the aTC-inducible system (PAS689) are able to produce BDSF

176 at a level, that is sufficient to inhibit *C. albicans* hypha formation.
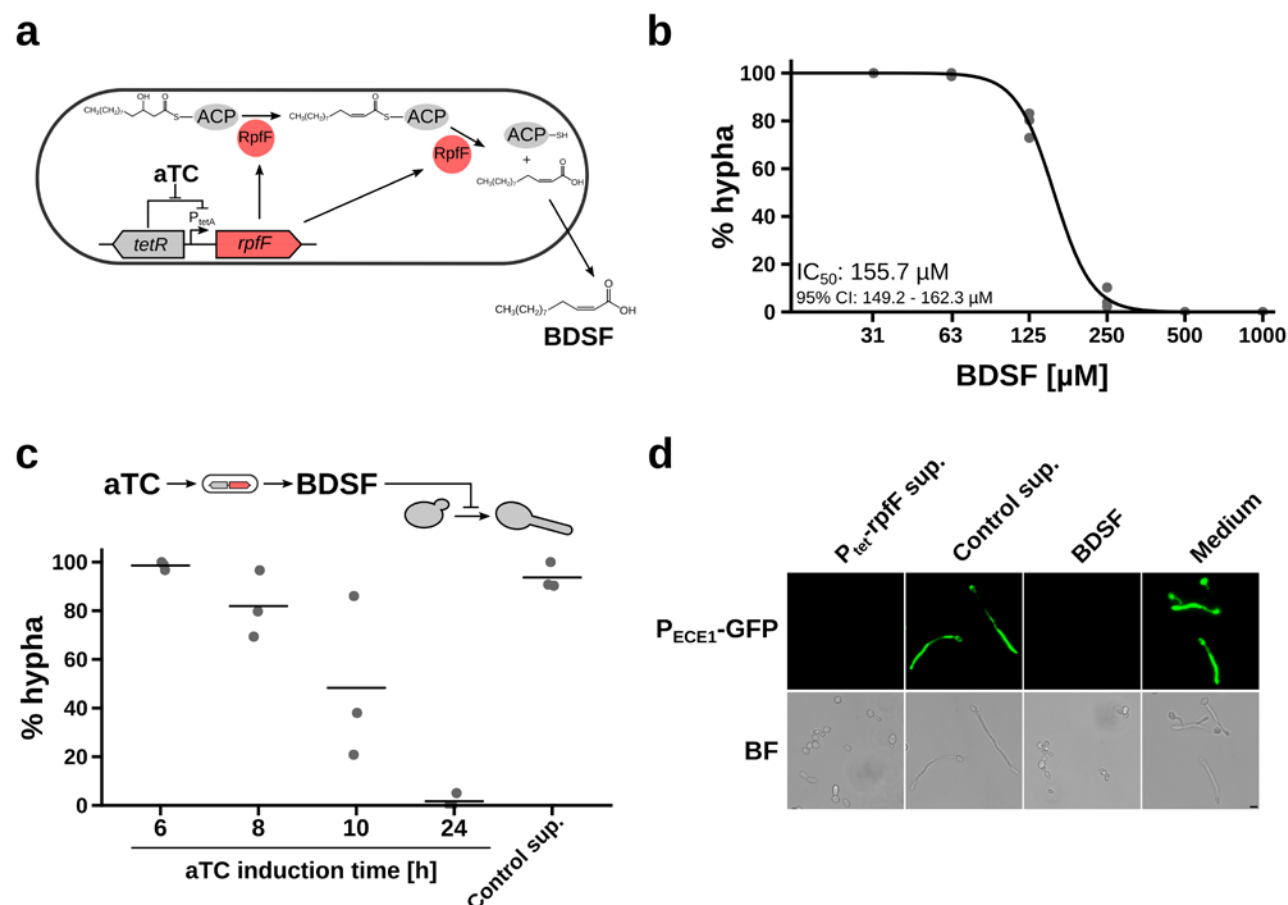
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Figure 3. Filament inhibition by engineered *E. coli*. (a) Anhydrotetracycline- (aTC-)inducible system for the production of BDSF (cis-2-dodecenoic acid). Addition of aTC induces expression of RpfF, which converts 3-hydroxydodecanoic acid-acyl carrier protein (ACP) to BDSF. (b) Filament inhibition by BDSF. *C. albicans* yeast cells were treated with BDSF under hypha inducing conditions in DMEM medium with 5% FBS at 37°C and morphology was determined after 3 hours. A four-parameter log-logistic dose-response model was fitted to the data. 95% CI: 95% confidence interval; (c) *C. albicans* yeast cells were treated with supernatants from RpfF expressing *E. coli* cells (PAS689) under hypha inducing conditions in DMEM with 5% FBS at 37°C and morphology was determined after 3 hours. Control supernatants were obtained from MG1655 cultures. Means of biological replicates are indicated. (d) *C. albicans* cells containing an *ECE1* promoter-GFP reporter (CA58) were treated as described in (c) with *E. coli* supernatants after 24 hours aTC induction or with 1 mM BDSF. Scale bar represents 5 µm.

193   BDSF produced by *E. coli* is able to inhibit virulence gene expression in *C. albicans*.

194 We used a fungal reporter strain containing GFP fused to the promoter of the *ECE1* gene

195 (CA58) encoding the precursor of Candidalysin[8]. We determined GFP expression levels upon

196 hypha induction with fetal bovine serum (FBS) at 37°C and observed strong *ECE1* induction

197 (**Figure 3d**). However, upon treatment with BDSF or aTC-induced *E. coli* supernatants we

198 observed no GFP induction. These data indicate that *E. coli* cells producing BDSF can inhibit

199 hypha formation and virulence gene expression in *C. albicans*.

200

201 **Protection of epithelial cells by engineered bacteria**

202 BDSF protects epithelial cells from killing by *C. albicans*. We incubated monolayer of Caco-2

203 epithelial cells with *C. albicans* in the absence or presence of BDSF. Without inhibitor fungal

204 cells start to filament extensively in the coculture, whereas in the presence of BDSF only

205 yeast cells were observed after 9 hours and 24 hours interaction, respectively (**Figure 4a**). To

206 determine epithelial cell damage we measured lactate dehydrogenase (LDH) release in

207 mammalian-fungal cocultures treated with increasing concentrations of BDSF. As expected,

208 we observed a dose-dependent decrease in *C. albicans*-induced cytotoxicity with increasing

209 BDSF concentrations (**Figure 4b**). Furthermore, the concentration required for epithelial cell

210 protection matches the concentration needed for hypha inhibition (**Figure 3b**). Interestingly,

211 we observed a similar protective effect of BDSF when we cultured epithelial cells in transwell

212 plates and treated them with *C. albicans*. The decrease in transepithelial electrical resistance

213 (TEER) caused by *C. albicans* was markedly reduced in the presence of 500 μM BDSF

214 (**Figure S1d**). In addition, the increase in epithelial permeability upon treatment with fungal

215 cells was also reduced in the presence of BDSF (**Figure S1e**). Furthermore, BDSF alone did

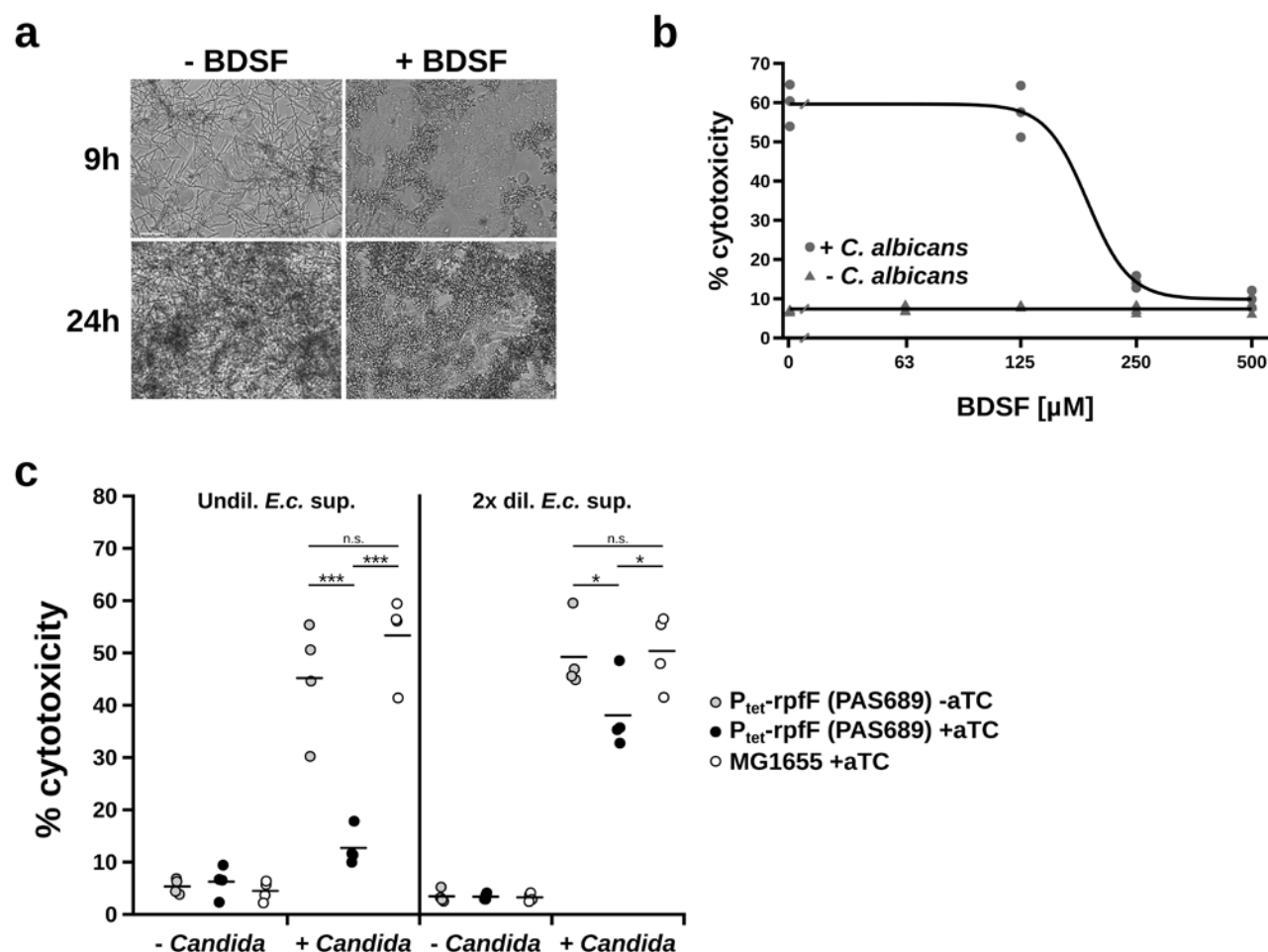216 not show any cytotoxicity (**Figure 4b** and **Figure S1d**).

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Figure 4. BDSF produced by *E. coli* protects epithelial cells from *C. albicans*-induced damage.
(a) Caco-2 epithelial cells were treated with *C. albicans* in the presence or absence of 1 mM BDSF for 9 hours and 24 hours, respectively. Scale bar represents 50 µm. (b) Caco-2 cells were treated with *C. albicans* in the presence of increasing concentrations of BDSF and cytotoxicity was determined after 20 hours by measuring LDH release. A four-parameter log-logistic dose-response model was fitted to the data. (c) Caco-2 cells were treated with *C. albicans* in the presence of *E. coli* supernatants and cytotoxicity was determined after 20 hours. Means of biological replicates are indicated. One-tailed Student's t-test: *P<0.05; ***P<0.001; n.s.: not significant;

BDSF producing *E. coli* protect epithelial cells from being damaged by *C. albicans*. We treated epithelial cells with *C. albicans* and *E. coli* culture supernatants and measured LDH release after interaction. As expected, in the presence of fungal cells we observed increased cytotoxicity when treated either with wild-type *E. coli* control supernatants from bacteria not

233 carrying *rpfF* or with supernatants from uninduced bacterial cultures (**Figure 4c**). However,

234 treatment with supernatants from aTC-induced PAS689 cells strongly reduced cytotoxicity in a

235 dose-dependent manner. Thus, our data indicate that bacterial cells producing BDSF can

236 protect epithelial cells from *C. albicans*-induced damage.

237

238 **Sensing and hypha inhibition by engineered *E. coli***

239 The *C. albicans* sensing and inhibiting activities can be combined to create a sense-and-

240 respond system by coupling the 4-HPA sensor to the *rpfF* gene (**Figure 5a**). To determine if

241 the system can work in commensal strains, the sense-and-respond system was tested in a

242 mouse commensal *E. coli* strain NGF-1[31]. Upon induction with 4-HPA the BDSF concentration

243 in supernatants of cells carrying the sense-and-respond system (PAS694) increased over

244 time (**Figure 5b**). Furthermore, induction with *C. albicans* culture supernatants led to a similar

245 increase in BDSF levels whereas for uninduced cells the inhibitor levels stayed low. To

246 determine if bacteria carrying the sense-and-respond system are able to inhibit hypha

247 formation, we treated *C. albicans* cells under hypha inducing conditions with *E. coli* culture

248 supernatants that had been induced with either 4-HPA or fungal supernatants and determined

249 fungal cell morphology. *E. coli* PAS694 was able to efficiently inhibit hypha formation only

250 upon induction with 4-HPA or fungal supernatants (**Figure 5c**). In contrast, treatment with

251 control supernatants from PAS693 cells lacking RpfF or cells that were not treated with HPA

252 or fungal supernatants allowed extensive filamentation.

253   Bacteria carrying the sense-and-respond system can protect epithelial cells from *C.*

254 *albicans*-mediated killing. We incubated fungal cells with Caco-2 cell monolayers in the

255 presence or absence of bacterial culture supernatants and determined cytotoxicity by

256 measuring LDH release. A clear reduction in cytotoxicity compared to control supernatants

257 from uninduced cells or cells lacking RpfF was observed only in the presence of supernatants

258 from *E. coli* PAS694 cells, which have been induced by 4-HPA or fungal supernatants prior to

259 harvest (**Figure 5d**). Decreased cytotoxicity and hypha formation in 4-HPA-induced samples

260 compared to samples treated with *C. albicans* supernatants (**Figure 5c and d**) is due to

261 higher BDSF levels upon 4-HPA induction (**Figure 5b**).

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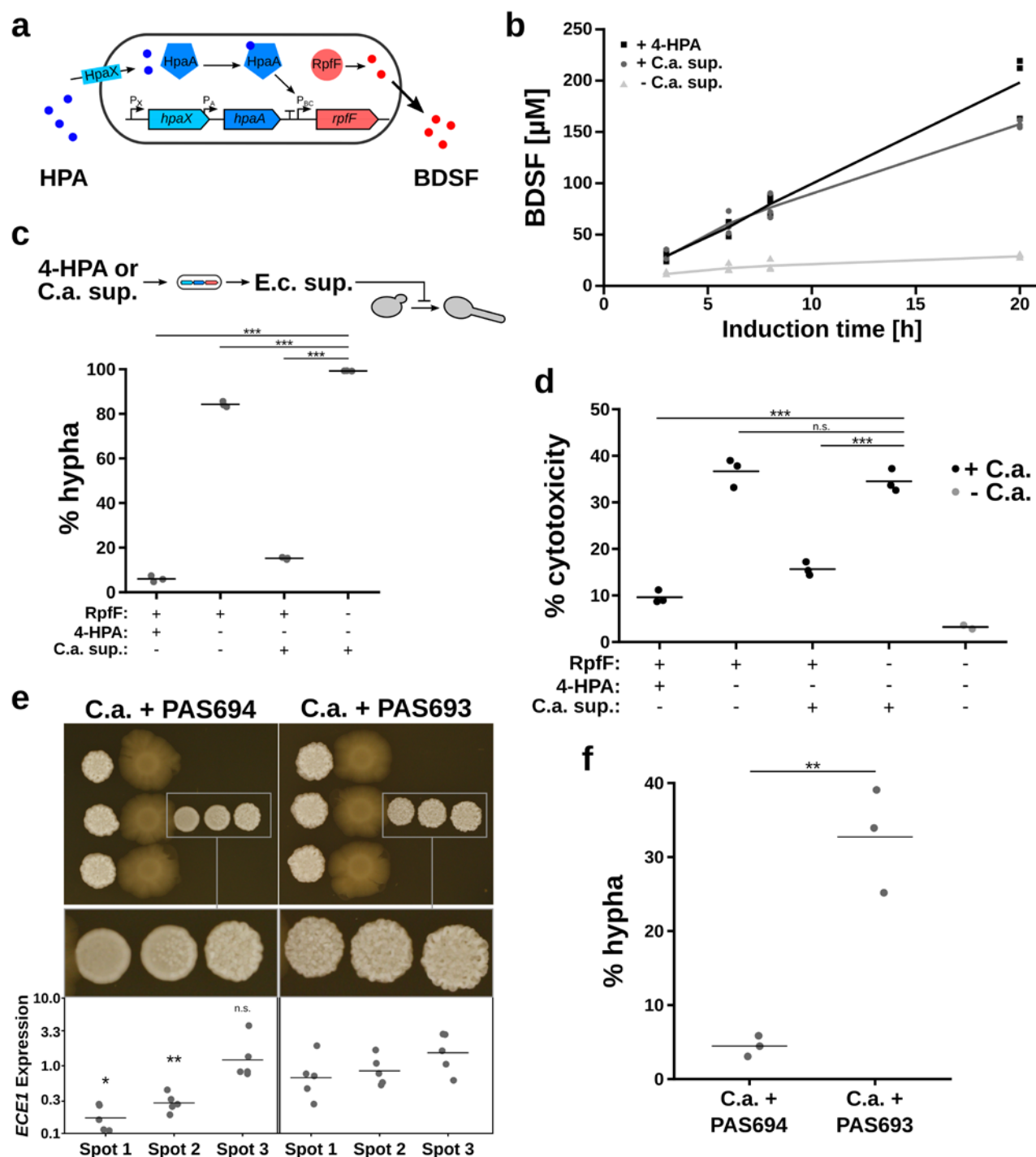
Figure 5. Engineered _E. coli_ can sense _C. albicans_, inhibit hypha formation and protect epithelial cells. (a) Design of a _C. albicans_ sense-and-respond system. Engineered _E. coli_ can import HPA produced by _C. albicans_ via the transporter HpaX. HpaA binds to HPA and induces expression of RpfF, which produces the hypha inhibitor BDSF. (b) BDSF levels in supernatants of _E. coli_ cells carrying the _C. albicans_ sense-and-respond system (PAS694) were determined by HPLC after induction with 100 µM 4-HPA or 0.1x _C. albicans_ culture supernatant. (c) _E. coli_ PAS694 supernatants

269 harvested 20 hours after induction as described in (b) were incubated with *C. albicans* yeast cells
270 under filament-inducing conditions in minimum medium with glycerol at 37°C and morphology was
271 determined after 3 hours. *E. coli* PAS693 (- RpfF) served as control. (d) *E. coli* PAS694 and PAS693
272 supernatants prepared as described in (c) were used to treat epithelial cells infected with *C. albicans*.
273 Epithelial damage was determined by measuring LDH release. (e) *C. albicans* and *E. coli* PAS694
274 were cocultured on agar plates under filament-inducing conditions. Filament inhibition was determined
275 based on colony morphology. *E. coli* PAS693 served as control. *C. albicans* spots were harvested and
276 used for RNA isolation and RT-qPCR analysis of *ECE1* expression levels. Spot 1 is closest to *E. coli*.
277 (f) *C. albicans* and *E. coli* PAS694 and PAS693, respectively, were cocultured on agar plates as
278 described in (e). *C. albicans* spots closest to *E. coli* were harvested and cell morphology was
279 determined microscopically. (b-f) Means of biological replicates are indicated. One-tailed Student's t-
280 test: *P<0.05; **P<0.01; ***P<0.001; n.s.: not significant relative to PAS693 control;

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284   *E. coli* cells carrying the sense-and-respond system are able to inhibit hypha formation
285 directly. *C. albicans* growth on agar plates yields an easy readout of filamentation based on
286 colony morphology; smooth colonies indicate the presence of yeast cells whereas wrinkling
287 indicates filamentation[32]. First, fungal cells were spotted on agar plates and incubated for 24
288 hours to allow the production of HPA. Then, engineered bacteria and subsequently again *C.*
289 *albicans* were spotted in proximity onto the same agar plate and incubated for additional 48
290 hours prior to imaging. Only *E. coli* PAS694 carrying the sense-and-respond system were
291 able to inhibit filamentation as indicated by a smooth fungal colony morphology in proximity of
292 the bacterial spots (**Figure 5e**). The inhibiting activity decreased with increased distance to
293 the bacterial cells indicating a gradient of BDSF production by the engineered *E. coli*.
294 Furthermore, we observed no significant inhibition with the PAS693 control strain lacking
295 RpfF.
296   To determine virulence gene expression, we harvested *C. albicans* cells upon coculture
297 on agar plates and determined the expression levels of the well-known hypha-specific
298 virulence genes *ECE1* (Candidalysin-encoding gene)[8] and *HWP1* (adhesin)[32] by RT-qPCR
299 (**Figure 5e** and **Figure S1f**). Only in proximity of *E. coli* cells carrying the sense-and-respond
300 system (C.a. + PAS694 spot 1 and 2) a significant repression of virulence genes was
301 observed. *E. coli* PAS693 served as control. To quantify hypha inhibition, we harvested *C.*
302 *albicans* cells upon 24 hour coculture on agar plates and determined cell morphology by

303 microscopy. Upon coculture with *E. coli* cells carrying the sense-and-respond system
304 (PAS694) we observed a significant reduction in *C. albicans* hypha formation compared to
305 PAS693 control cells (**Figure 5f**).

306   Taken together, these data indicate that *E. coli* cells carrying the *C. albicans* sense-
307 and-respond system are able to induce BDSF production in the presence of the fungus
308 thereby inhibiting hypha formation as well as virulence factor expression and protecting
309 epithelial cells from fungal-mediated damage.

310

311 **Discussion**

312 In this study, we present the construction of a synthetic sense-and-respond system directed
313 against a human fungal pathogen. We identified HPA as a novel secreted molecule produced
314 by *C. albicans*, one of the most common fungal pathogens infecting humans. Furthermore, we
315 built a sensor able to detect fungal HPA and coupled it to the production of the hypha inhibitor
316 BDSF. Finally, we were able to show that *E. coli* cells carrying the sense-and-respond system
317 can inhibit hypha formation in *C. albicans* and protect epithelial cells from fungal-mediated
318 damage.

319   To the best of our knowledge, there have been no reports describing the production of
320 HPA by *C. albicans* or any other *Candida* species so far. Similar to tyrosol, HPA production in
321 *C. albicans* is increased in the presence of tyrosine indicating that both molecules might be
322 produced from the same precursors. In *Saccharomyces cerevisiae* HPA and tyrosol are
323 produced via the Ehrlich pathway[33]. *C. albicans* uses the same pathway for production of
324 tyrosol[34]. Thus, HPA might also be produced via the Ehrlich pathway in *C. albicans*. Tyrosol is
325 able to reduce the lag phase in growth upon dilution into minimal medium and it can stimulate
326 hypha induction in *C. albicans*[35]. Given the structural similarity between 4-HPA and tyrosol as
327 well as the possibility of common precursors in biosynthesis the two molecules might have
328 overlapping biological functions. Interestingly, the 4-HPA sensor can be found not only in *E.*
329 *coli* W strains but also in the human commensal *E. coli* strain HS[36]. Thus, HPA might also
330 have a function in bacterial-fungal communication for example in the human intestine. In fact,
331 4-HPA has been detected in human fecal samples, although its source remains to be
332 determined[37,38].

333   Targeting filamentation of *C. albicans* as an antifungal strategy has gained interest
334 more recently. A number of substances inhibiting the transition from the yeast to the hyphal
335 morphology have been described[39]. Among them, BDSF has been shown to efficiently inhibit

336   hypha formation[13]. We could show that *E. coli* cells expressing RpfF are able to produce the

337   inhibitor at levels sufficient for efficient hypha inhibition. In addition, by engineering fatty acid

338   metabolism, BDSF production could potentially be even further increased[30]. Interestingly,

339   BDSF has also been used to inhibit virulence gene expression in *Pseudomonas aeruginosa*[40].

340   It inhibits expression of a type III secretion system and has been used to protect epithelial

341   cells as well as zebrafish from *P. aeruginosa*-mediated killing. Thus, our engineered sense-

342   and-respond system could potentially be easily adapted to be effective against *P. aeruginosa*

343   by coupling expression of RpfF to existing sensors for this important bacterial pathogen[24].

344   Various members of the human microbiota have been shown to produce inhibitors of

345   *C. albicans* hypha formation or virulence[13,14,16]. Often the actual inhibitory molecule is not

346   known and constitutively produced[41–43]. These observations suggest that bacteria inhabiting

347   the gastrointestinal tract along with *C. albicans* may utilize an inhibitory strategy to enhance

348   their ability to co-exist with *C. albicans* in their common niche. Our sense-and-respond system

349   represents the first engineered system able to exploit this strategy.

350   Filamentation is critical for active penetration of epithelia, which is the main mechanism

351   used by *C. albicans* to invade intestinal epithelial cells[44,45]. Thus, inhibiting hypha formation

352   could avoid *C. albicans* invasion of the intestinal epithelium. However, filamentation is not

353   absolutely required for intestinal colonization since cells lacking the transcription factor Efg1,

354   which are defective in hypha formation, show higher colonization levels in mice compared to

355   wild-type cells at least within 3 days upon gavage (Pierce, 2012)[46]. Thus, our approach would

356   not affect fungal growth and colonization in general, but inhibit epithelial penetration and

357   translocation into the bloodstream.

358   Our engineered bacterium detects a fungal pathogen and induces production of an

359   inhibitor of *C. albicans* virulence factors. The system has the ability to protect epithelial cells

360   from *C. albicans*-mediated damage. Engineered probiotics could lower treatment costs since

361   the active substance is produced by the microbe[17]. Furthermore, inhibiting virulence factors

362   instead of growth can lower the risk of resistance development[7,17]. Thus, our approach

363   represents a novel strategy to prevent fungal infections with the potential to reduce treatment

364   costs and counteract the development of antifungal resistance.

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369 **Methods**

370

371 **Bacterial and fungal strains and growth conditions**

372 Bacterial and fungal strains used in this study are listed in Table S1. *E. coli* strains were

373 routinely grown in LB medium at 37°C. *C. albicans* was routinely grown in YPD medium at

374 30°C. Minimum medium as described in Boon *et al*.[13] was used for sensor induction with *C.*

375 *albicans* supernatants and coculture experiments and was supplemented with 0.4% glycerol

376 or *N*-acetylglucosamine when indicated. M9 medium supplemented with 0.1% casamino acids

377 and 0.4% glycerol was used for dose-response experiments with 4-HPA and tyrosol. For solid

378 media 1.5% agar was added for LB plates and 2% agar for YPD plates. Antibiotics were used

379 at the following concentrations: 100 μg/mL spectinomycin, 25 μg/mL chloramphenicol, 50

380 μg/mL kanamycin and 100 μg/mL ampicillin.

381

382 **Mammalian cell culture**

383 Caco-2BBE human colorectal carcinoma-derived (Caco-2) intestinal epithelial cells (obtained

384 from Harvard Digestive Disease Center) were routinely cultured in Dulbecco's Modified Eagle

385 Medium (DMEM) containing 4.5 g/L glucose (ATCC) supplemented with 10% fetal bovine

386 serum (FBS), 100 units/mL penicillin and 100 μg/mL streptomycin (Pen/Strep) at 37°C in a

387 humidified incubator under 5% CO_2 in air.

388

389 **Plasmid and strain construction**

390 Plasmids and oligonucleotides used in this study are listed in Tables S2 and S3, respectively.

391 *E. coli* NEB5α was used for cloning. For the construction of pTet a *tetR-P$_{tetA}$* fragment was

392 amplified from the PAS132 genome with primers tet_XmaJI_fw & tet_BamHI_re and

393 BamHI/XmaJI cloned into the pBAD His A backbone amplified with primers pBAD_fw &

394 pBAD_XmaJI_re. Then, pTet-GFP was constructed by inserting the gfpmut2 gene from

395 pUA66[47] into pTet via BamHI/PstI. The *hpaXA-P$_{BC}$* fragment was amplified from the

396 ATCC11105 genome with primers hpaX_XmaJI_fw & PhpaBC_XbaI_re and XmaJI/XbaI

397 cloned into pTet-GFP replacing the *tetR-P$_{tetA}$* part to obtain pHpaXA-GFP. For the construction

398 of pCDF-HpaXA-GFP a *hpaXA*-GFP fragment was amplified from pHpaXA-GFP with primers

399 hpaX_XmaJI_fw & pHpaXA_ApaI_re and XmaJI/ApaI cloned into the pCDFDuet-1 backbone

400 amplified with primers pACYC_ApaI_fw & pACYC_XmaJI_re. The *hpaX* gene was removed

401 from pCDF-HpaXA-GFP by amplification with primers hpaA_XmaJI_fw & pACYC_XmaJI_re

402   and religation upon XmaJI digest to obtain pCDF-HpaA-GFP. The *B. cenocepacia rpfF* gene

403   with an N-terminal 6xHis-tag was obtained as gBlock from Integrated DNA Technologies. For

404   the construction of pCDF-HpaXA-rpfF the 6xHis-*rpfF* fragment was amplified from the gBlock

405   with primers Bcam0581_oe_fw & Bcam0581_oe_re and cloned into the pCDF-HpaXA-GFP

406   backbone amplified with primers PBC_oe_re & pCDF_oe_fw to replace the *gfp* gene.

407   Plasmids were transformed into different *E. coli* strains via heat shock.

408   To obtain pKD3-Ptet-rpfF the *tetR-P_{tetA}* fragment was amplified from the PAS132

409   genome with primers tet_fw & tet_re, the pKD3 vector backbone was amplified with primers

410   pKD3_fw & pKD3_tetR_re and both fragments were combined with the 6xHis-*rpfF* gBlock

411   using Gibson assembly. For the construction of PAS689 the Cm^R-*tetR-P_{tetA}-rpfF* fragment was

412   amplified from pKD3-Ptet-rpfF with primers pKD3int_fw & pKD3int_re and integrated into the

413   *araB-araC* locus of *E. coli* TB10 as described[48]. The locus was then transferred to *E. coli*

414   MG1655 by P1*vir* transduction as described[31]. The streptomycin-resistance mutation *rpsL*

415   (*lys42arg*) was transferred into *E. coli* NGF-1 as described[31].

416

417   **Tyrosol and HPA measurement by HPLC-MS**

418   *C. albicans* SC5314 supernatants were prepared by diluting overnight cultures to an OD_{600} of

419   0.25 in minimum medium. For tyrosine supplementation 1 mM tyrosine was added to the

420   medium. For hypha induction the medium included 4 g/L *N*-acetylglucosamine. Fungal

421   supernatants were harvested by centrifugation after incubation at 30°C for yeast cultures and

422   37°C for hyphal cultures for 24 hours with shaking and sterile filtered using 0.22 μm filters.

423   Samples were acidified by addition of 100 mM H_2SO_4 and extracted using Sep-Pak Light C18

424   cartridges (Waters). Samples were reduced in a speedvac benchtop concentrator at 50°C

425   prior to HPLC-MS analysis.

426   HPLC-MS analysis of standards and extracts was carried out using an Agilent 1260

427   Infinity HPLC system equipped with an Agilent Eclipse Plus C18 column (100 × 4.6 mm,

428   particle size 3.5 μm, flow rate: 0.3 mL/min, solvent A: dd.H_2O/0.1% (v/v) NH_4OH, solvent B:

429   acetonitrile, injection volume: 7.5 μL) connected to an Agilent 6530 Accurate-Mass Q-TOF

430   instrument. The following gradient was used for separation and analysis (time/min, %B): 0, 0;

431   13, 100; 18, 100; 19, 0, 25, 0. The mass spectrometer was operated in negative ESI mode

432   and the autosampler was kept at 4°C. MS-fragmentation experiments were carried out using

433   collision-induced dissociation (CID) fragmentation.

434   To quantitate tyrosol and HPA levels in extracts, a standard curve was recorded using

435   freshly prepared tyrosol and HPA standards dissolved in dd.H2O/acetonitrile (85/15 (v/v)).

436   Samples were kept on ice or at 4°C. After HPLC-MS analysis, extracted ion current (EIC)

437   peaks (m/z (tyrosol): 137.05-137.07, m/z (HPA): 151.0-151.05) were automatically integrated

438   using the MassHunter Workstation Software (version: B.07.00). A plot of peak area versus

439   concentration was used to generate a linear fit and quantitate levels found in extracts.

440

441   **BDSF measurement by HPLC**

442   *E. coli* PAS694 and PAS693 overnight cultures were diluted 1:100 in minimum medium with

443   0.4% glycerol and spectinomycin and incubated at 37°C for 4 hours. Glycerol

444   supplementation was done to enhance *E. coli* growth. For induction 100 µM 4-HPA or 0.1x *C.*

445   *albicans* supernatant in minimum medium with tyrosine prepared as described above was

446   added and cultures were incubated at 37°C with shaking. Supernatants were harvested at

447   different time points and sterile filtered using 0.22 µm filters. BDSF extraction was done

448   essentially as described[49] with some modifications. First, 200 µL culture supernatant were

449   acidified by addition of 0.3 M HCl and extraction was done twice with 200 µL ethyl acetate.

450   Extracts were dried in a speedvac benchtop concentrator at 40°C and dissolved in 100 µL

451   methanol. Five µL were used for analysis on an Agilent 1200 HPLC system using a Zorbax

452   SB-C18 column (3.0 x 150 mm, 3.5 µm). The mobile phase was 80% methanol with 0.1%

453   formic acid and the flow rate was 0.4 mL/min. BDSF was detected using a diode array

454   detector set to 220 nm (4 nm band width). Quantification was done using BDSF standards

455   spiked into medium and extracted as described above.

456

457   **Dose-response analysis**

458   For dose-response analysis with 4-HPA and tyrosol as inducers, *E. coli* overnight cultures

459   were diluted 1:100 in M9 medium containing different concentrations of 4-HPA or tyrosol and

460   incubated at 37°C for 20 hours with shaking. GFP levels and OD_{595} were determined using a

461   Victor3V plate reader (PerkinElmer). After blank correction GFP levels were normalized for

462   OD_{595} to obtain RFU values. A four-parameter log-logistic dose-response model was fitted to

463   the data using R version 3.2.3 and the *drc* package.

464   *C. albicans* culture supernatants for dose-reponse analysis were prepared as

465   described above. *E. coli* overnight cultures were diluted 1:100 in minimum medium

466   supplemented with 0.4% glycerol. Fungal culture supernatants were added at different

467 dilutions and cultures were incubated at 37°C for 20 hours with shaking. GFP and OD_{595}

468 measurement as well as RFU calculation was done as described above.

469

Bacterial-fungal coculture

471 To determine sensor induction on agar plates, *C. albicans* SC5314 overnight cultures were

472 washed once and diluted in PBS buffer to OD_{600} of 0.1 and 1 µL was spotted on LB agar

473 plates supplemented with 0.4% glycerol to support fungal growth and spectinomycin. Plates

474 were incubated for 24 hours at 37°C. *E. coli* PAS691 and PAS697 overnight cultures were

475 diluted to OD_{600} of 0.1 in PBS buffer and 1 µL spotted next to *C. albicans*. Plates were imaged

476 after 24 hours incubation at 37°C. Images were taken using an imaging station equipped with

477 a Canon EOS Rebel T3i camera and GFP excitation/emission filters.

478 To determine hypha inhibition on agar plates, *C. albicans* SC5314 suspensions were

479 spotted on YPGlcNacSpec plates containing 10 g/L yeast extract, 20 g/L peptone and 4 g/L *N*-

480 acetylglucosamine allowing hypha formation. Cell suspensions were prepared as described

481 above. Plates were incubated for 24 hours at 37°C. Then *E. coli* PAS694 and PAS693

482 suspensions were spotted next to *C. albicans* as described above and plates were further

483 incubated at 37°C for 6 hours. Finally, *C. albicans* suspensions were spotted in different

484 distances to the *E. coli* spots and plates were incubated at 37°C for 48 hours prior to imaging

485 as described above. Quantification of hyphal cells was done by resuspending *C. albicans*

486 spots after 24 hours coculture in 0.5 ml PBS containing 0.05% Tween 20. Cells were

487 sonicated for 10 seconds using a tip sonicator to disperse clumps prior to microscopy using

488 an inverted Zeiss Axiovert 200M microscope. At least 800 cells were counted per sample.

489 For bacterial-fungal cocultures in liquid medium *C. albicans* SC5314 overnight cultures

490 were washed twice and diluted in minimum medium with spectinomycin to OD_{600} of 0.12. *E.*

491 *coli* PAS691 overnight cultures were diluted to OD_{600} of 0.0063 in minimum medium in the

492 absence or presence of *C. albicans*. Cells were incubated in CellView glass bottom culture

493 dishes at 37°C and images were taken using an inverted Nikon Ti2 fluorescence microscope

494 over time in 15 minute intervals.

495

Hypha inhibition assay

497 *C. albicans* SC5314 overnight cultures were washed once and diluted to OD_{600} of 0.2 in PBS

498 buffer. Five µL cell suspension were added to 100 µL DMEM medium with 5% FBS and

499 different concentrations of BDSF in clear bottom 96 well plates. Cells were incubated at 37°C

500 for 3 hours to induce hypha formation prior to imaging on an inverted Nikon TE2000

501 microscope to determine cell morphology. More than 50 cells were counted for each condition

502 and the experiment was repeated 3 times. The percentage of cells showing hyphal

503 morphology was determined. A four-parameter log-logistic dose-response model was fitted to

504 the data using R version 3.2.3 and the drc package.

505 For hypha inhibition by *E. coli* supernatants upon aTC induction PAS689 and MG1655

506 overnight cultures were diluted to OD_{600} of 0.05 in DMEM medium without phenol red and

507 Pen/Strep in the absence or presence of 100 ng/mL aTC and incubated at 37°C with shaking.

508 Supernatants were harvested at different time points, sterile filtered using 0.22 μm filters and

509 100 μL aliquots transferred to clear bottom 96 well plates. Upon addition of *C. albicans* as

510 described above and 5% FBS to induce hypha formation cells were incubated at 37°C for 3

511 hours. Imaging and analysis was done as described above.

512 For hypha inhibition by *E. coli* supernatants upon 4-HPA or *C. albicans* induction

513 PAS694 and PAS693 overnight cultures were diluted 1:100 in minimum medium with glycerol

514 and spectinomycin and incubated at 37°C for 4 hours. For induction 100 μM 4-HPA or 0.1x *C.*

515 *albicans* supernatant in minimum medium with tyrosine prepared as described above was

516 added and cultures were incubated for 20 hours. Supernatants were harvested, sterile filtered

517 and 50 μL aliquots transferred to clear bottom 96 well plates. Upon addition of *C. albicans* as

518 described above and 50 μL 2x minimum medium with 8 g/L *N*-acetylglucosamine to induce

519 hypha formation cells were incubated at 37°C for 3 hours. Imaging and analysis was done as

520 described above.

521

522 **Analysis of virulence gene expression**

523 *C. albicans* CA58 overnight cultures were washed once and resuspended at OD_{600} of 0.25 in

524 DMEM medium or aTC-induced PAS689 and MG1655 supernatants prepared as described

525 above and 5% FBS were added to induce hypha formation. For BDSF treated samples the

526 inhibitor concentration was 1 mM. Samples were incubated at 37°C with shaking for 3 hours

527 prior to imaging on an inverted Nikon TE2000 fluorescence microscope.

528 For analysis of *ECE1* and *HWP1* expression by RT-qPCR fungal cells were

529 resuspended in 1 ml PBS and harvested by centrifugation. RNA extraction, DNAse treatment

530 and qPCR analysis was done as described previously[50]. *PAT1* and *RIP1* reference genes

531 were used for normalization[51].

532

Fungal-mammalian coculture and cytotoxicity assay

Caco-2 cells were seeded at 5 x 10^4 cells/well in 96 well plates and incubated for 3 days to form a monolayer. *C. albicans* SC5314 overnight cultures were diluted to OD_{600} of 0.1 in YPD medium and incubated at 30°C until they reached OD_{600} 1.0. Cells were harvested, washed twice in PBS buffer and resuspended in DMEM medium. To determine inhibition of cytotoxicity by BDSF the Caco-2 medium was changed to DMEM 5% FBS without phenol red containing different concentrations of BDSF. Then, 1 x 10^4 *C. albicans* yeast cells per well were added and samples were incubated at 37°C 5% CO_2 for 20 hours. Finally, LDH release into supernatants was determined using the CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega) according to the manufacturer's instructions. To determine inhibition of cytotoxicity by PAS689, aTC-induced culture supernatants were prepared as described above for hypha inhibition assays. Caco-2 medium was replaced by *E. coli* supernatants supplemented with 5% FBS. After addition of *C. albicans* yeast cells samples were incubated and LDH release determined as described above.

To determine inhibition of cytotoxicity by PAS694, 4-HPA-induced and *C. albicans*-induced bacterial supernatants were prepared as described above for hypha inhibition assays. PAS693 supernatants served as controls. Supernatants were diluted 4x in DMEM 10% FBS medium without phenol red and added to Caco-2 cells. After addition of *C. albicans* yeast cells samples were incubated and LDH release determined as described above.

For imaging of Caco-2 cells treated with *C. albicans* in the absence and presence of BDSF, samples were prepared as described above. Images were taken after 9 hours and 24 hours interaction, respectively, using an inverted Leica DM IL LED microscope.

Transwell culture conditions and epithelial barrier measurements

Caco-2 cells were seeded at 1 x 10^5 cells/cm^2 in collagen-coated transwell inserts (Corning) containing 200 μL DMEM medium in the apical compartment and 800 μL in the basal compartment. Cells were incubated at 37°C 5% CO_2 for 18-20 days and the medium was changed every 2-3 days. *C. albicans* SC5314 cells were prepared as described above for cytotoxicity assays and 1 x 10^5 cells were added to the apical compartment in the absence or presence of 500 μM BDSF. The medium in both compartments was removed after 12 and 24 hours. Fresh DMEM was added to the basal compartment and DMEM with BDSF was used for the apical compartment. Transepithelial electrical resistance and permeability measurements were used to determine epithelial barrier function exactly as described[52].

566

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575

Author contributions

M.T. and P.A.S. designed the project. M.T., T.W.G. and L.M. performed experimental work. M.T. and T.W.G. analyzed the results. C.A.K. gave technical and conceptual advice. M.T. and P.A.S. wrote the manuscript and T.W.G. and C.A.K. edited the manuscript.

580

Competing financial interests

The authors declare no competing financial interests.

583

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A synthetic system that senses *Candida albicans* and inhibits virulence factors

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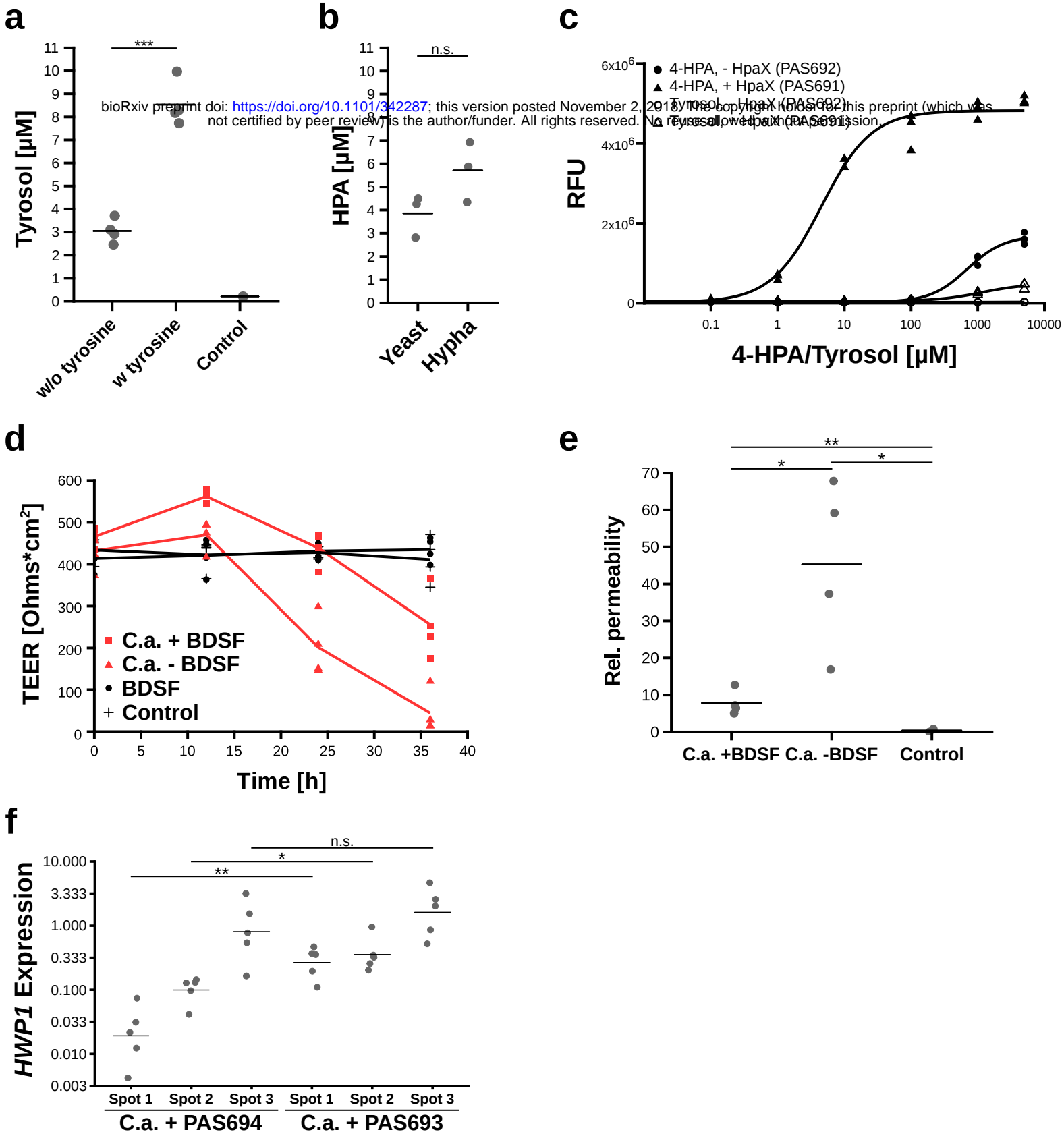
Supplementary Information

Figure S1. (a) Tyrosol levels in *C. albicans* culture supernatants were determined by HPLC-MS. Addition of tyrosine increases tyrosol production. (b) HPA levels in *C. albicans* yeast and hyphal culture supernatants were determined by HPLC-MS. (c) Dose-response of an *E. coli* 4-HPA sensor to 4-HPA and tyrosol. GFP was placed under the control of a 4-HPA inducible promoter, which is induced by HpaA. Expression of the 4-HPA transporter HpaX increases sensitivity. A four-parameter log-logistic dose-response model was fitted to the data. (d) Caco-2 cells cultured in transwell plates were treated with *C. albicans* -/+ 500 µM BDSF in the apical compartment and transepithelial electrical resistance (TEER) was measured at the indicated time points. (e) Caco-2 cells were treated as described in (d) and epithelial permeability was determined after 36 hours interaction. (f) *C. albicans* and *E. coli* PAS694 and PAS693, repestively, were cocultured on agar plates under hypha-inducing conditions as described in Figure 5e. *C. albicans* spots were harvested and used for RNA isolation and RT-qPCR analysis of *HWP1* expression levels. Spot 1 is closest to *E. coli*. (a-f) Means of biological replicates are indicated. One-tailed Student's *t*-test: *$P<0.05$; **$P<0.01$; n.s.: not significant;

Table S1: Bacterial and fungal strains used in this study.

Strain designation	Strain name	Plasmid	Parent strain	Relevant genotype	Marker	Source
	NEB5α					New England Biotech
PAS697	NEB5α pCDFDuet-1	pCDFDuet-1	NEB5α		Spec	This study
	MG1655					ATCC #700926
	TB10		MG1655	nadA::Tn10 λcI^{857} Δ(cro-bioA)	Tet	1
PAS132			MG1655	araB::Cm^R-tetR-P$_{tetA}$-cro mphR::Kan^R-O$_L$-rexBA-cI^{ind}-O$_R$-cro::lacZ rpsL lys42arg	Kan, Cm	2
PAS222	NGF-1					2
PAS688	ATCC11105					ATCC #11105
PAS689	MG1655-P$_{tet}$-rpfF		MG1655	araB-Cm^R-tetR-P$_{tetA}$-rpfF-araC	Cm	This study
PAS690	NGF-1 Str^R		NGF-1	rpsL lys42arg	Strep	This study
PAS691	NEB5α pCDF-HpaXA-GFP	pCDF-HpaXA-GFP	NEB5α		Spec	This study
PAS692	NEB5α pCDF-HpaA-GFP	pCDF-HpaA-GFP	NEB5α		Spec	This study
PAS693	NGF-1 pCDF-HpaXA-GFP	pCDF-HpaXA-GFP	PAS690	rpsL lys42arg	Spec, Strep	This study
PAS694	NGF-1 pCDF-HpaXA-rpfF	pCDF-HpaXA-rpfF	PAS690	rpsL lys42arg	Spec, Strep	This study
PAS695	SC5314			wt		3
PAS696	CA58		SC5314	ECE1/ece1::GFP-SAT1	SAT1	4

Table S2: Plasmids used in this study.

Plasmid name	Relevant inserts	Marker	Source
pBAD HisA		Amp	LifeTech
pCDFDuet-1		Spec	EMD Millipore
pUA66	GFP	Kan	5
pTet	tetR-PtetA from PAS132 XmaJIBamHI cloned into pBAD His A	Amp	This study
pTet-GFP	*gfpmut2* from pUA66 BamHIPstI cloned into pTet	Amp	This study
pHpaXA-GFP	*hpaXA-P$_{BC}$* XmaJIXbaI cloned into pTet-GFP	Amp	This study
pCDF-HpaXA-GFP	*hpaXA-P$_{BC}$-gfpmut2* from pHpaXA-GFP XmaJIApaI cloned into pCDFDuet-1	Spec	This study
pCDF-HpaA-GFP	*hpaX* removed from pCDF-HpaXA-GFP by amplification and religation after XmaJI cut	Spec	This study
pCDF-HpaXA-rpfF	*gfpmut2* in pCDF-HpaXA-GFP replaced by 6xHis-*rpfF* amplified from gBlock	Spec	This study
pKD3	FRT-Cm^{R}-FRT	Amp, Cm	Addgene #45604
pKD3-Ptet-rpfF	*tetR-P$_{tetA}$* from PAS132 and 6xHis-*rpfF* gBlock cloned into pKD3 via Gibson assembly	Amp, Cm	This study

Table S3: Oligonucleotides used in this study.

Name	Sequence	Description
tet_XmaJI_fw	atatcctaggCACATTAAGACCCACTTTCAC	pTet construction
tet_BamHI_re	acaaggatccACAACCTCCTTAGTACATTTCAC	pTet construction
pBAD_fw	ACCTGACGCTTTTTATCG	pTet construction
pBAD_XmaJI_re	gcaacctaggAGTTGTCATAATTGGTAACG	pTet construction
hpaX_XmaJI_fw	cagacctaggAGAACTTACCGCAGATTC	pHpaXA-GFP construction
PhpaBC_XbaI_re	atattctagaGTCGACCTCTACAGTATTAATCG	pHpaXA-GFP construction
pHpaXA_ApaI_re	atatgggcccCAAAAGAGTTTGTAGAAACGC	pCDF-HpaXA-GFP construction
pACYC_ApaI_fw	atatgggcccCCGCTGAGCAATAACTAGC	pCDF-HpaXA-GFP construction
pACYC_XmaJI_re	cactcctaggACCCAGTCAGCTCCTTCC	pCDF-HpaXA-GFP construction
hpaA_XmaJI_fw	atatcctaggGTATTGCGGTGATCAACGCC	pCDF-HpaA-GFP construction
Bcam0581_oe_fw	TTAATACTGTAGAGGTCGACATGAAACACCATCAC	pCDF-HpaXA-rpfF construction
Bcam0581_oe_re	CCCATATGGTACCAGCTGCATTACACTGTACGAAG	pCDF-HpaXA-rpfF construction
PBC_oe_re	TGATGGTGATGGTGTTTCATGTCGACCTCTACAGTATTAATCGG	pCDF-HpaXA-rpfF construction
pCDF_oe_fw	CGAAACTTCGTACAGTGTAATGCAGCTGGTACCATATGGG	pCDF-HpaXA-rpfF construction
tet_fw	CACATTAAGACCCACTTTCAC	pKD3-Ptet-rpfF construction
tet_re	ACAACCTCCTTAGTACATTTCAC	pKD3-Ptet-rpfF construction
pKD3_fw	CCATGGCTAATTCCCATGTC	pKD3-Ptet-rpfF construction
pKD3_tetR_re	CATATGCGGATTAGAAAAACAACTTAAATGTGAAAGTGGGTCTTAATGTGTCCATATGAATATCCTCCTTAG	pKD3-Ptet-rpfF construction
pKD3int_fw	AGGATCCGCTAATCTTATGGATAAAAATGCTATGGCATAGCAAAGTGTGAGTCTTGAGCGATTGTGTAGG	pKD3-Ptet-rpfF integration
pKD3int_re	AAGTGTCTATAATCACGGCAGAAAAGTCCACATTGATTATTTGCACGGCGGACATGGGAATTAGCCATGG	pKD3-Ptet-rpfF integration
RT5_PAT1	GCCACCTCAGTATCCTTATCCAG	qPCR
RT3_PAT1	TTGCTGTTGCTGTTGAGGAG	qPCR
RT5_RIP1	TGCTGACAGAGTCAAGAAACC	qPCR
RT3_RIP1	GAACCAACCACCGAAATCAC	qPCR
RT5_ECE1	CTGTTGTTGCTGGTATCATTGCTG	qPCR
RT3_ECE1	CAACGTCATCATTAGCTCCATCTC	qPCR
RT5_HWP1	GAAACCTCACCAATTGCTCCAG	qPCR
RT3_HWP1	GTAGAGACGACAGCACTAGATTCC	qPCR

Supplemental References

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