1 # Contact-dependent traits in *Pseudomonas syringae* B728a

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11 **Short Title: Contact-dependent traits**

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15 # Abstract

16 Production of the biosurfactant syringafactin by the plant pathogen *Pseudomonas syringae*

17 B728a is a surface contact-dependent trait. Expression of *syfA*, as measured using a *gfp* reporter

18 gene fusion was low in planktonic cells in liquid cultures but over 4-fold higher in cells

19 immobilized on surfaces as varied as glass, plastic, paper, parafilm, agar, membrane filters, and

20 leaves. Induction of *syfA* as measured by GFP fluorescence was rapid, occurring within two

21 hours after immobilization of cells on surfaces. Comparison of the global transcriptome by RNA

22 sequencing of planktonic cells in a nutrient medium with that of cells immobilized for 2 hours on

23 filters placed on this solidified medium revealed that, in addition to *syfA*, 3156 other genes were

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24   differentially expressed. Genes repressed in immobilized cells included those involved in

25   quaternary ammonium compound (QAC) metabolism and transport, compatible solute

26   production, carbohydrate metabolism and transport, organic acid metabolism and transport,

27   phytotoxin synthesis and transport, amino acid metabolism and transport, and secondary

28   metabolism. Genes induced in immobilized cells included *syfA* plus those involved in translation,

29   siderophore synthesis and transport, nucleotide metabolism and transport, flagellar synthesis and

30   motility, lipopolysaccharide (LPS) synthesis and transport, energy generation, transcription,

31   chemosensing and chemotaxis, replication and DNA repair, iron-sulfur proteins,

32   peptidoglycan/cell wall polymers, terpenoid backbone synthesis, iron metabolism and transport,

33   and cell division. That many genes are rapidly differentially expressed upon transfer of cells

34   from a planktonic to an immobilized state suggests that cells experience the two environments

35   differently. It seems possible that surface contact initiates anticipatory changes in *P. syringae*

36   gene expression, which enables rapid and appropriate physiological responses to the new

37   environmental conditions. Such responses could help cells survive transitions from aquatic

38   habitats fostering planktonic traits to attachment on surfaces, conditions that alternatively occur

39   on leaves.

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41   **Introduction**

42   *Pseudomonas syringae* has adapted to live in a variety of different environments. While most

43   studies of this taxon have focused on its life as a plant colonist in which it grows on the surfaces

44   of leaves and subsequently in the apoplast where it can cause disease, many strains apparently

45   inhabit other habitats. For instance, Morris *et al.* [1] described *P. syringae* in snow, rain, and in

46   lakes and rivers, suggesting that this bacterium is disseminated through the water cycle. All

47 bacteria are intrinsically aquatic, although many have adapted to survive in periodically dry

48 environments, often forming biofilms on surfaces in contact with water. The very different

49 chemical and physical properties of aquatic environments versus surfaces, which in the case of *P.*

50 *syringae* are frequently dry, presumably select for coordinated gene expression that optimizes

51 fitness across these different conditions. Leaf surfaces are relatively harsh habitats that exhibit

52 strong spatially and temporally varying conditions. Water and nutrients are unevenly dispersed

53 across leaf surfaces [2,3] and leaves experience rapid temporal fluctuations in temperature,

54 humidity, and liquid water availability [3]. Leaf surfaces also experience high ultraviolet

55 radiation flux [3]. In a bacterial population inhabiting such a setting, some cells might exhibit a

56 planktonic existence within water drops containing nutrients, while other cells must contend with

57 relatively dry surfaces.

58 Previous studies have revealed the role of syringafactin, a hygroscopic biosurfactant whose

59 production by *P. syringae* is encoded by *syfA*, as an adaptation to the frequent absence of liquid

60 water on leaf surfaces [4,5]. By binding water vapor trapped within the humid boundary layer

61 and/or by binding liquid water that is transiently present on leaves, syringafactin can alleviate

62 water stress by expanding the conditions under which liquid water is available for *P. syringae* [6].

63 Furthermore, syringafactin does not seem to disperse in water, perhaps due to its strong

64 hydrophobic characteristics, but instead remains immobilized onto leaf surfaces near the cells

65 that produced it [5]. Syringafactin is thus unlikely to benefit cells immersed in water, such as

66 those occurring in the planktonic state in aquatic environments involved in the water cycle [1].

67 Instead, syringafactin production might maximally benefit cells in non-aquatic settings where it

68 would improve *P. syringae* survival on surfaces subject to frequent drying. Interestingly, Burch

69 *et al*. [7] found that cells grown on agar surfaces produced much more syringafactin and

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70   expressed *syfA* at a much higher level than those in broth cultures. This observation suggests two

71   competing hypotheses. First, *syfA* differential gene expression may be instigated by the

72   differences in chemical or physical conditions found on an agar surface compared to that in a

73   similar liquid culture. Alternatively, immobilization of cells on a surface may be used as a cue to

74   immediately indicate the current or anticipated presence of environmental conditions common to

75   leaf surfaces where the production of syringafactin would be maximally beneficial to the cell.

76   Since Burch *et al*. [7] examined *syfA* expression only after growth of bacteria in different

77   conditions for extended time periods (>24 hours), they could not unambiguously distinguish

78   between these two models of gene regulation.

79   To distinguish between these two hypotheses, we determined how quickly *syfA* expression

80   increased after planktonic cells of *P. syringae* contacted a surface and whether the nature of the

81   surface it interacted with determined the extent of any response. It seemed likely that cells of *P.*

82   *syringae* might benefit from anticipatory patterns of gene expression, where certain cues might

83   be used to indicate large and rapid changes in environmental conditions. Thus, rapid or prior

84   expression of certain traits would be highly beneficial in adapting to large changes in habitat

85   characteristics. In this case, we predicted that changes in gene expression associated with

86   transition from a planktonic to a surface-associated habitat such as a leaf would be linked to

87   perception of a surface that might be imminently dry. While there has been interest in such

88   surface sensing in bacteria [8, 9, 10, 11, 12] little is known of the mechanism by which cells

89   sense surfaces and there are few studies that have examined rapid changes in gene expression

90   that could be associated with perception of a surface [13]. We therefore explored the rapid

91   changes in the global transcriptome of B728a soon after planktonic cells became sessile on a

92   surface. The large number of genes that we find to undergo changes in gene expression upon the

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93 transition from planktonic to sessile life suggest that surface sensing could be a major cue

94 controlling adjustment of *P. syringae* to the varying habitats it colonizes on leaf surfaces and

95 elsewhere.

Materials and methods

Bacterial strains and growth conditions

98 *Pseudomonas syringae* B728a strains were grown on King's medium B (KB) plates containing

99 1.5% technical agar or in KB broth lacking agar [14]. Antibiotics were used at the following

100 concentrations (µg/ml): spectinomycin (100), kanamycin (50), and tetracycline (15) as

101 appropriate.

Quantification of GFP fluorescence in individual bacterial cells

103 *P. syringae* B728a cells harboring a plasmid in which the *syfA* promoter was fused to a

104 promoterless *gfp* reporter gene [7], were grown in KB broth until they reached a density of 10^8

105 cells/ml as determined by Optical Density ($\lambda = 600$ nm). 10 µl of cells were then placed onto

106 various surfaces. To subsequently remove cells from these surfaces, moistened cotton swabs

107 were used to scrub off the cells. The swabs were then placed into microcentrifuge tubes

108 containing 100 µl of water and vortexed to re-suspend the cells. 5 µl of cell suspension from

109 each treatment was applied to glass slides for fluorescence microscopy to quantify the GFP

110 fluorescence of individual cells. Cells were visualized at 100X magnification with a M2

111 AxioImager. A GFP filter set was used to view cells, and images were captured in black and

112 white format using a 12-bit Retiga camera. The software iVision was used to identify all

113   bacterial cells in an image and to quantify the average pixel intensity of each object identified as

114   in other studies [6]. Aggregates of bacterial cells and extraneous particles were identified by

115   visual examination and marked for exclusion before image processing.

RNA isolation

117   Bacterial cells were grown with shaking in KB broth at 28°C until cultures reached a cell density

118   of 5×10^8 cells/ml. Three replicate cultures were used. 300 µl of each culture was then applied to

119   a 0.4 µm Isopore® membrane filter and excess liquid was removed by exposing the filter to a

120   vacuum source for 5 seconds. Filters were then immediately placed onto KB plates to incubate at

121   28°C for two hours while broth cultures were returned to a shaker and incubated at 28°C for two

122   more hours. Cells in broth cultures were harvested by pipetting 1 ml of suspension into a 15 mL

123   conical tube containing 125 µl ice-cold EtOH/Phenol stop solution (5% water-saturated phenol

124   (pH<7.0) in ethanol). Filters were immersed in the EtOH/Phenol stop solution and cells were

125   harvested by sonication for 30 seconds followed by vortexing for 20 seconds to ensure complete

126   cell detachment from the filters. Cells were then collected by centrifugation at 12,000 rpm

127   (13,800 x g) for five minutes at 4°C. Supernatant was decanted and the cells were frozen in

128   liquid nitrogen and stored at -80°C until RNA isolation. RNA isolation was performed using a

129   Direct-zol™ RNA Kit (Zymo Research). To isolate RNA, 600 µl of TRI Reagent® (Sigma-

130   Aldrich) was then added to the pelleted cells. An equal volume of ethanol (100%) was added to

131   the cells in TRI Reagent® and mixed thoroughly. The mixture was transferred into a Zymo-

132   Spin™ IICG Column (Zymo Research) in a collection tube and centrifuged. The flow-through

133   was discarded and the column was transferred into a new collection tube. For DNase I treatment,

134   400 µl RNA Wash Buffer (Zymo Research) was added to the column which was then

135   centrifuged. 5 µl DNase I (6 U/µl) (Zymo Research) and 75 µl DNA Digestion Buffer (Zymo

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136   Research) were then added to the tube and mixed. The mix was added directly to the column

137   matrix and incubated at room temperature (20-30°C) for 15 minutes. 400 µl Direct-zol™ RNA

138   PreWash (Zymo Research) was then added to the column and then centrifuged. The flow-

139   through was discarded and the step was repeated once more. 700 µl RNA Wash Buffer (Zymo

140   Research) was then added to the column and subsequently centrifuged for two minutes. The

141   column was then transferred into a RNase-free tube. To elute RNA, 50 µl of DNase/RNase-Free

142   Water (Zymo Research) was added directly to the column matrix which was then centrifuged.

143   RNA was stored frozen at -80°C.

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mRNA sequencing

146   1 µl of each sample was diluted into 4 µl of RNase free water and submitted to the Vincent J.

147   Coates Genomics Sequencing Laboratory at UC Berkeley where Ribo-Zero was used for rRNA

148   removal. RNA abundance and purity were determined using a 2100 Bioanalyzer (Agilent

149   Technologies) and quantified using Qubit (Invitrogen). After reverse transcription, shearing of

150   cDNA, size fractionation, and Illumina library production, the Vincent J. Coates Genomics

151   Sequencing Laboratory samples were sequenced using an Illumina HiSeq4000 platform with 50

152   base pair, single-end reads. Three biological replicates were sequenced per treatment. Reads

153   were uploaded to Galaxy [15] and cleaned using Trimmomatic [16]. Reads were aligned to the

154   *Pseudomonas syringae* B728a genome [17] using Salmon Transcript Quantification [18] in

155   Galaxy. The program edgeR in R was then used to assess the statistical significance of

156   differential gene expression [19]. Gene expression levels were normalized using a weighted

157   trimmed mean of M values (TMM; where M is the log expression ratio per gene between

158   treatments) [20]. Empirical Bayes estimation and tests based on the negative binomial

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159   distribution were then used to determine significance [20]. A gene was considered significantly

160   differentially regulated if the p-value for the difference in relative expression between the filter

161   treatment and the liquid treatment was less than 0.001.

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Statistical analysis

164   The hypergeometric distribution was performed in R [21] to test for significance of functional

165   category enrichment [22]. All p-values were adjusted using the Bonferroni correction [23] and

166   the Benjamini-Hochberg correction [24] in R.

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Results

SyfA induction occurs rapidly upon surface contact

170   If the *syfA* gene is regulated directly as a response to contact with a surface, we would expect

171   gene induction to occur shortly after surface contact. To test how rapidly *syfA* was induced after

172   planktonic cells were transferred to various surfaces, we monitored the expression of *syfA* in *P.*

173   *syringae* B728a harboring a plasmid containing the *syfA* promoter fused to a promoterless *gfp*

174   reporter gene by assessing the GFP fluorescence of individual cells by epifluorescence

175   microscopy. Planktonic cells from broth cultures were applied to three surfaces: (1) agar-

176   solidified King's medium B (KB) media, (2) a 0.4 μm polycarbonate Isopore® filter that was

177   placed on KB agar, and (3) a 0.4 μm Isopore® filter floated on the surface of a small quantity of

178   KB broth. Since nutrient solution diffused through the filters, wetting the filters in these settings,

179   we presume that the cells experienced similar levels of most nutrients while immobilized on the

180   filters as those remaining in broth cultures but experienced different physical conditions.

181   Planktonic cells that remained in broth cultures served as a control. We compared gene

182   expression of cells on filters placed on KB agar and KB broth to test whether chemical

183   components of the agar, rather than simply the physical change it elicited, contributed to

184   induction of *syfA* expression as seen by Burch *et al*. [7]. By two hours after transfer to each of

185   the solid surfaces, cells exhibited significantly greater GFP fluorescence than those remaining in

186   the broth cultures (Fig 1). Similar levels of *syfA* induction occurred on all surfaces (Fig 1). Thus,

187   *syfA* induction clearly occurs rapidly upon encountering a surface and elevated expression on

188   agar appears to be due to the exposure of cells to a surface rather than any chemical components

189   of the agar.

190   **Fig 1. Induction of *syfA* expression in cells of *Pseudomonas syringae* B728a as a function of**

191   **time.**

192   Gene expression is estimated as the mean GFP fluorescence of individual cells harboring a

193   plasmid in which the *syfA* promoter was fused to a *gfp* reporter gene when harvested from these

194   surfaces at the various times shown on the abscissa. 7,038 cells were evaluated in total for

195   control cells that remained in a broth culture (blue); 3,068 cells were evaluated after application

196   to agar (green); 10,184 cells were evaluated after application to filters on an agar surface

197   (yellow); and 3,791 cells were evaluated after application to a filter placed on plastic (orange).

198   The vertical bars represent the standard error of the mean GFP fluorescence for a given cell.

SyfA is induced in cells on a variety of solid surfaces

200   Given that *syfA* induction quickly followed transfer of planktonic cells to various surfaces with

201   abundant nutrient resources, we explored whether any particular physical parameters such as

202   hydrophobicity or roughness were associated with the induction process. Since *P. syringae*

203   B728a was isolated from leaf surfaces and is highly fit as an epiphyte [25, 26], we tested the

204   hypothesis that leaf surfaces would confer the most rapid or largest contact-dependent induction

205   of this gene. By exposing cells to various types of solid surfaces we further explored whether

206   *syfA* expression was regulated by contact with a surface per se as opposed to a chemical cue.

207   Planktonic cells from KB broth cultures were applied to polycarbonate, paper, parafilm, excised

208   leaves, water agar, and Isopore®, Durapore®, and Teflon® filters. Cells all exhibited at least 2-

209   fold greater GFP fluorescence than those of the planktonic cells when assessed six hours after

210   application to these surfaces (Fig 2). Little difference in GFP fluorescence was observed among

211   cells placed on the various solid surfaces, suggesting that neither surface type-dependent

212   chemical cues nor the physical properties of the surface on which cells were immobilized

213   strongly influenced the induction of *syfA*.

214   **Fig 2. The expression of *syfA* increases rapidly after immobilization of cells of *Pseudomonas***

215   ***syringae* B728a.**

216   Gene expression is estimated as the mean GFP fluorescence of individual cells harboring a

217   plasmid in which the *syfA* promoter was fused to a *gfp* reporter gene when harvested 6 hours

218   after application to glass (green), polycarbonate plastic (yellow), paper (orange), parafilm (red),

219   bean leaves (purple), agar (grey), Isopore filters (pink), Durapore filters (aqua), Teflon filters

220   (brown), or recovered from broth cultures at the time of application to surfaces or after 6 hours

221   (blue). The vertical bars represent the standard error of the mean GFP fluorescence for a given

222   cell.

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224 RNA sequencing of *P. syringae* B728a reveals many genes

225 differentially regulated in cells transferred to a filter surface versus

226 in liquid culture

227 Given that cells of *P. syringae* exhibited large increases in *syfA* expression within two hours of

228 transfer to a variety of solid surfaces, we performed RNA sequencing to determine the extent to

229 which other genes exhibit contact-dependent gene expression. The transcriptome of cells grown

230 in KB broth was compared to those placed on filters atop KB agar for 2 hours. RNA sequencing

231 returned between 20 and 40 million reads per sample (Fig S1). As expected, a wide range of

232 reads were recovered for a given gene (Fig S2). A heatmap revealed that while patterns of gene

233 regulation were similar among replicates in which cells were either planktonic or immobilized on

234 a filter, gene expression differed strongly between cells in these two conditions (Fig 3). Much of

235 the variation in relative gene expression levels of the average gene was thus associated with the

236 environment of the cells before RNA was harvested rather than other factors.

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238 **Fig 3. Heat map illustrating different patterns of gene expression in cells of *Pseudomonas***

239 ***syringae* B728a.**

240 Heat map illustrating different patterns of gene expression in cells of *Pseudomonas syringae*

241 B728a 2 hours after application to a filter surface compared to that in broth culture. Differential

242 up-regulation of genes is shown in yellow while down-regulation is depicted in red.

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244 Expression of 1,390 genes was up-regulated in cells on the filter surface compared to those in

245 broth media, while expression of 1,766 genes was down-regulated on filter surfaces compared to

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246   that in broth (Fig 4). Of the 1,390 genes that were induced on filter surfaces, 881 were induced

247   more than 2-fold while 509 exhibited lesser induction. Of the 1,766 genes that were repressed on

248   filter surfaces, 1,138 were repressed more than 2-fold while 628 were repressed less than 2-fold.

249   In total, 3,156 genes (60.46% of the *P. syringae* B728a genome) were found to be differentially

250   expressed in cells on the filter surface compared to those in broth culture (Fig 4). There was no

251   apparent relationship between the absolute level of expression of a gene and the likelihood that it

252   would exhibit differential expression in these two settings (Fig S3).

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254   **Fig 4. Volcano plot illustrating the differential expression of genes in *Pseudomonas syringae***

255   **B728a.** Volcano plot illustrating the differential expression of genes in *Pseudomonas syringae*

256   B728a 2 hours after application to a filter surface. Genes that are significantly differentially

257   expressed compared to that in broth culture are shown in red, and those that do not differ are

258   shown in black.

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260   Functional category analysis

261   We grouped the differentially regulated genes into functional categories to better establish those

262   processes that were most differentially expressed in planktonic and immobilized cells (Tables 1

263   and 2). Twice as many functional gene categories were enriched in genes that were significantly

264   up-regulated in immobilized cells than those that were down-regulated. The following gene

265   functional categories were examined in further detail:

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268 **Table 1. Functional gene categories preferentially up-regulated in cells of *Pseudomonas***

269 ***syringae* B728a 2 hours after application to a filter surface.**

Gene Category	Bonferroni adjusted p-value	Benjamini-Hochberg adjusted p-value
Translation	1.11E-18	1.11E-18
Siderophore synthesis and transport	4.75E-09	2.38E-09
Nucleotide metabolism and transport	1.12E-06	3.73E-07
Flagellar synthesis and motility	2.44E-03	6.11E-04
Lipopolysaccharide synthesis and transport	9.16E-03	1.83E-03
Energy generation	0.02	2.63E-03
Transcription	0.04	0.04
Chemosensing and chemotaxis	0.06	7.15E-03
Replication and DNA repair	0.12	0.01
Iron-sulfur proteins	0.19	0.02

Peptidoglycan/cell wall polymers	0.19	0.02
Terpenoid backbone synthesis	0.24	0.02
Iron metabolism and transport	0.25	0.02
Cell Division	0.59	0.04

270   The significance of functional category enrichment was assessed using the hypergeometric distribution

271   (Castillo-Davis and Hartl, 2003).

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281 **Table 2. Functional gene categories of *Pseudomonas syringae* B728a preferentially down-**

282 **regulated in cells 2 hours after application to a filter surface.**

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Gene Category	Bonferroni adjusted p-value	Benjamini-Hochberg adjusted p-value
Quaternary ammonium compound metabolism and transport	2.19E-08	2.19E-08
Compatible solute synthesis	2.33E-06	1.17E-06
Carbohydrate metabolism and transport	1.72E-05	5.74E-06
Organic acid metabolism and transport	8.84E-05	2.21E-05
Phytotoxin synthesis and transport	1.16E-04	2.32E-05
Amino acid metabolism and transport	0.01	1.77E-03
Secondary metabolism	0.01	1.01E-03

284 The significance of functional category enrichment was assessed using the hypergeometric distribution

285 (Castillo-Davis and Hartl, 2003).

286 **Translation.** Many genes encoding the 30S and 50S ribosomal protein subunits were induced

287 on the filter surface, as were genes encoding the elongation factor proteins Ts, P, Tu, and G.

288 Many genes encoding t-RNA synthetases were also induced. It thus appeared that translation as a

289 whole may have accelerated upon transition of cells from a planktonic to an immobilized state.

290 **Siderophore synthesis and iron metabolism.** Many genes involved in siderophore

291 synthesis and transport were induced on the filter surface. Many genes such as *pvdS*, *pvdG*, *pvdL*,

292 *pvdI*, *pvdJ*, *pvdK, pvdD*, *pvdE*, *pbdO*, *pvdN*, *pvdT*, and *pvdR* were involved in regulation of

293 pyoverdine production and its transport. Many genes involved in achromobactin regulation,

294 synthesis, and transport including *acsG*, *acsD*, *acsE*, *yhcA*, *acsC*, *acsB*, *acsA*, *carA-2*, *cbrB-2*,

295 and *cbrC-2* were also induced. Many genes involved in iron metabolism and transport were also

296 induced on the filter surface. This included the genes *fecE*, *fecD*, *fecC*, *fecB*, *fecA*, *fecR*, and the

297 RNA polymerase ECF sigma factor *fecI*. These results suggest that iron became less available on

298 the filter surfaces than in broth, perhaps due to diffusional limitations associated with the lack of

299 mixing of cells as would occur in a liquid medium. Alternatively, iron might commonly be less

300 available on the natural surfaces on which *P. syringae* typically inhabits, such as leaf surfaces,

301 and the filter mimicked physical cues that the bacteria might use to anticipate transition into such

302 low iron environments.

303 **Nucleotide metabolism and transport.** Numerous genes involved in nucleotide

304 metabolism and transport were induced in immobilized cells. This included many genes involved

305 in purine and pyrimidine metabolism such as *purA*, *purT*, *purC*, *purF*, *purB*, *purM*, *purN*, *purU-3*,

306 *purH*, *purD*, *purK*, *purE*, and *pyrB*, *pyrR*, *pyrH*, *pyrG*, *pyrF*, *pyrD*, *pyrC-2* respectively. As with

307 the apparent increase in translational activity seen upon transition of planktonic cells to those on

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308   surfaces, increased transcription might be expected to also be linked to such increases, requiring

309   higher rates of nucleotide synthesis. Almost all genes with significant differential expression

310   involved in cell division were also induced on the filter surface. This included the cell division

311   proteins FtsK, FtsQ, and FtsL as well as the rod-shape determining proteins MreD and MreC.

312   MrdB, a cell cycle protein, was also induced.

313   **Flagellar synthesis and motility.** Many genes encoding flagellar biosynthesis proteins,

314   flagellar basal body proteins, and flagellar hook-associated proteins were induced on the filter

315   surface. The gene encoding the anti-sigma-28 factor FlgM was also induced more than 2-fold.

316   Genes encoding the flagellar motor proteins MotA, MotB, MotC, and MotD were induced as

317   well. While it would be expected that planktonic cells of *P. syringae* would be motile, higher

318   levels of expression of motility genes on leaves compared to that in broth cultures has been

319   previously noted [26] and would likely require higher levels of flagellar production and repair [9].

320   **Lipopolysaccharide synthesis and transport.** All the genes with significant differential

321   expression that are involved in lipopolysaccharide (LPS) synthesis and transport were induced in

322   cells immobilized on filters except *arnB* and *arnA*. Most of these induced genes are involved in

323   LPS transport and lipid A biosynthesis.

324   **Energy generation.** Many genes encoding proteins involved in oxidative phosphorylation

325   were expressed at a higher level on filter surfaces than in broth cultures. Such genes included

326   *cyoA*, *cyoB*, *cyoC,* and *cyoD*, all of which encode cytochrome c oxidase subunits, as well as *ccoN*,

327   *ccoO,* and *ccoP* which encode cytochrome c oxidase cbb3-type subunits. Many genes encoding

328   F0F1 ATP synthase subunits were induced as well.

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329   **Transcription.** Similar to that seen for nucleotide synthesis, nearly all of the genes involved

330   in transcription that were differentially expressed were induced on filters compared to that in

331   broth cultures, with the exception of Psyr_4263. Genes up-regulated on surfaces included those

332   encoding the transcription termination factor Rho, the transcription elongation factors GreB and

333   GreA, the transcription anti-termination proteins NusB and NusG, and the DNA-directed RNA

334   polymerase subunits RpoA, RpoC, and RpoB.

335   **Chemosensing and chemotaxis.** Many genes involved in chemosensing and chemotaxis

336   were induced in cells attached to on the filter surface. Many of the induced genes encoded

337   histidine kinases. Interestingly, Psyr_1306, Psyr_1307 and Psyr_1308, which encode homologs

338   to WspD, WspE, and WspF respectively, were all down-regulated on the filter surface.

339   Psyr_1309, which encodes the homolog for WspR, was also down-regulated. However, this latter

340   gene was assigned to the cyclic diguanylate (cyclic di-GMP) cyclase proteins functional group.

341   **Replication and DNA repair.** Most of the genes involved in replication and DNA repair

342   were induced on the filter surface. This included Pysr_1408, Psyr_1409, and Psyr_1410 which

343   encode RuvC, RuvA, and RuvB respectively. These genes are also involved in homologous

344   recombination in addition to DNA repair.

345   **Iron-sulfur proteins.** Many genes encoding iron-sulfur proteins were induced on the filter

346   surface. This included *dsbE* which is involved in cytochrome synthesis.

347   **Peptiodoglycan/cell wall polymers.** All of the genes involved in encoding

348   peptidoglycan/cell wall polymers that were differentially expressed on filters compared to broth

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349   cultures were induced on the filter surface. Most of these genes are involved in peptidoglycan

350   biosynthesis.

351   **Terpenoid backbone synthesis.** Many genes involved in terpenoid backbone synthesis

352   were induced in cells applied to filter surfaces. This included genes that are part of the

353   deoxyxylulose pathway of terpenoid biosynthesis.

354   **Quaternary ammonium compound metabolism and transport.** Surprisingly, all

355   genes with differential expression that are required for quaternary ammonium compound (QAC)

356   metabolism and transport were repressed on the filter surface. This included genes encoding

357   proteins involved in glycine betaine, choline, and carnitine metabolism and transport. The gene

358   *betI*, encoding the transcriptional regulator of choline degradation, was also down-regulated.

359   Similar to that seen for genes involved in QAC metabolism, all of the genes that had significant

360   differential expression that are involved in compatible solute synthesis were repressed on the

361   filter surface. Many of these genes contribute to either trehalose or N-acetylglutaminylglutamine

362   amide (NAGGN) synthesis. These compounds are solutes that are part of the cellular response to

363   water stress.

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365   **Carbohydrate metabolism and transport.** Many of the genes involved in carbohydrate

366   metabolism and transport were repressed in immobilized cells on filters. This included genes

367   involved in trehalose, mannose, fructose, ribose, arabinose, maltose, manitol, and sorbitol

368   transport as well as genes involved in the pentose phosphate pathway.

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370 **Organic acid metabolism and transport.** Many of the genes involved in organic acid

371 metabolism and transport were also repressed on the filter surface. This included the genes *phnF*,

372 *phnG*, *phnH*, *phnI*, *phnJ*, *phnK*, *phnL*, *phnM*, *phnN*, and *phnP* which are all involved in

373 phosphonate metabolism and transport. The transcriptional regulator of vanillate metabolism,

374 *vanR*, was also down-regulated.

375 **Phytotoxin synthesis and transport.** All of the differentially expressed genes involved in

376 phytotoxin synthesis and transport were repressed in cells applied to filters. These genes included

377 *salA,* which is the regulator of syringomycin, as well as *slyA,* the regulator of syringolin A

378 production. Other genes involved in syringolin synthesis and transport (*slyB*, *slyC*, *slyD*, and

379 *slyE*), syringomycin synthesis and transport (*syrE*, *syrC*, *syrB1*, *syrP*, and *syrD*), and

380 syringopeptin synthesis and transport (*sypA*, *sypB*, and *sypC*). Syringomycin and syringopeptin

381 secretion proteins PseA and PseB were also down-regulated in immobilized cells on filters.

382 **Amino acid metabolism and transport.** Many of the genes involved in amino acid

383 metabolism and transport were repressed on the filter surface. This included genes involved in

384 gamma-aminobutyric acid (GABA) metabolism (*gabT-2*, *gabD-2*, *gabT-1*, *gabD-1*, *gabD-3*, and

385 *gabP*).

386

387 **Secondary metabolism.** All of the differentially expressed genes involved in secondary

388 metabolism were repressed on the filter with the noteworthy exception of Psyr_2575, Psyr_2576,

389 and Psyr_2577 which encode SyfR, SyfA, and SyfB, responsible for the regulation of and

390 production of syringafactin, respectively. It was therefore intriguing to find that syringafactin

391   production was the sole example of secondary compounds that were not down-regulated when

392   cells transitioned from a planktonic to a sessile state.

Discussion

394   The remarkably strong and rapid induction of *syfA* in *P. syringae* B728a in cells transferred from

395   broth culture to any of several different types of surfaces encouraged us to test the hypothesis

396   that a variety of other traits would exhibit similar surface-dependent changes in expression. The

397   rapidity with which *syfA* induction occurred on all of the various surfaces makes it unlikely that

398   cells modified their local microhabitat in any substantial way. We thus presume that assessment

399   of transcriptional changes within two hours after transfer to these surfaces reflected contact-

400   specific gene regulation rather than changes responsive to altered microenvironments that have

401   been seen in studies of biofilms. Such studies typically examined bacteria many hours after

402   attachment, usually after a thick biofilm had formed on surfaces [27, 28, 29]. We also presume

403   that regulatory shifts that occurred shortly after contact with a surface also were distinct from

404   those conditioned by other secondary events such as cell-cell contact or cell density-dependent

405   regulatory processes. Given the brief period of immobilization of cells on a filter surface, we

406   were surprised to find such a high proportion of the genes in *P. syringae* B728a to respond to this

407   transition. In addition to induction of the genes conferring syringafactin production and its

408   regulation (*syfA*, *syfB*, and *syfR*), it was striking that genes involved in many other cellular

409   processes such as flagellar synthesis and motility, LPS synthesis and transport, chemosensing

410   and chemotaxis, siderophore synthesis and transport, and DNA replication and repair underwent

411   changes in expression. While coherent arguments could be made for why some of these

412   processes should exhibit contact-dependent expression, the responses of many other processes

21

413   remain enigmatic. The induction of many genes required for the synthesis of peptidoglycan/cell

414   wall polymers and LPS synthesis and transport seen in the immobilized cells (Table 1) suggests

415   that, like that of other bacteria for which surface contact-dependent gene expression has been

416   investigated [30, 31], *P. syringae* may also utilize a mechanosensitive pathway for sensing a

417   surface that involves disruption and repair of the cell envelope. Previous studies have suggested

418   that cells experience cell wall damage when they contact a surface [8, 30]. Peptidoglycan and

419   cell wall synthesis might be induced to repair any damage that resulted from such an encounter.

420   Moreover, the bacterial outer membrane also contains abundant lipopolysaccharides that could

421   also be disrupted during physical binding of cells to a hard surface, making LPS synthesis

422   essential [9, 32, 33]. Interestingly, this initial cell wall stress may also be a cue for the

423   differential expression of other surface-regulated genes as suggested by others [8].

424   Historically, it has been common to study bacteria in broth cultures. While it is presumed that

425   such cultures are more homogeneous and facilitate studies of coordinated patterns of gene

426   expression etc., many aspects of the manner in which microorganisms interact with their

427   environment cannot be studied in such a setting [8, 11, 12]. For example, low nutrient conditions

428   in broth cultures are typically associated with both high cell densities and low oxygen levels. In

429   contrast, cells on the leaf surface would typically experience low nutrient conditions in a fully

430   aerobic environment. Although *P. syringae* B728a can be found in aquatic environments, it also

431   colonizes the surface of leaves, a decidedly different environment where it encounters both low

432   nutrient concentrations and high oxygen levels [1, 3, 34]. Furthermore, the study of bacterial

433   adaptations to life on surfaces has focused almost entirely on the process of biofilm formation in

434   aquatic settings [27, 28, 29]. The three-dimensional aggregates of bacteria that often form in

435   such aqueous environments, in which nutrients are provided by flowing liquids, are almost

436   certainly very different from the monolayers of bacteria that typically develop on leaf surfaces

437   [35, 36]. Delivery of soluble nutrients in flowing liquids is probably an exceptional situation on

438   leaves [2, 3]. In addition, biofilm formation in a flowing liquid environment occurs over

439   relatively long periods of time. Cells are acquired by, or develop within, a biofilm over a long

440   period of time, during which the nature of the environment within the biofilm changes

441   dramatically, with large spaciotemporal variations [27]. For both practical and other reasons,

442   studies of aquatic biofilms typically have examined gene regulation only 24 hours or later after

443   biofilm initiation [27, 29]. Few studies have addressed very early stages of biofilm formation

444   such as this study.

445   Genes involved in both transcription and translation were typically induced upon cell

446   immobilization (Table 1). Given that the transcription of a large number of genes was rapidly

447   increased upon transitioning from a planktonic to a sessile state (Figs 3 and 4), there would need

448   to be a corresponding increase in translation to produce the corresponding proteins. Likewise,

449   genes linked to nucleotide metabolism and transport, transcription, and replication and DNA

450   repair (Table 1) were also induced upon immobilization of cells. It would follow that higher

451   rates of nucleotide synthesis or procurement would be needed to support RNA synthesis

452   associated with the elevated transcription. The apparent elevated DNA replication associated

453   with cell immobilization would also require increased expression of genes enabling nucleotide

454   metabolism.

455   Studies have suggested that cells on leaf surfaces and attached to apoplastic surfaces experience

456   oxidative stresses resulting from plant defenses [37, 38, 39]. These compounds, including

457   hydroxyl radicals created when iron reacts with H_2O_2, can be damaging to iron-sulfur proteins

458   and DNA [39]. Genes involved in iron-sulfur protein synthesis and DNA repair would be

23

459   expected to be induced on surfaces, as we observed here, if cells anticipated such a chemical

460   assault on plant surfaces and linked their expression with surfaces per se.

461   Genes required for siderophore synthesis and transport as well as iron metabolism and transport,

462   typically activated under conditions of low iron availability [26] were also induced in

463   immobilized cells (Table 1), suggesting that iron was less available to cells on the filter surface

464   than in broth media. Access to the limited iron in any environment would be expected to be

465   determined by its delivery to the vicinity of the cell by diffusion. Such a process would be

466   diminished in the two-dimensional environment on the surface of filters compared to the three-

467   dimensional habitat of cells immersed in a liquid medium. Cells affixed to a surface also would

468   be unable to move in response to local resource depletion to gain access to such molecules [13].

469   Given that iron is commonly present in low amounts on the natural surfaces that *P. syringae*

470   typically inhabits, such as leaves [40, 41] immobilization of cells on filters might have mimicked

471   this physical cue to anticipate transition into such low-iron environments.

472   The many genes involved in flagellar synthesis and motility and chemosensing and chemotaxis

473   that were surface induced (Table 1) would be important in the colonization of leaf surfaces that

474   harbor limited amounts of nutrient resources that are also heterogeneously dispersed [2, 42, 43].

475   Moreover, pathogenic bacteria such as *P. syringae* often eventually colonize the leaf apoplast - a

476   process requiring cells to move towards and enter a stomate or breaks in the cuticular surface of

477   leaves to access this intercellular habitat [34]. The up-regulation of motility and chemotaxis

478   genes was also observed in a study examining the transcriptome of *P. syringae* B728a on leaf

479   surfaces and apoplast when compared to that in a liquid medium *in vitro* [26]. Direct support for

480   the importance of cell motility of *P. syringae* on leaves was provided by studies of Haefele and

481   Lindow [43] who showed that non-motile mutants were much less fit than motile cells.

482   Surprisingly, genes involved in QAC metabolism and compatible solute synthesis tended to be

483   repressed in cells on filter surfaces (Table 2). Such compounds often serve as compatible solutes

484   whose purpose is to maintain equilibrium of water availability between the inside of the cell and

485   the outside environment. Thus, genes involved in compatible solute synthesis would be expected

486   to be up-regulated in cells experiencing osmotic or matric stresses [26]. Previous studies have

487   suggested that the leaf surface is often sufficiently dry that compatible solute synthesis is needed

488   to combat matric stress [44, 45]. However, since this experiment was performed on filters placed

489   on nutrient agar, the water status of cells upon such a surface is uncertain. While one might

490   imagine such a surface to be a drier habitat than what cells suspended in the corresponding broth

491   medium might experience, it is likely that this surface would be moister than leaf surfaces

492   typically are. The disruption of the gel matrix that would occur by application of a filter on an

493   agar surface might release free water in a process known as syneresis [46]. Indeed, filters

494   immediately appeared transiently wet after application to the agar surface. While the leaf surface

495   is composed of a waxy, hydrophobic cuticle to prevent the release of water vapor [47, 48], a

496   membrane filter is by definition quite porous and would enable the movement of water onto the

497   filter surface where it could wet cells.

498   The down-regulation of genes encoding carbohydrate metabolism and transport, organic acid

499   metabolism and transport, and amino acid metabolism and transport (Table 2) upon

500   immobilization of cells was surprising. While we expected nutrients to move from the agar

501   matrix through the filter to the attached cells on top, it is likely that the rate at which nutrients are

502   replenished by this diffusional process would be slower than that occurring during mixing of

503   cells in a planktonic state in the corresponding shaken broth medium. It might thus be expected

504   that the immobilized cells could experience a locally more nutrient-limited environment than

25

505   those of the planktonic cells. The common observation of slower growth of bacteria on the

506   surface of membranes placed on agar media than in shaken broth of the same composition also

507   supports such a model. Interestingly it has been noted that many genes in *P. syringae* involved in

508   amino acid metabolism and transport were repressed on the leaf surface [26].

509   Surprisingly, genes involved in phytotoxin synthesis and transport as well as many other genes

510   enabling production of other secondary metabolites were repressed on the filter surface (Table

511   2). Such compounds often act as virulence factors. We had expected that contact with a surface

512   would serve as a cue to *P. syringae* that it had encountered a potential host plant after

513   transitioning from a planktonic existence, and thus one would have expected such compounds to

514   be expressed. However, the large majority of *P. syringae* cells in the phyllosphere of healthy

515   leaves usually occur only as epiphytes on the surface of leaves rather than within the apoplast

516   [49]. Thus, when *P. syringae* colonizes the leaf surface it seldom interacts directly with living

517   plant cells and thus would not be expected to benefit from the production of various toxins.

518   Rather, such toxins would prove beneficial only after it had entered the leaf apoplast [34] and

519   their expression is highly elevated in the apoplast, but not on the leaf surface [26]. We

520   hypothesize that a secondary signal, indicative of conditions within the leaf, and not a surface

521   itself, is required for expression of such virulence factors. Arbutin and other compounds that are

522   not common on leaf surfaces have also been shown to be required for the induction of toxins

523   such as syringomycin in *P. syringae* [50]. Such secondary cues would prevent the costly

524   production of toxins when they would not be beneficial.

525   One of the few studies that have examined gene expression in bacterial cells soon after surface

526   attachment assessed gene expression in *Escherichia coli* strain CSH50 by microarray analysis

527   one, four, and eight hours after planktonic cells had attached to mannose agarose beads

528   submerged in cultures [13]. This study revealed that changes in gene expression upon

529   immobilization of cells were both very rapid and often transient as many genes that had become

530   induced by one hour of surface attachment exhibited subsequent decreases in expression by four

531   hours of attachment. By eight hours of attachment many of these genes exhibited continued

532   decreases in expression while others only then became induced. Interestingly, we found similar

533   functional genes in *P. syringae* to be up-regulated soon after cells attached to a surface, including

534   *emrB* encoding a drug resistance transporter as well as other genes in this operon, and *glnH*,

535   involved in the transport of glutamine (Fig S4). The gene *marA,* involved in resistance to a

536   variety of antimicrobial compounds and *marR* encoding its regulator as well as *ahpF, grxA*, and

537   *katG* and their regulator *oxyR,* collectively mediating oxidative stress tolerance were also up-

538   regulated in both systems (Fig S4). Genes involved in attachment and DNA repair were up-

539   regulated in response to attachment in both systems as well. Taken together, these results suggest

540   that both *E. coli* and *P. syringae* experience chemical stresses to which these defensive responses

541   are necessary soon after mobilization on surfaces. Alternatively, surface attachment is taken as a

542   cue to alert cells that they have arrived at a surface upon which such chemical stresses could be

543   anticipated. *E. coli* can experience chemical stresses associated with innate resistance responses

544   of animal cells [51] as well as plants [37, 39]. Likewise, *P. syringae* experiences defensive

545   chemicals as well as oxidative stress in both compatible and incompatible host plants [38, 52]

546   and induces defensive genes in response [26]. Our work suggests that many such traits might be

547   induced in *P. syringae* merely upon contact with the leaf surface itself. Thus, stress response

548   traits, presumably less important in an open, aquatic environment, are rapidly induced in both *P.*

549   *syringae* and *E. coli.* when host defenses might otherwise prove lethal.

550   In another study, Siryaporn *et al*. [31] examined rapid differential expression of genes in

551   *Pseudomonas aeruginosa* UCBPP-PA14 one hour after attaching to a surface, finding that *pilY1*

552   plays a central role in its surface-dependent induction of virulence and that the expression of

553   many genes was rapidly changed. Despite finding little surface-mediated induction of this

554   homolog in *P. syringae*, the homologs of several other genes were found to be induced in *P.*

555   *syringae* (Figs S4, S5, S6, S7). Among these genes were those involved in flagellar synthesis and

556   motility. A variety of genes involved in cell wall synthesis also exhibited similar regulatory

557   responses to surfaces in both bacteria. The genes *mraY* and *murA,* involved in peptidoglycan

558   synthesis, as well as *lpxC* and *lpxD*, involved in LPS synthesis, were induced upon

559   immobilization of both *P. syringae* and *P. aeruginosa* (Fig S7), suggesting that cell wall damage

560   is common upon interacting with surfaces [8, 30, 53]. Likewise, *ispZ*, involved in cell division,

561   was also induced on surfaces in both studies (Fig S7). It thus appears that there are many

562   common features of the initial interaction of a variety of bacteria taxa with surfaces, and that the

563   nature of the surface with which they interact may play a subservient role in dictating such

564   responses. A better understanding of the initial interaction of bacteria with surfaces hopefully

565   will lead to better manage their interactions in both agricultural and biomedical settings [12].

566

Acknowledgements

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717

Supporting Information

719   **Figure S1. Number of sequencing reads.** Number of sequencing reads obtained for the various

720   replicate samples of *Pseudomonas syringae* B728a cells recovered 2 hours after application to

721   filters or from cells in broth cultures used to inoculate the filters.

722   **Figure S2. Distribution of number of sequencing reads.** Distribution of number of sequencing

723   reads assigned to a given gene following RNA sequencing of *Pseudomonas syringae* B728a cells

724   recovered 2 hours after application to filters or from cells in broth cultures used to inoculate the

725   filters.

726   **Figure S3. Smear plot documenting the differential expression of genes in *Pseudomonas***

727   ***syringae* B728a.** Smear plot documenting the differential expression of genes in *Pseudomonas*

728   *syringae* B728a 2 hours after inoculation onto filter surfaces compared to that in planktonic cells

729   as a function of their levels of expression. Shown is the proportion of the genes that were

730   differentially expressed on the filter surface (red) and those not differentially expressed on a

731   surface (black).

732 **Figure S4. Comparison of differential expression of homologs of genes in *Pseudomonas***

733 ***syringae* B728a and in *E. coli* CSH50.** Comparison of differential expression of homologs of

734 genes in *Pseudomonas syringae* B728a 2 hours after application to membrane surfaces and of *E.*

735 *coli* CSH50 1 hour after attachment to mannose agarose beads in the study of Bhomkar *et al.*

736 (2010). Differential up-regulation of genes is shown in green, down-regulation is depicted in red,

737 and no change in expression is shown in yellow.

738 **Figure S5. Relationship between magnitude (fold-change) of differential expression of gene**

739 **homologs in *Pseudomonas syringae* B728a and in *E. coli* CSH50**. Relationship between

740 magnitude (fold-change) of differential expression of gene homologs in *Pseudomonas syringae*

741 B728a 2 hours after transfer to a filter surface and that of *E. coli* CSH50 1 hour after attachment

742 on agarose beads in the study of Bhomkar *et al.* (2010).

743 **Figure S6. Comparison of the number of gene homologs in *Pseudomonas syringae* B728a**

744 **and in *Pseudomonas aeruginosa* UCBPP-PA14.** Comparison of the number of gene homologs

745 in *Pseudomonas syringae* B728a (blue) and *Pseudomonas aeruginosa* UCBPP-PA14 (red) that

746 were up-regulated when transferred to filter surfaces for 2 hours or were attached to a glass

747 surface for 1 hour in the study of Siryaporn *et al.* (2014), respectively. Using the hypergeometric

748 distribution, this overlap was determined to be significant with a p-value of 1.65E-05 (Castillo-

749 Davis and Hartl, 2003).

750 **Figure S7. Comparison of differential expression of homologs of genes in *Pseudomonas***

751 ***syringae* B728a and in *Pseudomonas aeruginosa* UCBPP-PA14.** Comparison of differential

752 expression of homologs of genes in *Pseudomonas syringae* B728a 2 hours after application to

753 membrane surfaces and of *Pseudomonas aeruginosa* UCBPP-PA14 wild-type cells attached to a

754 glass surface for 1 hour in the study of Siryaporn *et al.* (2014). Differential up-regulation of

755   genes is shown in green, down-regulation is depicted in red, and no change in expression is
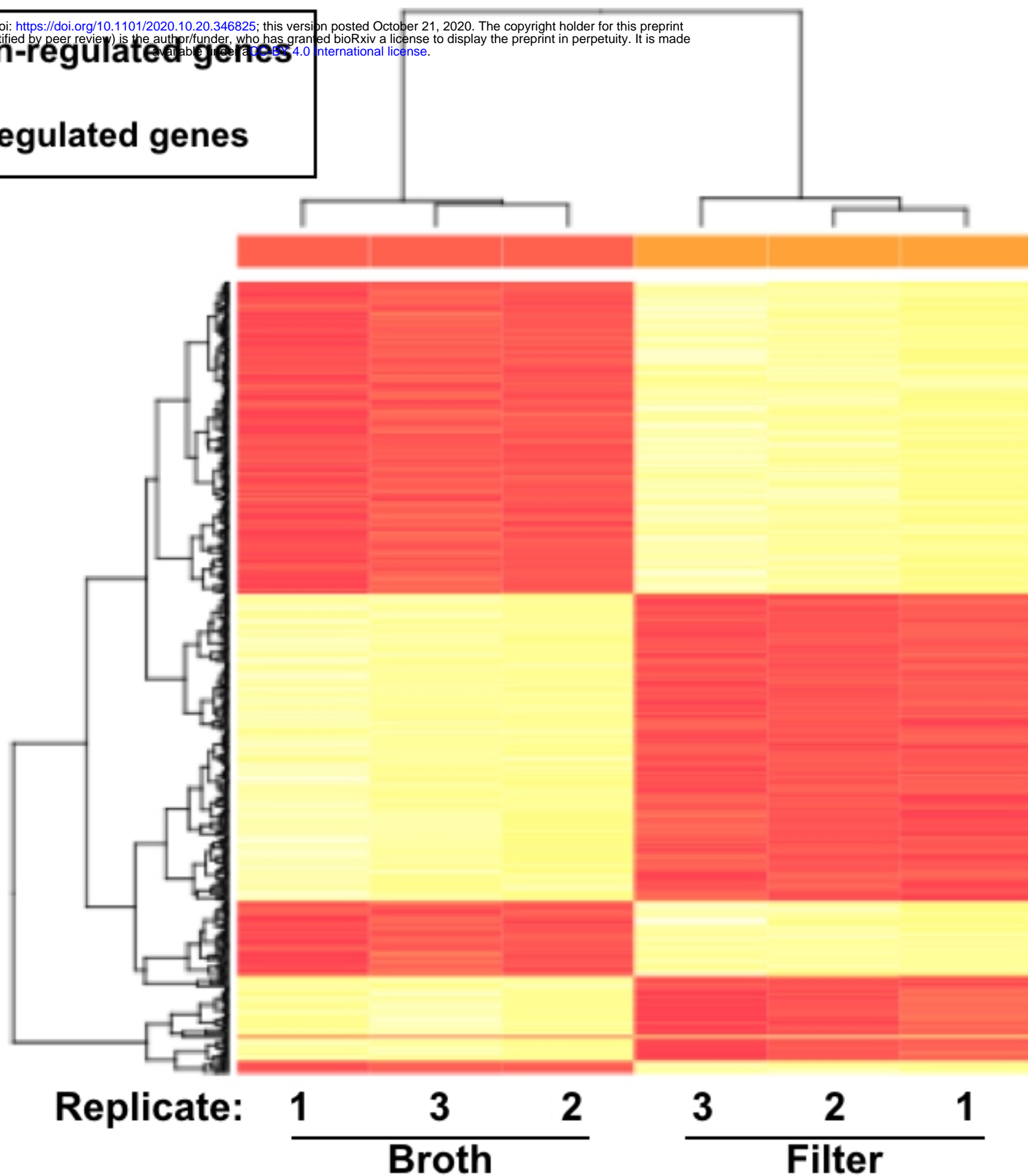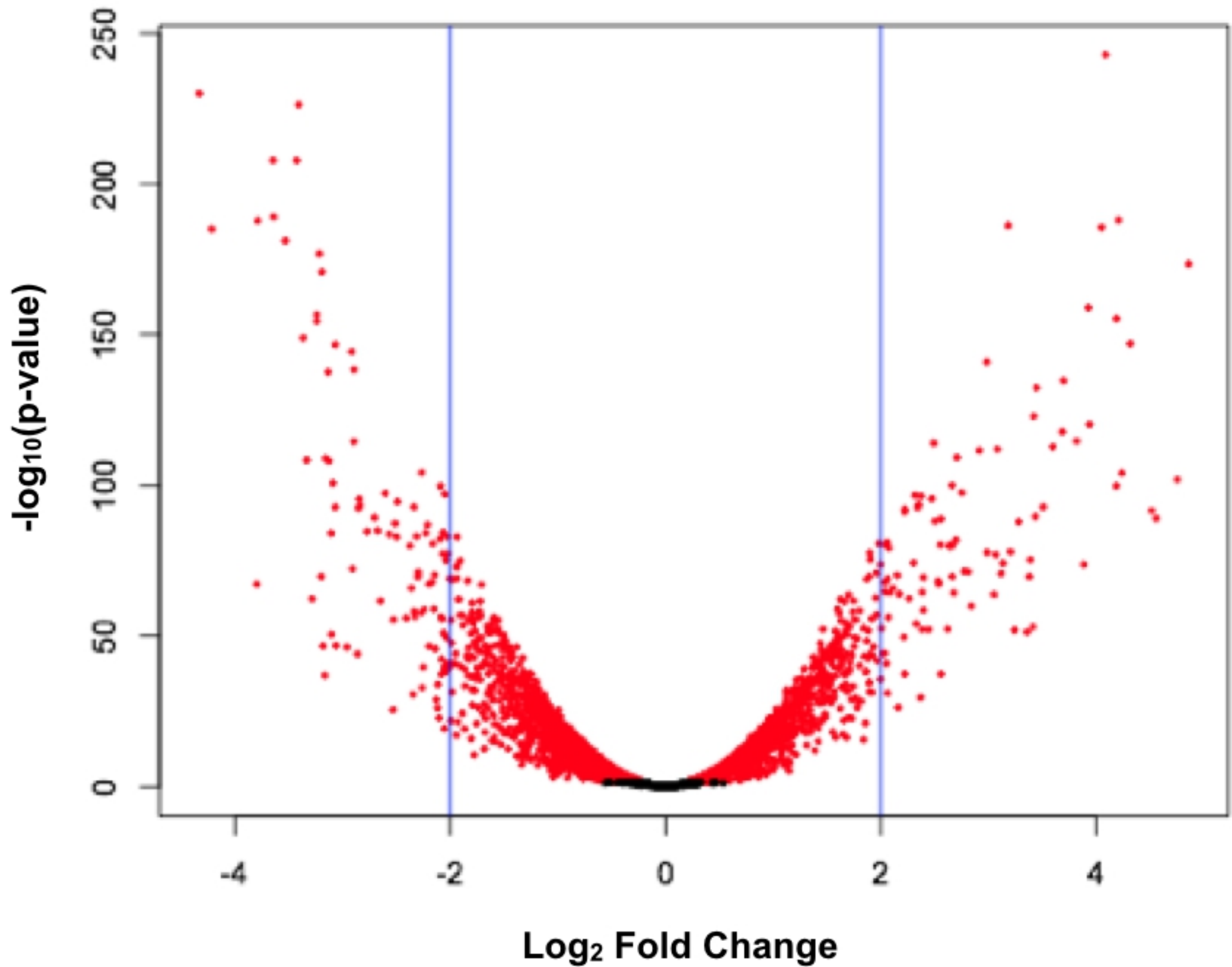
756   shown in yellow.
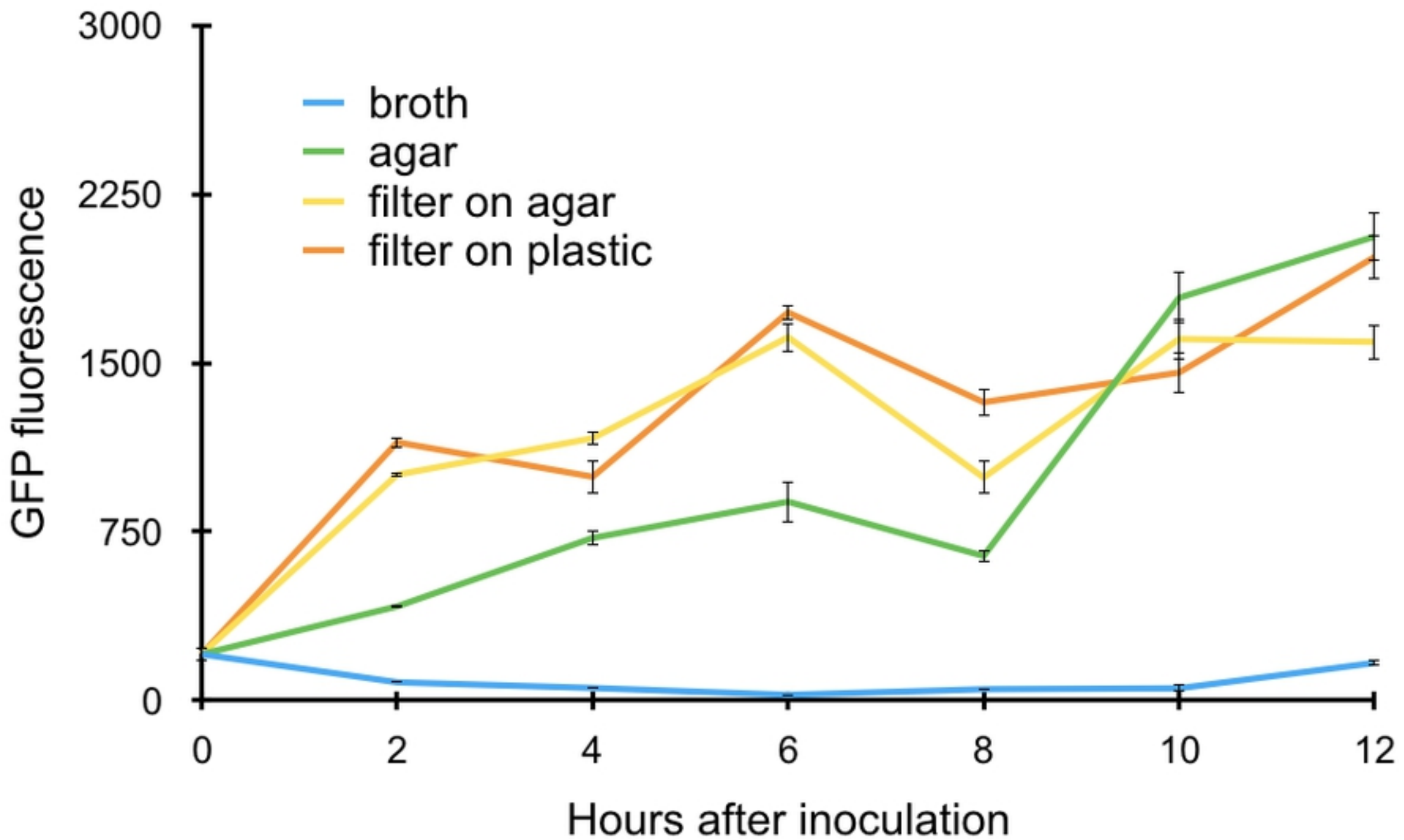
Figure
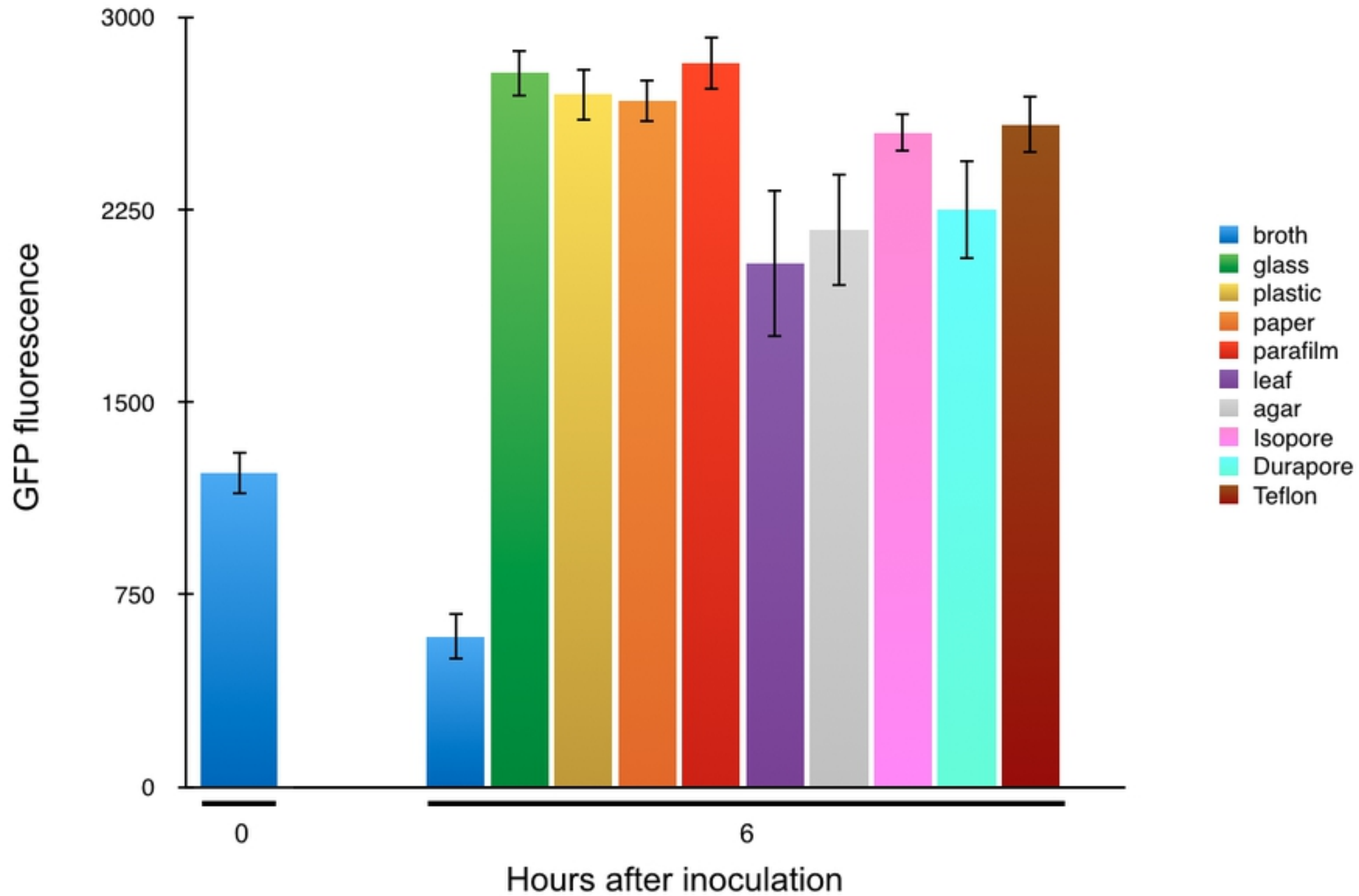
Figure

■ = down-regulated genes

□ = up-regulated genes

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