Identification of host pathways targeted by bacterial effector proteins using yeast toxicity and suppressor screens
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Dear Editor,

We would like to submit a revised version of our manuscript (JoVE60488) "Identification of host pathways targeted by bacterial effector proteins using yeast toxicity and suppressor screens" for consideration by JoVE.

We have carefully reviewed the manuscript and have addressed all the concerns raised by the editor and peer-reviewers. We hope that our manuscript is found to be acceptable by JoVE, the editors, and peer-reviewers. If there is anything you need in relation to this resubmission, please don't hesitate to ask. We look forward to hearing back from you soon.

Sincerely,

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1   **TITLE:**
2   Identification of Host Pathways Targeted by Bacterial Effector Proteins using Yeast Toxicity and
3   Suppressor Screens
4
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16   **KEYWORDS:**
17   bacterial effector protein, yeast suppressor, effector protein function, yeast screens, *Chlamydia*
18   *trachomatis*, type III secreted effector protein, pathway identification
19
20   **SUMMARY:**
21   Bacterial pathogens secrete proteins into the host that target crucial biological processes.
22   Identifying the host pathways targeted by bacterial effector proteins is key to addressing
23   molecular pathogenesis. Here, a method using a modified yeast suppressor and toxicity screen
24   to elucidate host pathways targeted by toxic bacterial effector proteins is described.
25
26   **ABSTRACT:**
27   Intracellular bacteria secrete virulence factors called effector proteins into the host cytosol that
28   act to subvert host proteins and/or their associated biological pathways to the benefit of the
29   bacterium. Identification of putative bacterial effector proteins has become more manageable
30   due to advances in bacterial genome sequencing and the advent of algorithms that allow in silico
31   identification of genes encoding secretion candidates and/or eukaryotic-like domains. However,
32   identification of these important virulence factors is only an initial step. Naturally, the goal is to
33   determine the molecular function of effector proteins and elucidate how they interact with the
34   host. In recent years, techniques like the yeast two-hybrid screen and large-scale
35   immunoprecipitations coupled with mass spectrometry have aided in the identification of
36   protein-protein interactions. Although identification of a host binding partner is the crucial first
37   step toward elucidating the molecular function of a bacterial effector protein, sometimes the
38   host protein is found to have multiple biological functions (e.g., actin, clathrin, tubulin), or the
39   bacterial protein may not physically bind host proteins, depriving the researcher of crucial
40   information about the precise host pathway being manipulated. A modified yeast toxicity screen
41   coupled with a suppressor screen has been adapted to identify host pathways impacted by
42   bacterial effector proteins. The toxicity screen relies on a toxic effect in yeast caused by the
43   effector protein interfering with the host biological pathways, which often manifests as a growth
44   defect. Expression of a yeast genomic library is used to identify host factors that suppress the

45 toxicity of the bacterial effector protein and thus identify proteins in the pathway that the
46 effector protein targets. This protocol contains detailed instructions for both the toxicity and
47 suppressor screens. These techniques can be performed in any lab capable of molecular cloning
48 and cultivation of yeast and *Escherichia coli*.
49
50 **INTRODUCTION:**
51 The first report of procedures similar to those presented here characterized the *Legionella*
52 *pneumophila* type IV effector SidD, a deAMPylase that modifies Rab1[1]. Comparable techniques
53 were used for the characterization of several *L. pneumophila* effectors[1–3]. The assay was adapted
54 to characterize a *Coxiella burnetii* type IV effector protein[4], and recently the utility of this
55 technique was expanded for the characterization of *Chlamydia trachomatis* inclusion membrane
56 proteins[5].
57
58 This protocol can be broken into two major parts: 1) the yeast toxicity screen, in which the
59 bacterial effector protein of interest is expressed in yeast and clones are screened for a toxic
60 phenotype as evidenced by a growth defect, and 2) the yeast suppressor screen, in which the
61 toxic phenotype is suppressed by expression of a yeast genomic library in the toxic strain. Thus,
62 the toxicity screen is a screen for toxic phenotypes that manifest as growth defects when the
63 bacterial effector of interest is overexpressed. Toxic clones, successfully transformed with and
64 expressing the bacterial effector, are selected and saved for the next step. The second major step
65 involves overexpressing a partially digested yeast genomic library in the toxic yeast clone.
66 Plasmids making up the yeast genomic library suggested for the use in this protocol carry 5−20
67 kb inserts, usually corresponding to 3−13 yeast open reading frames (ORF) of an average gene
68 size of ~1.5 kb across all plasmids, representing the whole yeast genome covered approximately
69 10x. This part of the assay is called the suppressor screen, as the goal is to suppress the toxicity
70 of the bacterial effector protein. Potential suppressor plasmids are isolated from yeast,
71 sequenced, and the suppressing ORFs identified. The rationale underlying the suppressor screen
72 is that the effector protein binds, interacts with, and/or overwhelms components of the host
73 pathway it targets, and that providing those host proteins back in excess can rescue the toxic
74 effect on the pathway and thus, the growth defect. Thus, identified ORFs that suppress toxicity
75 often represent multiple participants of a host pathway. Orthogonal experiments are then
76 performed to verify that the bacterial effector indeed interacts with the implicated pathway. This
77 is especially necessary if a binding partner such as clathrin or actin has been identified, because
78 these proteins are involved in a multitude of host processes. Further experiments can then
79 elucidate the physiological function of the effector protein during infection. The toxicity and
80 suppressor screens are also powerful tools for deciphering the physiological function of bacterial
81 effector proteins that do not physically bind host proteins with affinities sufficient to detect by
82 immunoprecipitation or that interact with the host in enzymatic hit-and-run interactions that
83 may not be detected by a yeast-two hybrid screen.
84
85 Although the suppressor screen can be a powerful method to reveal potential physiological
86 interactions between bacterial effector proteins and host pathways, the bacterial effector
87 protein must induce a growth defect in yeast, otherwise using it in the suppressor screen will be
88 of little use. Furthermore, the toxic phenotype must result in at least a 2−3 $\log_{10}$ deficit in growth

89 or it will be difficult to identify suppressors. If a laboratory is set up for cell culture, screening
90 effector proteins for toxicity in common cell lines such as HeLa can often give insight as to
91 whether it is worth the effort to proceed with the yeast toxicity screen. Ectopic expression of the
92 effector protein in HeLa cells sometimes results in toxicity that correlates very strongly with
93 toxicity in the yeast strain used for these screens[4]. Observable hallmarks of stress in HeLa cells
94 include loss of stress fibers, cell detachment from the plate, and nuclear condensation indicating
95 apoptosis. Any visual indication of stress in HeLa cells make the protein of interest a good
96 candidate for inducing a growth defect in yeast, which replicate much more rapidly and are thus
97 more responsive to perturbation of essential pathways.
98
99 It should be noted that the suppressor screen does not always identify host binding partners as
100 suppressors, but it can still implicate critical components of the host pathway(s) targeted,
101 yielding a holistic view of the biological processes being hijacked by the bacterial effector protein.
102 On the surface, this seems counterintuitive, because providing the binding partner of the effector
103 protein in excess would be expected to rescue the growth defect. In efforts to identify pathways
104 targeted by the *C. trachomatis* effector protein CT229 (CpoS), which binds to at least 10 different
105 Rab GTPases during infection[5], none of the Rab binding partners suppressed the toxicity of CT229.
106 However, numerous suppressors involved in clathrin-coated vesicle (CCV) trafficking were
107 identified, which led to further work demonstrating that CT229 specifically subverts Rab-
108 dependent CCV trafficking. Similarly, when investigating the *C. burnetii* effector protein Cbu0041
109 (CirA) several Rho GTPases that rescued the yeast growth defect were identified, and it was later
110 found that CirA functions as a GTPase activating protein (GAP) for RhoA[4].
111
112 The usefulness of the yeast suppressor screen for elucidating host pathways targeted by bacterial
113 effector proteins cannot be overstated, and other researchers attempting to characterize
114 intracellular bacterial effector proteins can greatly benefit from these techniques. These assays
115 are of value if immunoprecipitations and/or yeast-two hybrid screens have failed to find a binding
116 partner and can elucidate which pathways are targeted by the bacterial effector protein. Here,
117 detailed protocols for the toxicity and suppressor screens to identify host biological pathways
118 targeted by intracellular bacterial effector proteins are provided, as well as some of the common
119 obstacles experienced when using these assays and their corresponding solutions.
120
121 **PROTOCOL:**
122
123 **1. Preparation of media and reagents**
124
125 NOTE: Plates should be prepared before the day of the assay and are good for 1 month. Media
126 and reagents can be made at any point and are good for 1 month.
127
128 1.1. Prepare 1 L of the glucose solution (10% w/v) by dissolving 100 g of D-(+)-glucose in 800 mL
129 of distilled water in a 1, 000 mL beaker. Adjust the volume to 1 L with distilled water. Filter
130 through a 0.2 µm sterile filter into a sterile 1 L media storage bottle.
131
132 1.2. Prepare 1 L of the galactose solution (10% w/v) by dissolving 100 g of D-(+)- galactose in 800

133 mL of distilled water in a 1 L beaker. Adjust the volume to 1 mL using distilled water and filter
134 through a 0.2 μm sterile filter into a sterile 1,000 mL media storage bottle.

136 1.3. Prepare 1 L of yeast extract peptone dextrose (YPD) agar by dissolving 10 g of yeast extract,
137 20 g of peptone, 20 g of glucose, and 20 g of agar in 1,000 mL of distilled water. Autoclave for 20
138 min and cool in 56 °C water bath until cooled. Pour into 100 mm plates using ~20 mL of media
139 per plate.

141 1.4. Prepare 1 L of YPD broth by dissolving 10 g of yeast extract, 20 g of peptone, and 20 g of
142 glucose in 1,000 mL of distilled water. Autoclave for 20 min.

144 1.5. Prepare the synthetic dropout (SD) uracil (Ura⁻) glucose agar. For 1 L, dissolve 6.7 g of yeast
145 nitrogen base without amino acids, 1.9 g of dropout supplement without uracil, and 15 g of agar
146 in 800 mL of distilled water. Autoclave for 20 min and cool in a 56 °C water bath until the
147 temperature is ~50–60 °C. Using a 50 mL serological pipette or a sterile graduated cylinder, add
148 200 mL of the glucose solution prepared in step 1.1. Mix well by gently swirling or place on stir
149 plate. Pour into 100 mm plates using ~20 mL of media per plate.

151 1.6. Prepare the synthetic dropout (SD) uracil (Ura⁻) galactose agar. For 1 L, dissolve 6.7 g of yeast
152 nitrogen base without amino acids, 1.9 g of dropout supplement without uracil, and 15 g of agar
153 in 800 mL of distilled water. Autoclave for 20 min and cool in a 56 °C water bath until the
154 temperature is ~50–60 °C. Using a 50 mL serological pipette or a sterile graduated cylinder, add
155 200 mL of the galactose solution prepared in step 1.2. Mix well by gently swirling or place on the
156 stir plate. Pour into 100 mm plates using ~20 mL of media per plate.

158 1.7. Prepare the synthetic dropout (SD) uracil (Ura⁻) glucose broth. For 1 L, dissolve 6.7 g of yeast
159 nitrogen base without amino acids and 1.9 g of dropout supplement without uracil in 800 mL of
160 distilled water. Autoclave for 20 min and cool in a 56 °C water bath until the temperature is
161 ~50–60 °C. Using a 50 mL serological pipette or a sterile graduated cylinder, add 200 ml of the
162 glucose solution prepared in step 1.1.

164 1.8. Prepare the synthetic dropout (SD) uracil (Ura⁻) leucine (Leu⁻) glucose agar. For 1 L, dissolve
165 6.7 g of the yeast nitrogen base without amino acids; 1.9 g of dropout supplement without uracil,
166 leucine, and tryptophan; 15 g of agar; and 0.076 g of tryptophan in 800 mL of distilled water.
167 Autoclave for 20 min and cool in a 56 °C water bath until the temperature is ~50–60 °C. Using a
168 50 mL serological pipette or a sterile graduated cylinder, add 200 mL of the glucose solution
169 prepared in step 1.1. Pour into 100 mm plates using ~20 mL of media per plate.

171 1.9. Prepare synthetic dropout (SD) uracil (Ura⁻) leucine (Leu⁻) galactose agar. For 1 L, dissolve 6.7
172 g of yeast nitrogen base without amino acids; 1.9 g of dropout supplement without uracil,
173 leucine, and tryptophan; 15 g of agar; and 0.076 g of tryptophan in 800 mL of distilled water.
174 Autoclave for 20 min and cool in a 56 °C water bath until the temperature is ~50–60 °C. Using a
175 50 mL serological pipette or a sterile graduated cylinder, add 200 mL of the galactose solution
176 prepared in step 1.2. Mix well by gently swirling or placing on a stir plate. Pour into 100 mm plates

177   using ~20 mL of media per plate.

178

179   1.10. Prepare synthetic dropout (SD) uracil (Ura⁻) leucine (Leu⁻) glucose broth. For 1 L, dissolve

180   6.7 g of yeast nitrogen base without amino acids; 1.9 g of dropout supplement without uracil,

181   leucine, tryptophan; and 0.076 g of tryptophan in 800 mL of distilled water. Autoclave for 20 min

182   and cool in a 56–60 °C water bath until the temperature is ~50 °C. Using a 50 mL serological

183   pipette or a sterile graduated cylinder, add 200 mL of the glucose solution prepared in step 1.1.

184

185   1.11. Prepare polyethylene glycol (PEG) solution. Add 50% w/v of polyethylene glycol 3350 in

186   distilled water. Sterilize by autoclaving.

187

188   1.12. Prepare 1 M lithium acetate (LiAc) by dissolving 10.2 g of lithium acetate dehydrate in 200

189   mL of distilled water. Sterilize by autoclaving.

190

191   1.13. Prepare herring sperm DNA. Dilute 10 mg/mL herring sperm DNA to 2 mg/mL using distilled

192   water. Heat at 100 °C for 5 min and immediately place on ice for 5 min.

193

194   **2. Cloning the gene of interest into the yeast toxicity plasmid pYesNTA-Kan**

195

196   NOTE: Currently, there are a variety yeast toxicity vectors available both commercially and

197   academically. The yeast suppressor screen can be used in conjunction with many of these vectors

198   provided the plasmid expressing the effector protein of interest uses a dropout selection other

199   than uracil and an antibiotic marker other than Bla^R. This work used a modified pYesNTA vector[4,5]

200   that includes a kanamycin resistance cassette for easier screening for potential suppressors. The

201   cloning scheme must allow for the effector protein to be in-frame with the Gal promoter and His-

202   Tag. The *Chlamydia trachomatis* inclusion membrane protein CT229 was used as a proof of

203   principle for these assays.

204

205   2.1. Use PCR to amplify CT229 from *C. trachomatis* genomic DNA following the manufacturer's

206   instructions using CT229 +1 Kpn F (CCGGTACCAATGAGCTGTTCTAATGTTAATTCAGGT) and CT229

207   XhoI R (CCCTCGAGTTTTTTACGACGGGATGCC) primers. Use the following PCR conditions: (1) 98

208   °C for 30 s; (2) 98 °C for 10 s, 55 °C for 30 s, 72 °C for 2 min; (3) repeat step 2 for a total of 25x;

209   (4) 72 °C for 10 min; and (5) 4 °C hold.

210

211   2.2. Analyze 5 µL of the PCR product on a 1% agarose gel (**Figure 1**).

212

213   2.3. Purify the remaining 45 µL of DNA using a PCR purification kit following the manufacturer's

214   instructions.

215

216   2.4. Digest the 50 µL of purified PCR insert and pYesNTA-Kan (**Figure 2**) for 1 h at 37 °C in a water

217   bath using KpnI-HF and XhoI-HF.

218

219   NOTE: For pYesNTA-Kan, digest 5 µg of plasmid using 5 µL of KpnI-HF, 5 µl of XhoI-HF, and 6 µL

220   of buffer in a total volume of 60 µL. For the insert, digest the entire 50 µL of purified PCR product

221   using 2 µL of KpnI-HF, 2 µL of XhoI-HF, and 6 µL of buffer.
222
223   2.5. Run the entire plasmid digest on a 1% agarose gel. Perform purification of the digested
224   plasmid using a gel extraction kit following the manufacturer's instructions.
225
226   2.6. Purify the digested PCR insert using a PCR purification kit following the manufacturer's
227   instructions.
228
229   2.7. Clone the insert into pYesNTA-Kan using 2 µL of pYesNTA-Kan (step 2.5), 2 µL of DNA ligase
230   buffer, 1 µL of T4 ligase, and 15 µL of insert (step 2.6). Incubate at room temperature for 1 h.
231
232   2.8. Add the entire cloning reaction to 50 µL of *E. coli* competent cells and perform the
233   transformation reaction.
234
235   2.9. Plate the entire transformation reaction on an LB plate containing 100 µg/mL of carbenicillin.
236
237   2.10. Inoculate 10 mL of the LB containing 100 µg/mL of carbenicillin with three individual
238   colonies. Incubate overnight at 37 °C with shaking at 150 rpm.
239
240   2.11. Isolate the plasmid using a plasmid miniprep kit following the manufacturer's instructions.
241
242   2.12. Sequence the isolated plasmids using gene-specific primers (step 2.1).
243
244   **3. Test the protein of interest for toxicity in yeast**
245
246   3.1 Streak *Saccharomyces cerevisiae* W303 on YPD agar (step 1.3) to obtain isolated colonies.
247   Incubate at 30 °C for 24 h.
248
249   3.2 Inoculate 10 mL of YPD broth (step 1.4) with a single colony from the agar plate (step 3.1).
250   Incubate at 30 °C with shaking at 150 rpm overnight.
251
252   3.3 Add 0.5 mL of the overnight culture to 10 mL of YPD broth (step 1.4) and incubate at 30 °C
253   with shaking at 150 rpm for 4 h.
254
255   3.3.1 Pellet the 10 mL of culture at 3,000 x *g* for 10 min at 4 °C.
256
257   3.3.2 Resuspend the pellet in 1 mL of sterile water, transfer to the microcentrifuge tube, and
258   pellet at 3,000 x *g* for 1 min at room temperature.
259
260   3.3.3 Resuspend the pellet in 1 mL of 1 mM lithium acetate (LiAc) and pellet at 3,000 x *g* for 1
261   min at room temperature.
262
263   3.3.4 Repeat the LiAc wash 2x.
264

265   3.3.5 Remove the wash. Resuspend the pellet in 2.4 mL of 50% PEG 3350. Add 360 µL of 1M
266   LiAc, 500 µL of 2 mg/mL of herring sperm DNA, and 400 µL of sterile water.

267

268   NOTE: This is sufficient for 20 transformations.

269

270   3.3.6 Add 180 µL of the transformation mix from step 3.3.5 to a microcentrifuge tube
271   containing 5 µL of pYesNTA-Kan CT229 plasmid DNA (100–500 ng) from step 2.12.

272

273   3.3.7 Incubate in a water bath at 30 °C for 30 min.

274

275   3.3.8 Incubate in a water bath at 42 °C for 30 min.

276

277   3.3.9 Pellet at 3,000 x g for 1 min at room temperature.

278

279   3.3.10 Remove the transformation mix with the pipette. Resuspend the pellet in 100 µL of
280   sterile water. Plate the transformation on SD Ura⁻ agar with glucose (step 1.5).

281

282   3.3.11 Incubate plates at 30 ˚C for 48 h.

283

284   3.4 Inoculate 5 mL of SD Ura⁻ broth containing glucose (step 1.7) with a single colony from the
285   plate. Incubate overnight at 30 °C with shaking at 150 rpm. Include yeast transformed with the
286   vector alone as a negative control.

287

288   3.4.1 Add 180 µL of sterile water to 5 wells of a 96 well plate (A2–A6).

289

290   3.4.2 Vortex the overnight culture to mix.

291

292   3.4.3 Add 180 µL of yeast to the first well (A1). Serially dilute 1:10 (six samples in total including
293   undiluted).

294

295   3.4.4 Using a multichannel pipette, spot 5 µL of each dilution on SD Ura⁻ glucose (step 1.5) and
296   Ura⁻ galactose (step 1.6) agar plates. Incubate at 30 °C for 48 h.

297

298   3.5 Assess the toxicity by comparing the growth of the yeast expressing the effector protein of
299   interest grown on the galactose-containing media to the growth of yeast expressing the vector
300   alone.

301

302   3.6 Confirm the expression of the His-tag fusion protein by Western blotting.

303

304   **4. Transform toxic yeast with the yeast genomic library**

305

306   4.1 Inoculate 100 mL of SD Ura⁻ glucose broth (step 1.7) using 1 mL of the stock from step 3.4.
307   Incubate for 16–24 h at 30 °C with shaking at 150 rpm. Place 900 mL of SD Ura⁻ glucose broth at
308   30 °C overnight to prewarm the media.

309

310 4.2 Add the entire 100 mL of the overnight culture to the prewarmed 1 L flask. Incubate for 4–5
311 h at 30 °C with shaking at 150 rpm.

312

313 4.2.1 Pellet the culture at 6,000 x g for 10 min at 4 °C.

314

315 4.2.2 Discard the supernatant. Resuspend the pellet in 250 mL of sterile water. Pellet the culture
316 at 6,000 x g for 5 min at 4 °C.

317

318 4.2.3 Discard the supernatant. Resuspend the pellet in 250 mL of 1 mM LiAc. Pellet the culture at
319 6,000 x g for 5 min at 4 °C.

320

321 4.2.4 Remove LiAc and resuspend the pellet in 9.6 mL of 50% PEG 3350. Add 1.44 mL of 1M LiAc,
322 2 mL of 2 mg/mL herring sperm DNA, 50 µg of the pYep13 genomic library (ATCC 37323). Adjust
323 the volume to 15 mL with sterile water. Mix gently by inversion.

324

325 4.2.5 Incubate in a water bath at 30 °C for 30 min.

326

327 4.2.6 Add 750 µL of dimethyl sulfoxide (DMSO) to enhance the transformation efficiency.
328 Incubate in a water bath at 42 °C for 30 min. Mix by gentle inversion every 10 min.

329

330 4.2.7 Pellet the yeast at 3,000 x g for 5 min at room temperature.

331

332 4.2.8 Discard the supernatant and resuspend the pellet in 10 mL of sterile water. Pellet at 3,000
333 x g for 5 min at room temperature.

334

335 4.2.9 Resuspend the pellet in 8 mL of sterile water.

336

337 4.2.10 To determine the transformation efficiency, dilute 1:10 and plate 100 µL of each dilution
338 on SD Ura⁻ Leu⁻ glucose agar (step 1.8).

339

340 4.2.11 Plate 200 µL of the sample on SD Ura⁻ Leu⁻ galactose agar (step 1.9) plates. Use 50 plates
341 in total.

342

343 4.2.12 Incubate at 30 °C for 48–96 h or until colonies appear.

344

345 NOTE: Colonies generally appear on glucose agar plates at 48–72 h and galactose agar plates at
346 72–96 h.

347

348 4.3 Patch colonies (potential rescues) on SD Ura⁻ Leu⁻ galactose agar (step 1.9) to expand and
349 make stock. Incubate at 30 °C for 24–48h.

350

351 4.4 Inoculate 5 mL of SD Ura⁻ Leu⁻ glucose broth (step 1.10) using the part of the patch. Incubate
352 overnight at 26 °C with shaking at 150 rpm.

4.4.1 Add 180 µL of the sterile water to 5 wells of a 96 well plate (A2–A6).

4.4.2 Vortex the overnight culture to mix.

4.4.3 Add 180 µL of yeast to the first well (A1). Serially dilute 1:10 (six samples in total including undiluted).

4.4.4 Using a multichannel pipette, spot 5 µL of each dilution on SD Ura⁻ glucose and SD Ura⁻ galactose agar plates. Include toxic effector alone as a control.

4.4.5 Incubate at 30 °C for 48 h.

4.5 Compare the growth of the yeast expressing the toxic effector alone to yeast containing the potential suppressors. Only proceed with potential suppressors that have diminished toxicity compared to the yeast expressing the effector alone.

5. Identify and confirm suppressors

5.1 Inoculate 5 mL of SD Ura⁻ Leu⁻ glucose broth (step 1.10) with 100 µL of yeast from step 4.4 and incubate overnight at 30 °C with shaking at 150 rpm.

5.1.1 Pellet the yeast at 3,000 x g for 2 min at room temperature. Gently discard the supernatant using a pipette.

5.1.2 Isolate the plasmid with a yeast plasmid miniprep kit following the manufacturer's instructions.

5.2 Transform the isolated plasmid into the *E. coli* competent cells and plate the entire transformation on LB agar with 100 µg/mL of carbenicillin. Incubate at 37 °C for 24 h.

5.3 Inoculate 10 mL of LB broth containing 100 µg/mL of carbenicillin, with three colonies from the plate and incubate at 37 °C overnight with shaking at 150 rpm.

5.3.1 Pellet cultures at 3,000 x g for 10 min at room temperature. Isolate plasmid using a miniprep kit following the manufacturer's instructions.

5.4 Retransform the toxic yeast with the isolated plasmids from step 5.3.1 following the steps in part 3 of the protocol.

5.4.1 Inoculate 5 mL of SD Ura⁻ Leu⁻ glucose broth with a colony from the transformation plate. Incubate overnight at 30 °C with shaking at 150 rpm.

5.4.2 Spot on Ura⁻ Leu⁻ glucose and galactose agar to confirm suppression of toxicity.

397
398   5.5   Sequence   using   pYep13   F   (ACTACGCGATCATGGCGA)   and   pYep13   R
399   (TGATGCCGGCCACGATGC) primers to identify yeast ORFs.
400
401   **REPRESENTATIVE RESULTS:**
402   Before the actual yeast suppressor screen can be performed, the effector protein of interest must
403   be tested for toxicity in yeast. This is accomplished by expressing the protein of interest in yeast
404   under the control of a galactose-inducible promoter. Growth on glucose (noninducing conditions)
405   should first be compared to ensure toxicity is specifically due to the expression of the protein of
406   interest and is not a general defect. As shown in **Figure 3**, toxicity manifests as smaller colonies
407   and/or reduced growth. A 2–3 log decrease in yeast growth is ideal for the yeast suppressor
408   screens.
409
410   As shown in **Figure 4**, the toxicity and suppressor screen can be accomplished in eight steps using
411   the methods described in the protocol section. The gene of interest is cloned into a suitable
412   vector[1], and the resulting constructs are transformed into yeast to assess toxicity. This study used
413   CT229 as the gene of interest and pYesNTA-Kan as the vector. Additionally, the expression of the
414   His-tagged fusion protein was confirmed by Western blotting. Ideally, a 2–3 log reduction in yeast
415   grown on galactose-containing media should be observed. If the protein of interest is toxic to
416   yeast (**Figure 3** and **Figure 4**), the suppressor screen can be conducted by transforming the toxic
417   strain with the yeast genomic library pYep13. Transformants are plated on glucose agar to
418   determine transformation efficiency and galactose agar to identify potential suppressors. Using
419   this protocol, a minimum transformation efficiency of 5 x 10^5 was achieved with a total of 10–250
420   colonies on galactose agar. Patching these colonies on SD Ura$^-$ Leu$^-$ galactose agar (**Figure 4**) aided
421   in identification of true suppressors, because many false positives will not grow when patched.
422   Here, 50 colonies were patched and eight clones that suppressed effector toxicity were obtained
423   (**Figure 4**). Spotting suppressors on SD Ura$^-$ Leu$^-$ galactose agar was done to confirm suppression
424   of toxicity. As shown in **Figure 4**, pSup1 and pSup 2 suppressed the toxicity of the effector protein,
425   whereas pSup3 did not. Therefore, pSup3 was discarded. Plasmids were subsequently isolated
426   from the suppressors and transformed into *E. coli* to increase the plasmid yield. The plasmid can
427   then be retransformed into the toxic yeast to confirm that the isolated plasmid definitely
428   suppresses effector toxicity (**Figure 4**). While most of the isolated plasmids should rescue toxicity,
429   occasionally a plasmid that does not suppress toxicity when retransformed into the toxic yeast
430   strain is obtained. Those plasmids are discarded, and only those that suppress toxicity following
431   retransformation should be sequenced.
432
433   The isolated plasmid will contain multiple yeast ORFs[4]. To identify which is the true suppressor,
434   each ORF can be individually cloned and expressed in the toxic yeast strain as previously
435   described[1–4].
436
437   **FIGURE AND TABLE LEGENDS:**
438
439   **Figure 1: PCR amplification of target gene.** CT229 from *Chlamydia trachomatis* serovar L2 was
440   PCR amplified from genomic DNA. PCR products were analyzed on a 1% agarose gel stained with

441   ethidium bromide.

442

443   **Figure 2: pYesNTA-Kan plasmid map.** The target gene of interest was cloned into the multiple
444   cloning site (MCS) of pYesNTA-Kan. Kanamycin was used for selection in *E. coli* and uracil dropout
445   was used for selection in *S. cerevisiae.* Expression of the His-tagged fusion protein was verified
446   by Western blotting.

447

448   **Figure 3: Representative yeast toxicity results.** Effector proteins of interest were cloned into
449   pYesNTA-Kan. Sequence-verified plasmids were transformed into *S. cerevisiae* and transformants
450   were serially diluted and spotted on Ura⁻ glucose and galactose agar to assess toxicity.

451

452   **Figure 4: Overview of the yeast suppressor screen.** The target gene of interest was cloned into
453   pYesNTA-Kan and plasmids were transformed into *S. cerevisiae*. Transformants were serially
454   diluted and spotted on Ura⁻ glucose and galactose agar to assess toxicity. To identify the pathway
455   targeted by the toxic effector protein, the yeast strain expressing the toxic protein was
456   transformed with a yeast genomic library. Colonies were patched on Ura⁻ Leu⁻ galactose agar and
457   spotted to assess toxicity. Plasmids were isolated from those that suppress the toxicity of the
458   effector protein. Plasmids were retransformed into the toxic yeast strain to confirm that the
459   isolated plasmid is a suppressor. Plasmids from suppressors were sequenced to identify the yeast
460   ORFs present.

461

462   **DISCUSSION:**
463   This protocol outlines step-by-step procedures for identifying host biological pathways targeted
464   by bacterial effector proteins using a modified yeast toxicity and suppressor screen. The yeast
465   strain used, *S. cerevisiae* W303, is auxotrophic for both uracil and leucine. Uracil auxotrophy of
466   the strain is used to select yeast carrying the protein of interest on the pYesNTA-Kan vector while
467   leucine auxotrophy is used to select for the yeast genomic library vector pYep13. The yeast
468   genomic library plasmids carry 3–13 ORFs, so each ORF should be individually cloned into the
469   p415-ADH vector[6] or a similar vector and retransformed into the toxic yeast to identify which is
470   the true suppressor. It is typical to identify several suppressors representing a host biological
471   pathway. Suppressors can sometimes be direct binding partners of the bacterial protein of
472   interest, but not always.

473

474   Generating yeast clones carrying the bacterial protein of interest on pYesNTA-Kan is the first
475   major step and great care should be taken to preserve the integrity of these clones as soon as
476   possible because there is strong negative selection for yeast carrying toxic proteins, even without
477   intentional galactose induction, and they may lose the plasmid. Occasionally there is plasmid loss
478   even when the yeast is maintained under selection on dropout media. A method has been
479   previously reported to decrease the occurrence of plasmid loss by linearizing the vector and
480   integrating it within the yeast genome[1].

481

482   A major drawback of these techniques is that the suppressor screen only yields meaningful data
483   if overexpression of the bacterial effector protein triggers an observable and consistent growth
484   defect in yeast. The primary difficulty in this method is when no suppressors are identified. It is

485   hard to determine whether a failure to identify suppressors is due to biological reasons or
486   technical issues. From a biological perspective, there could be several causes: the protein of
487   interest could be so toxic that proteins from the genomic library are not able to significantly
488   suppress toxicity, the targeted pathway may be incapable of rescue, or numerous co-expressed
489   factors may be required to overcome the toxic effect. Approaching the biological explanation is
490   challenging, as there are many undefined variables to dissect. Because there is an established
491   protocol, exploring technical explanations is a much more tractable approach. From a technical
492   standpoint, a failure to identify suppressors is likely the result of low transformation efficiency of
493   the yeast genomic library. This protocol uses the ATCC *S. cerevisiae* AB320 partially digested
494   genomic library in pYEp13. Other libraries may be sufficient as well as other vectors, but this
495   protocol specifically uses the ATCC library and there are no studies currently published that
496   report alternate library or vector sources. It is strongly recommended that coverage of the yeast
497   genome should be at least 10x, or underrepresentation may hinder the identification of
498   suppressors. Decreased transformation efficiency can bring these numbers under 1x. Thus, parts
499   of the yeast genome may not be represented in the suppressor screen. Low transformation
500   efficiency can be due to many issues, including bad reagents or poor library preparation. This
501   method largely adheres to the DMSO/LiAc transformation method first put forth by Hill et al.[7]. It
502   is recommended that new DMSO, 50% PEG, and fresh herring sperm DNA are purchased and
503   prepared for the transformations and used exclusively for these assays to decrease
504   contamination or degradation of the quality of the reagents. DMSO is highly hygroscopic and
505   absorbs water from the atmosphere readily. Therefore, making many individual use aliquots is
506   recommended to avoid repeated opening of a container's lid and exposure to the atmosphere.
507   High quality herring sperm DNA can be stored at -20 °C and remains fit for use for several years,
508   although a fresh preparation should be prepared from the stock for each transformation. Once
509   boiled and cooled, the herring sperm DNA should not be reboiled or reused, so it is best to only
510   remove what is needed and avoid making more than is necessary for the current transformation.
511   Attention to detail can greatly improve the transformation efficiency of yeast and improve the
512   chances of identifying a suppressor.
513
514   Considering that the yeast pYep13 vector carries 3–13 different yeast ORFs, reducing the number
515   of false-positive plasmids is paramount to identifying suppressors. Yeast can take up and harbor
516   multiple copies of similar vectors, whereas the general wisdom associated with plasmid
517   incompatibility traditionally dictates that *E. coli* only maintains one type of plasmid with the same
518   origin of replication (ORI) at a time, although this is not always the case. This can become a major
519   issue when attempting to identify suppressor candidates. If suppressor yeast clones are
520   identified, the plasmids will be extracted and retransformed into *E. coli* for propagation and
521   subsequent sequencing for identification. Once the candidate suppressor plasmid has been
522   isolated and retransformed into *E. coli*, it is strongly recommended that at least five *E. coli*
523   colonies are picked for sequencing, because it is possible that individual clones may be carrying
524   different plasmids. Since *E. coli* should only be able to propagate one plasmid at a time, if the
525   yeast isolation yields multiple plasmids, different *E. coli* clones on the plate should carry only one
526   of them at a time. Therefore picking five colonies based on normal transformation efficiency in
527   *E. coli* should show whether the clones are carrying the same or different plasmids. If multiple
528   plasmids arise subsequent to sequencing of the plasmids from *E. coli*, then each one should be

529   transformed into the toxic yeast clone and assessed for rescue of the growth defect.
530   Furthermore, even if only a single suppressor plasmid is identified, for rigor and reproducibility
531   retransformation of the plasmid into the toxic clone is encouraged.
532
533   The yeast suppressor assay is a very large experiment and appropriate time and materials should
534   be planned accordingly. This protocol contains many details that must be adhered to strictly and
535   it is recommended that the researcher conducting these assays carefully read and watch the
536   protocol in full prior to starting an assay. This is a multipartite procedure including both bacteria
537   and yeast that need to be cultivated at different temperatures. Isolation of plasmids from yeast
538   can sometimes be difficult due to their tough cell wall and the use of DMSO helps achieve higher
539   transformation efficiency.
540
541   One of the largest advantages of the yeast toxicity and suppressor screen is that the bacterial
542   effector does not need to physically bind to the host substrates for any appreciable amount of
543   time to detect the putative pathway targeted. Only a small handful of studies have reported using
544   this or a similar technique[1–5]. However, there are reports of techniques that identify host
545   pathways and many techniques to screen for binding partners of bacterial effector proteins.
546   Kramer et al. reported a novel systems biology approach to identify pathways targeted by
547   bacterial effector proteins[8]. The technique used a haploid deletion strain collection of *S.*
548   *cerevisiae* to screen for mutants sensitive to the *Shigella* effector Osp4. Using this technique,
549   Osp4 was linked to regulation of MAPK signaling and attenuation of innate immunity in host cells.
550   Array-based, high-throughput, automated yeast-two hybrid screens can now rapidly identify
551   putative bacterial effector binding partners by screening many at the same time against millions
552   of host proteins[9]. Similarly, high-throughput immunoprecipitations coupled with mass
553   spectrometry are being used to mass identify putative binding partners of entire families of
554   bacterial effector proteins[10]. While many of these studies can accelerate the timetable of effector
555   protein characterization, they rarely provide insight as to what physiological processes are being
556   manipulated and what the holistic outcome of these interactions are. Thus, techniques such as
557   the yeast toxicity and suppressor screen are essential for illuminating the cellular processes being
558   manipulated by these effector proteins. At some point, observations identifying binding partners
559   or pathways targeted by bacterial effector proteins need to return to cellular infection models to
560   determine whether these in vitro findings hold true in physiologically relevant scenarios. Genetic
561   manipulation of obligate intracellular pathogens like *C. trachomatis* has been a major hurdle until
562   recently[11–15] and generation of site-specific mutants to study effector proteins was not possible.
563   In the last few years though, a revolution in generating chlamydial mutants, complementing
564   them, and overexpressing target proteins has occurred. These recent advances have greatly
565   enhanced the value of the information derived from studies using toxicity and suppressor screens
566   as it is now possible to go from yeast directly into infection to probe the validity of the results.
567

572

DISCLOSURES:
The authors declare that they have no competing financial interests.

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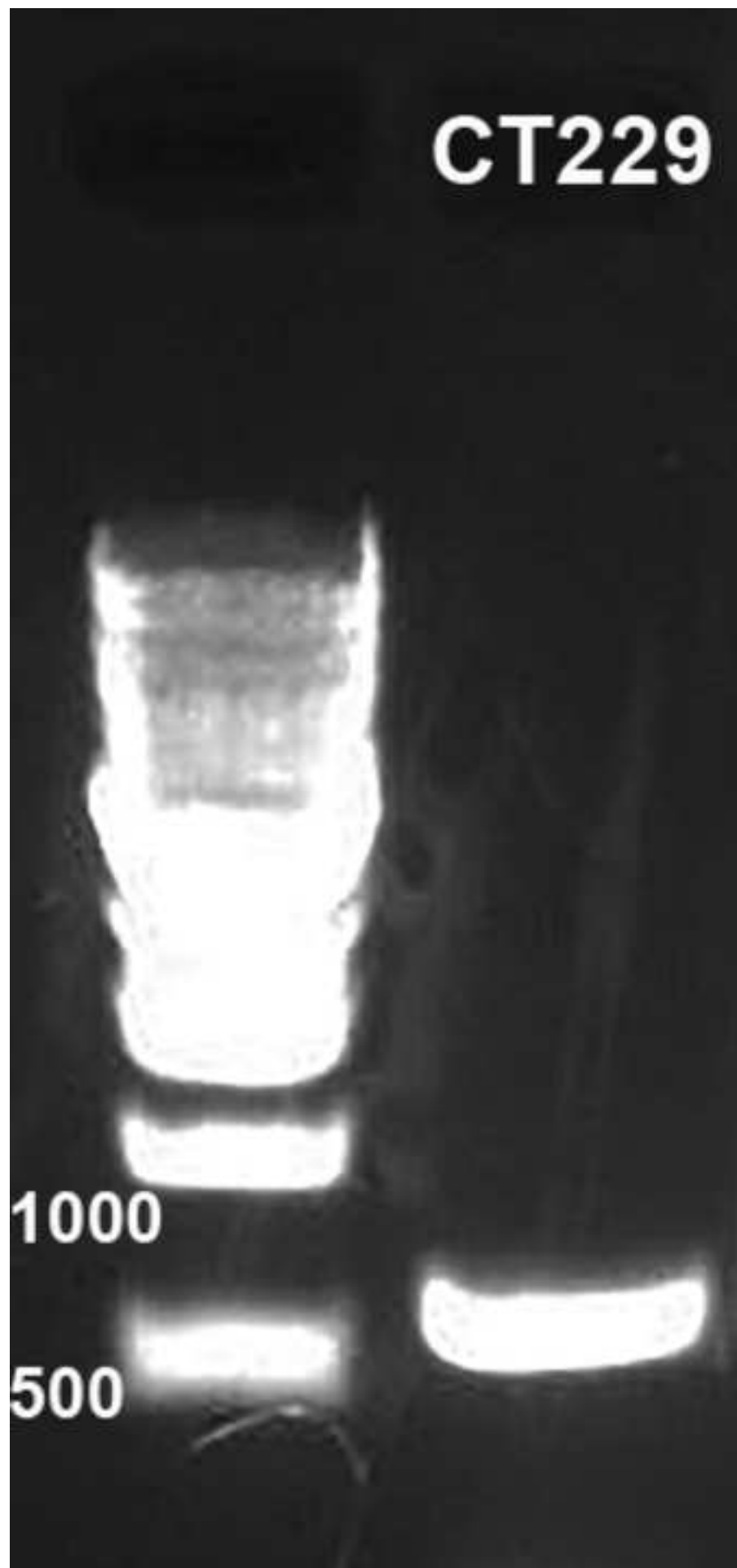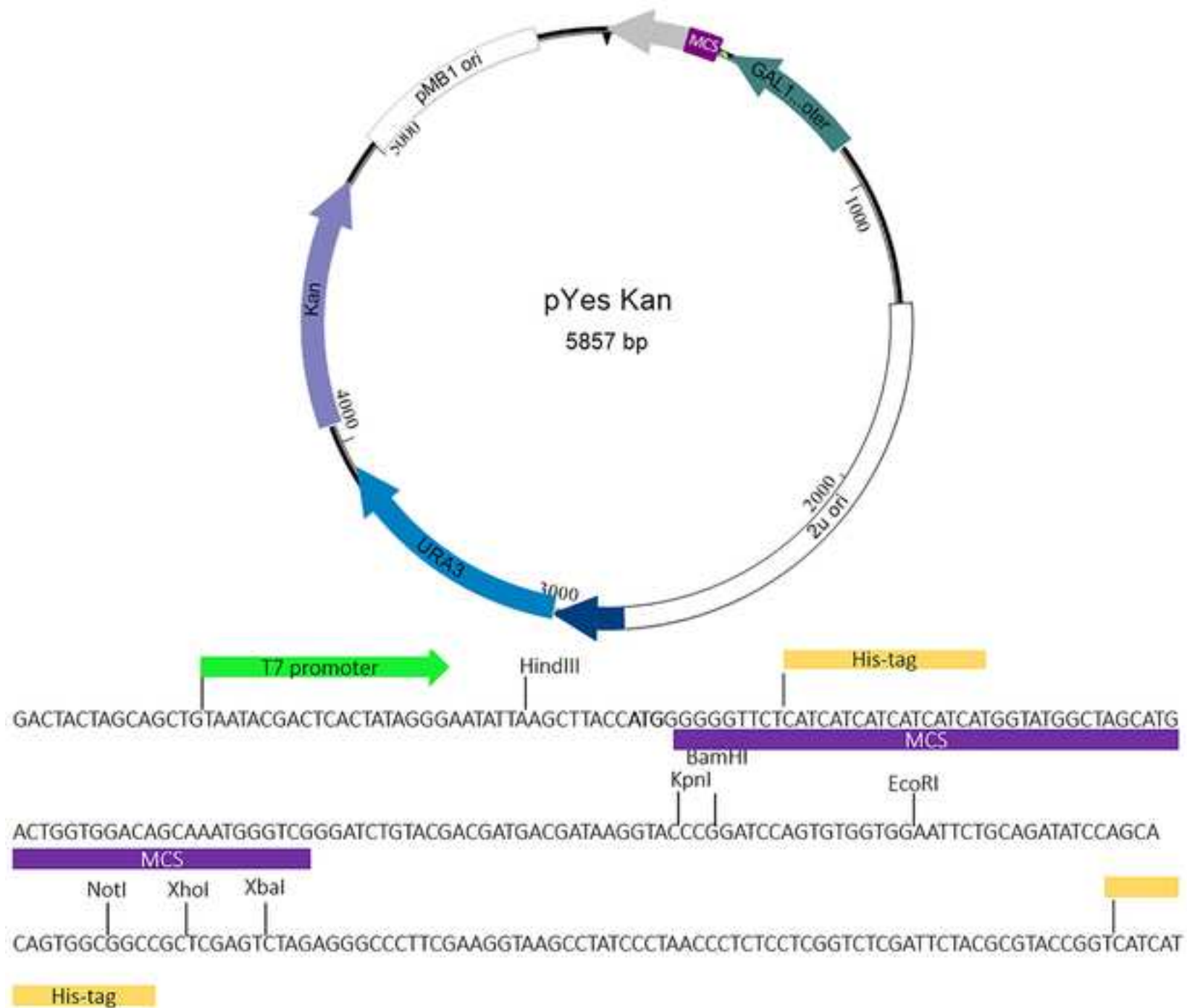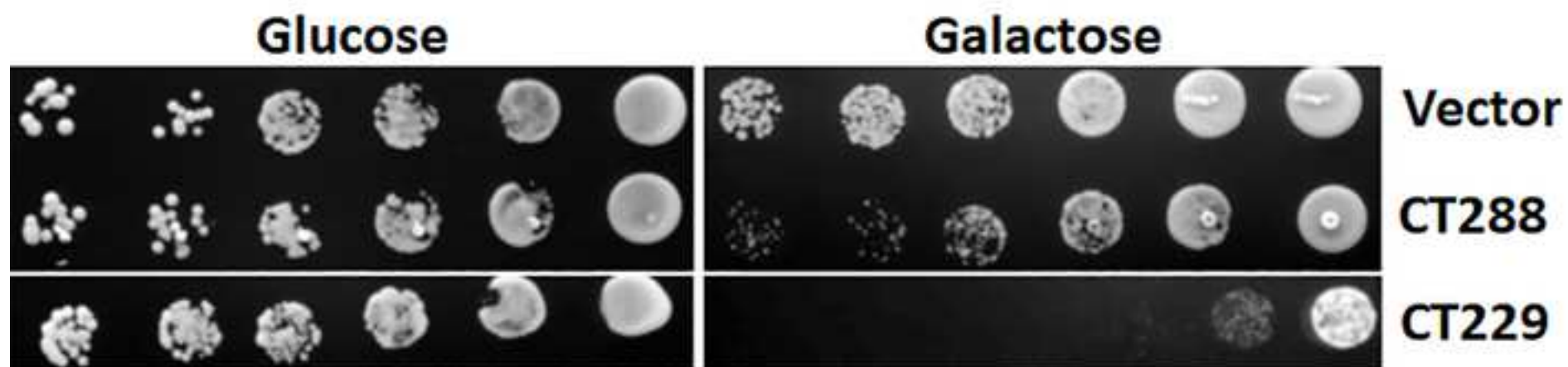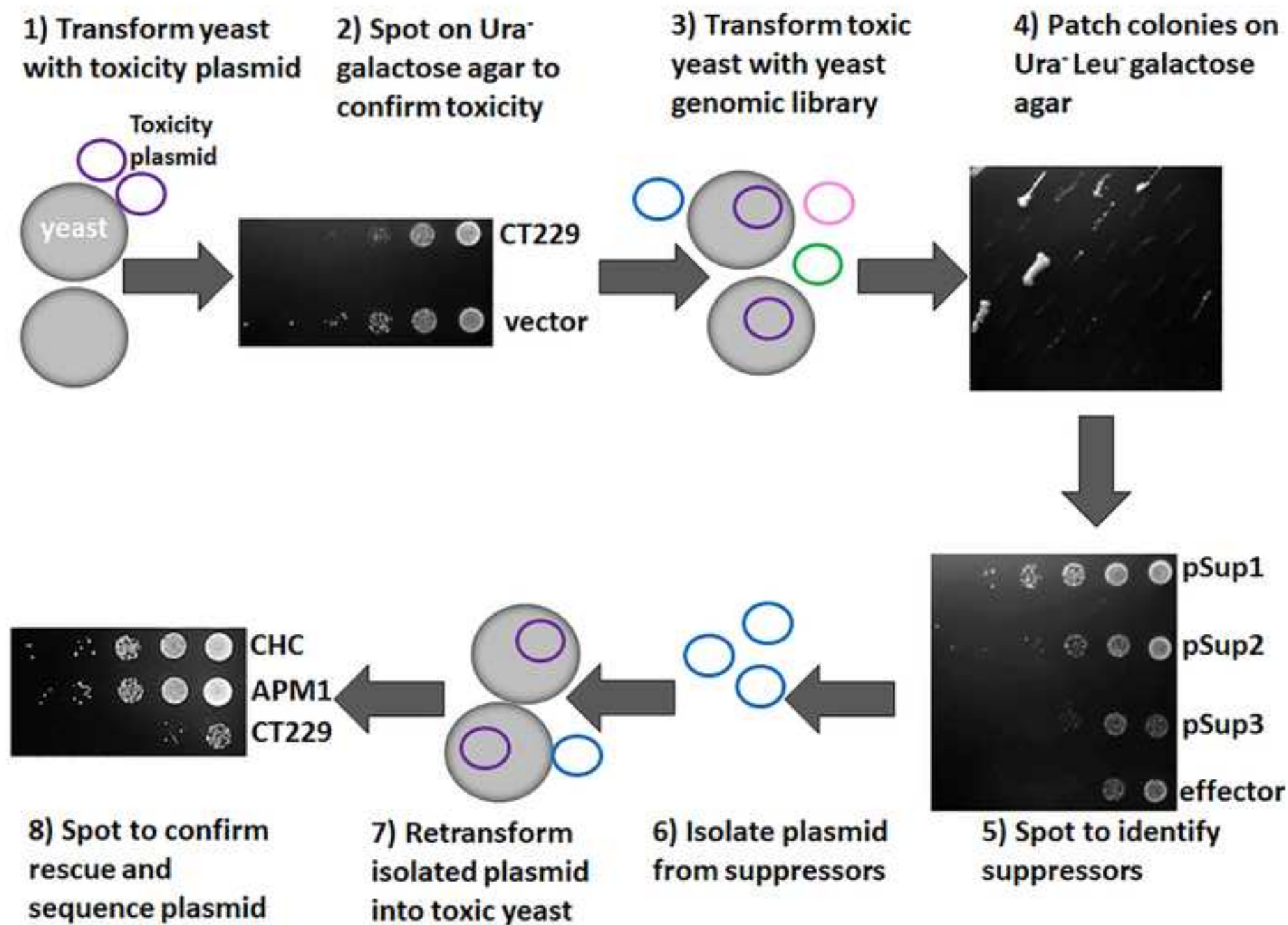
Figure 1

Figure 1

Figure 2   Click here to access/download;Figure;Figure 2.psd ⬇

Figure 2

Figure 3

Figure 4

1) Transform yeast with toxicity plasmid

2) Spot on Ura⁻ galactose agar to confirm toxicity

3) Transform toxic yeast with yeast genomic library

4) Patch colonies on Ura⁻Leu⁻ galactose agar

5) Spot to identify suppressors

6) Isolate plasmid from suppressors

7) Retransform isolated plasmid into toxic yeast

8) Spot to confirm rescue and sequence plasmid

Name of Material/ Equipment	Company	Catalog Number
Agar	Fisher Scientific	BP2641500
Galactose	MilliporeSigma	G0750-1KG
GeneJet Gel extraction kit	ThermoFisher Scientific	K0691
GeneJet PCR purification kit	ThermoFisher Scientific	K0701
GeneJet plasmid miniprep kit	Thermo	K0503
Glucose	MilliporeSigma	G8270-1KG
Herring Sperm DNA	Promega	D1811
KpnI-HF	New England Biolabs	R3142S
Lithium acetate dihydrate	MilliporeSigma	L6883-250G
Peptone	Fisher Scientific	
Phusion High-Fidelity DNA Polymerase	New England Biolabs	M0530
Poly(ethylene glycol) 3350	MilliporeSigma	1546547-1G
pYep13	ATCC	37323
T4 DNA ligase	New England Biolabs	M0202S
Tryptophan	MilliporeSigma	470031-1G
XhoI-HF	New England Biolabs	R0146S
Yeast extract	Fisher Scientific	BP1422-500
Yeast miniprep kit	Zymo	D2001
Yeast nitrogen base without amino acids	MilliporeSigma	Y0626-250G
Yeast Synthetic Drop-out Medium Supplements	MilliporeSigma	Y1501-20G
Yeast Synthetic Drop-out Medium Supplements	MilliporeSigma	Y1771-20G

Comments/Description

without uracil
without uracil, leucine, tryptophan

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5. The Protocol should contain only action items that direct the reader to do something.
We have confirmed the protocol is written in this format.

6. Please revise the protocol text to avoid the use of any personal pronouns in the protocol (e.g., "we", "you", "our" etc.).
We have confirmed the protocol is written in this format.

7. Please ensure that individual steps of the protocol should only contain 2-3 actions per step.
We have confirmed the protocol is written in this format.

8. Please ensure that you include all specific details associated with your protocol in a step by step manner. Please use complete sentences throughout.
We have confirmed the protocol is written in this format.

9. Please bring out the link between different subheadings of the protocol.
We have brought out the link between different subheadings in the protocol.

10. Please ensure you answer the "how" question, i.e., how is the step performed?
We have confirmed the protocol is written in this format.

11. 1: Please include what gene was amplified in your case, where was the nucleic acid obtained for amplification, what are the primers used, reaction set up and program used for amplification. Please also include a step by step protocol to show how the cloning was performed in the MCS of the plasmid used. Please include a short note stating why this plasmid was used. After cloning, did you perform restriction digestion, colony pcr, etc.
We have updated the protocol to include this information.

12. 2.3: Do you check the O.D in this case?
No

13. 2.3.6: What is the transformation mix- the mix obtained in the step 2.3.4? The plasmid DNA is from step 1? Please include this for clarity. Do you add the culture medium at this stage? Do you perform the incubations in the water bath?
Additional details have been added.

14. 3.1.2: Do you measure the O.D in this case?
No

15. 3.1.6: importance of pYep13?
pYep13 is the yeast genomic library. This information has been added to the protocol.

16. 3.1.7: Why is DMSO added at this stage?
The addition of DMSO significantly increases the transformation efficiency. This information has been added to the protocol.

17. 4.1.3: If this step needs filming, please describe the actions associated with it in brief.
This step does not need filming.

18. There is a 10-page limit for the Protocol, but there is a 2.75-page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Please adjust the highlights accordingly.
Highlights have been adjusted accordingly.

19. Please include representative results for all the sections in the protocol. For example, PCR results, positive clone screen is not included.
Representative results have been added.

20. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."
All figures are new, no copyright permission is needed.

21. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique
We have confirmed the discussion contains this information and have shortened it to 6 paragraphs.

22. Please sort the materials table in alphabetical order.
The materials are now listed in alphabetical order.

Reviewers' comments:
Reviewer #1:

Manuscript Summary:
This manuscript provides a detailed explanation of how to use a yeast toxicity-suppressor screen to elucidate eukaryotic pathways that may be targeted by bacterial effector proteins. The protocol is timely as it has recently been used in several high profile publications that have reported function of novel effectors.

Major Concerns:
No major concerns

Minor Concerns:
* the protocol makes no mention of checking for expression of the effector protein in yeast although this is mentioned in the discussion. This should be included in the protocol. Authors mention a His-tag - is it routine to include a tag to detect the effector? N- or C- terminal? Incorporated into the oligonucleotide for amplification of the effector?
The His-tag is on the plasmid and thus is not incorporated into the oligos for effector amplification. We have incorporated that effector expression should be confirmed in the protocol.

* The protocol refers to "agar" and "broth" in vague ways. I think the yeast media being used should be more clearly defined (can a recipe be included?). I assume it is a Yeast minimal media with other amino acids supplemented?
We have added a section that includes the recipes for all the media and reagents.

* what are the oligonucleotides used to sequence recovered pYep13 clones?
The oligonucleotides used for sequencing have been added.

Reviewer #2:
Manuscript Summary:
This manuscript by Faris and Weber describes a protocol to identify bacterial effectors toxic to eukaryotic cells via yeast viability and subsequently identify yeast proteins/pathways targeted by the toxic effector using an over-expression suppressor screen. Yeast are powerful model organisms to investigate bacterial effector protein function and this protocol will be of immense value to the scientific community. Minor comments are listed below.

Major Concerns:
None.

Minor Concerns:
1. Line 126: Is pYesNTA-Kan available commercially or from the authors upon request? Are there alternative plasmids that can be used?
We have added a note in the protocol. The pYesNTA-Kan is available from our laboratory and other commercially available plasmids can be used for the screen.

2. Are the restriction sites in frame with the 6xHis tag? Please revise Figure 1 so that the MCS shows the 6xHis codons.
The figure has been revised to include the 6X His-tag codons.

3. Step 2.3.1 describes a centrifugation step at 4˚C. Are subsequent incubations/centrifugation steps also done at the this temperature? No temperature is specified.
Temperature for all centrifugation steps have been added.

4. Lines 158-169: fix numbering for steps and highlighting.
The numbering has been corrected.

5. Line 210: do you mean the growth of yeast harboring the toxic effector?
Yes, we have clarified this in the text.

6. Line 219: typo: manufacturers
The type has been corrected.

7. The use of idioms such as "pipe-dream" and "foolhardy" may decrease readability for non-native English readers. Consider revising verbiage.
We have revised the verbiage.