Engineering of Robust Host Strains: Enhancing *Escherichia coli* Abiotic Stress Resistance through Ornithine Lipid Formation

Leidy Patricia Bedoya-Pérez[1], Alejandro Aguilar-Vera[1], José Utrilla[1*], Christian Sohlenkamp[1*]

[1]Centro de Ciencias Genómicas, Universidad Nacional Autónoma de México, Av. Universidad s/n Col. Chamilpa, C.P. 62210, Cuernavaca, Mor., Mexico.

*Corresponding authors

Name: José Utrilla: utrilla@ccg.unam.mx

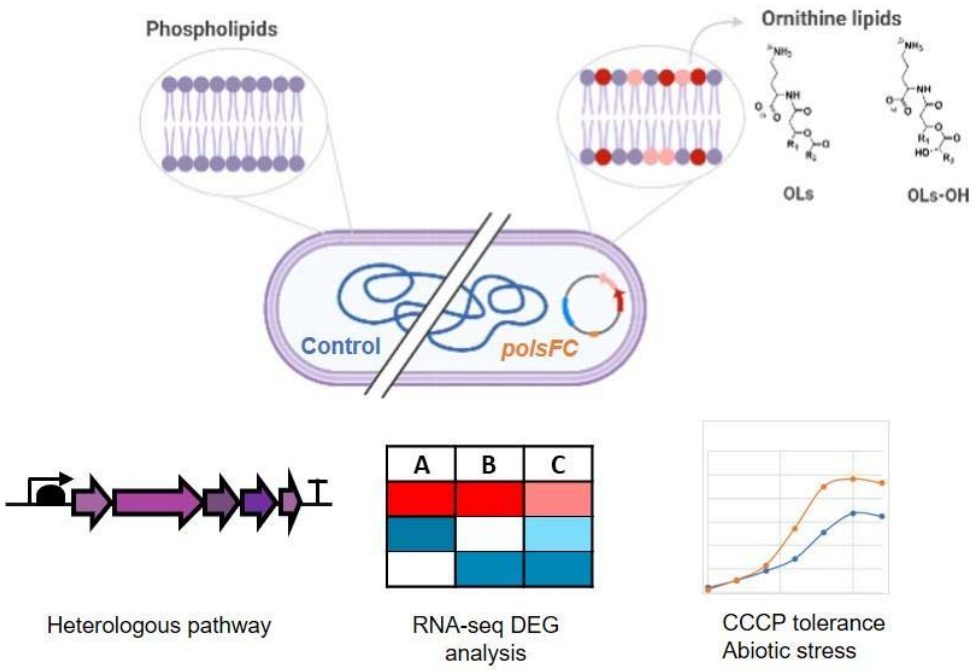
Name: Christian Sohlenkamp: chsohlen@ccg.unam.mx

Address: Centro de Ciencias Genómicas, Universidad Nacional Autónoma de México, Av. Universidad s/n, Col. Chamilpa, C.P. 62210, Cuernavaca, Mor., Mexico.

Abstract

Escherichia coli is a common host for biotechnology and synthetic biology applications. During growth and fermentation, the microbes are often exposed to stress conditions, like variations in pH or solvent concentrations and it has been described that bacterial membranes play a key role in response to abiotic stresses. Ornithine lipids (OLs) are a group of membrane lipids whose presence and synthesis have been related to stress resistance in different bacteria. In this study, we engineered different *E. coli* strains to produce unmodified OLs and hydroxylated OLs by expressing the synthetic operon *olsFC*. Our results showed that OL formation improved pH resistance and increased biomass under phosphate limitation. Transcriptome analysis revealed that OL-forming strains differentially expressed stress and membrane-related genes. OL-producing strains also showed better growth in the presence of the ionophore carbonyl cyanide 3-chlorophenylhydrazone (CCCP), suggesting reduced proton leakiness. Furthermore, our engineered strains showed improved heterologous violacein production at phosphate limitation and low pH. Overall, this study demonstrates the potential of engineering the *E. coli* membrane composition for constructing robust hosts for biotechnology and synthetic biology applications.

Keywords: synthetic biology, ornithine lipids, membrane engineering, acid stress response, phosphate limitation response, violacein production, proton motive force.



Graphical abstract. *Escherichia coli* strains forming ornithine lipids in addition to glycerophospholipids show increased resistance to acidic pH, improved growth under phosphate-limiting conditions, and allowed a higher production of violacein in a heterologous expression system. Transcriptional profiling by RNAseq showed expression changes that suggest reduced proton leakiness explained by CCCP tolerance.

INTRODUCTION

The use of microorganisms as cell factories plays an important role in biotechnology to produce bulk chemicals, proteins, biofuels, pharmaceuticals, etc. During these fermentations, the microbes are often exposed to stress conditions, like variations in pH conditions or solvent concentrations[1]. It can also be desirable to grow the production strain under conditions different from standard growth conditions, for example, to use less phosphate in the fermentation broth or to grow the microbe at an increased temperature. Microbes with both efficient production and tolerance to stress conditions are desirable for industrial production processes, however, strains combining different properties are rare to occur in nature [2]. Such adapted microorganisms are named "microbial chassis" [3] and could be engineered using synthetic biology tools [4].

Escherichia coli is among the most frequently used bacteria because of its easy genetic manipulation, fast growth to high cell densities, and safe use [5]. Efforts have been made to construct improved *E. coli* strains for biotechnological applications. Recently, the computational tool ReProMin (Regulation-based Proteome Minimization) was designed to identify genetic interventions required to divert cellular resources invested in the expression of non-essential genes toward the increase in biosynthetic potential [6]. These predictions allowed the design of two triple mutants called PFC and PYC, with mutations in *phoB, fhlC y cueR* genes, and mutations in *phoB, yedW, cusR* genes, respectively, allowing a theoretical release of 0.5% of the *E. coli* proteome. These strains exhibited increased availability of cellular resources that may be of interest in the biotech industry allowing a larger production of recombinant proteins [6]. Both triple

mutants, PFC, and PYC, lack the *phoB* gene encoding PhoB, a transcription factor central to the bacterial phosphate starvation response. PhoB activates the expression of 33 genes, mainly related to the use of alternative sources of phosphorus [7] [8]. Its absence in the two triple mutants could imply limitations in their use on a large scale in minimal media with low phosphate concentration.

On the other side, the bacterial membrane has an important function during the response of bacteria to abiotic stresses, with bacteria frequently adapting their membrane lipid compositions [9]. A major component of *E. coli* membranes are glycerophospholipids, which are composed of two fatty acids, a glycerol moiety, a phosphate group, and a variable head group [10] . In recent years, a much wider diversity of lipid structures has become evident in different bacteria. Although glycerophospholipids are the best-known membrane lipids, it is now known that many other lipids can be present in bacterial membranes, examples being hopanoids, ornithine lipids, betaine lipids, sulfonolipids or sphingolipids [10]. Most importantly, the lipid composition of a bacterial membrane in many cases is not static and the presence of some membrane lipids, specifically hopanoids and ornithine lipids, seems to form part of a stress response to (changing) environmental conditions in some bacteria.

Ornithine lipids (OLs) are phosphorus-free membrane lipids relatively common in bacteria but absent from eukaryotes and archaea [11]. They are composed of a 3-hydroxy fatty acid amide-bound to the α-amino group of ornithine and of a second fatty acyl group ester-linked to the 3-hydroxy position of the first fatty acid. This basic OL structure can be modified by hydroxylation in different positions, by *N*-methylation, or by taurine transfer. It was predicted that around 50% of sequenced bacterial species can synthesize OLs under certain growth

conditions. These conditions include limiting phosphate concentrations, acidic pH, and increased temperatures [10] [12,13] and several bacteria such as *Pseudomonas fluorescens*, *Serratia proteamaculans* and *Vibrio cholerae* only form OLs under phosphate-limiting conditions. In *Burkholderia cepacia,* an increased formation of hydroxylated OLs (OLs-OH) was observed in cells grown at 40 °C, and in the symbiotic alpha-proteobacterium *Rhizobium tropici* CIAT899 it has been described that the presence of OLs-OH improves growth at increased temperatures and under acidic conditions [12,14]. Especially, the hydroxylation introduced by the OL hydroxylase OlsC in the C2 position of the secondary fatty acid in OLs may increase hydrogen bonding between the lipid headgroups thereby increasing acid stress resistance [15]. In recent years, many enzymes involved in the synthesis and modification of OLs have been described [9]. One of them is OlsF, a bifunctional OL synthase that was first described in *S. proteamaculans*. When OlsF is expressed in *E. coli*, the resulting strains can form OLs in one step [13,16].

Wild-type *E. coli* cannot form OLs and in its inner membrane the glycerophospholipids, phosphatidylethanolamine (PE), phosphatidylglycerol (PG), and cardiolipin (CP) can be detected. This composition is relatively stable under different growth conditions. In the present study, we hypothesized that the formation of OLs and hydroxylated OLs (OLs-OH) in *E. coli* strains might improve the resistance of the hosts to different abiotic stress conditions. We constructed the synthetic operon pSEVA*olsFC* (p*olsFC*) composed of the genes o*lsF* from *S. proteamaculans* and *olsC* from *R. tropici* CIAT899 required to produce unmodified and hydroxylated OLs, respectively [16,17] and expressed it in different genetic backgrounds of *E. coli*. We observed increased biomass formation under

125 phosphate limitation and improved growth with a shortened lag phase at reduced
126 pH values. Transcriptome analysis revealed that OL-forming strains differentially
127 expressed stress and membrane-related genes, indicating a reduced proton
128 leakiness. Consistently, it was observed that OL-producing strains showed better
129 growth in the presence of the ionophore carbonyl cyanide 3-
130 chlorophenylhydrazone (CCCP). Finally, we could show that the formation of
131 OLs-OH in *E. coli* strains improved violacein formation in a heterologous
132 expression system compared to *E. coli* strain not forming OLs.

133

134

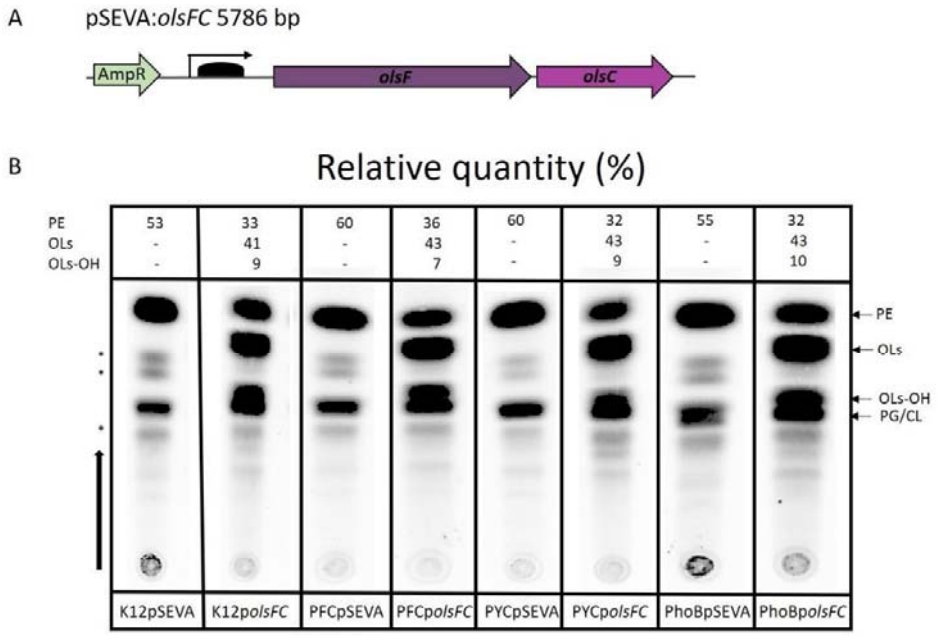
135 # RESULTS AND DISCUSSION

136 **Construction of *E. coli* strains synthesizing ornithine lipids and**
137 **hydroxylated ornithine lipids**

138 The success of many synthetic biology and biotechnology applications depends
139 on the presence of a robust microbial chassis. The modification of bacterial
140 membrane lipid composition is a promising way to obtain strains with improved
141 properties. OLs are a group of phosphorus-free bacterial membrane lipids that
142 have an important function during bacterial resistance to abiotic stress, including
143 acid stress. *E. coli* lacks genes encoding enzymes required for OL synthesis, but
144 *E. coli* strains forming OLs can be constructed [13,16]. We wanted to test how the
145 presence of OLs in *E. coli* would affect its stress resistance and its performance
146 as a host for synthetic biology applications. First, we constructed a plasmid-borne
147 synthetic operon containing the genes *olsF* and *olsC* required for the synthesis
148 of OLs and hydroxylated OLs (OLs-OH) [16,17] (Fig. 1A). This plasmid called
149 p*olsFC* and the empty control plasmid pSEVA631 (pSEVA) [18] were transformed
150 into four different *E. coli* strains: The wild-type strain *E. coli* K12 (BW25113), the
151 triple mutants PFC (*phoB, fhlC* and *cueR*) and PYC (*phoB, yedW, cusR*), which
152 have a reduced cellular proteome [6], and a *phoB*-deficient strain [19].

153 First, we wanted to determine if the *E. coli* strains expressing the synthetic operon
154 formed OLs and OLs-OH. The strains were grown in a phosphate-limited MOPS
155 medium, [^{14}C] acetate was added for lipid labeling, and total lipids were extracted
156 and analyzed by thin-layer chromatography (TLC). In all strains, the
157 glycerophospholipids phosphatidylethanolamine (PE), phosphatidylglycerol (PG)
158 and cardiolipin (CL) were detected. In addition to the lipids PG, PE, and CL, the

159 *E. coli* strains expressing the synthetic operon produced both, unmodified OLs
160 and OLs-OH (Fig. 1B). A few lipids of minor abundance which were not identified
161 were observed in the TLC (labeled with asterisks). Interestingly, in *E. coli* strains
162 accumulating OLs and OLs-OH the formation of phosphatidylethanolamine (PE)
163 was reduced relative to the control strains with the empty plasmid (Fig. 1B).



164

165 **Figure 1. Heterologous production of OLs and OLs-OH in *E. coli* strains.** (A)
166 Synthetic operon containing *olsFC* under the control of a C6 Acyl homoserine Lactone
167 (AHL) inducible promoter in plasmid p*olsFC*. (B) TLC analysis of total membrane lipids
168 of *E. coli* strains K12, PYC, PFC, and *phoB* harboring pSEVA or p*olsFC*. Total lipids were
169 labeled with [^{14}C]acetate, extracted and separated by one-dimensional TLC. PE:
170 phosphatidylethanolamine, PG: phosphatidylglycerol, CL: cardiolipin, OLs: unmodified
171 ornithine lipid, OLs-OH: hydroxylated ornithine lipid, *: undetermined lipid.

172 **OLs-OH production increases biomass yield under phosphate limitation**

In some bacteria such as *S. proteamaculans* or *V. cholerae,* OLs are not formed under phosphate-replete conditions, and their synthesis is induced by phosphate-limiting growth conditions [20]. It is thought that the zwitterionic OLs replace the zwitterionic phospholipid PE under conditions of phosphate limitation, reducing the requirement for phosphate for the membrane, and thereby allowing an alternative use for the phosphate. Phosphorus is a scarce mineral and one of the most expensive nutrients in bacterial cultures at an industrial level [21]. Three of the four strains we used in this study are deficient in the gene *phoB* and are therefore unable to induce a proper phosphate scavenging response. As described earlier, we observed a decrease in PE formation when OLs were accumulated in the *E. coli* strains expressing the synthetic operon. Therefore, we hypothesized that an *E. coli* strain being able to replace a fraction of its phospholipids with OLs could have a growth advantage under phosphate-limiting conditions, because phosphate normally used for membrane synthesis would be liberated for other cellular processes.

We performed growth kinetics of all *E. coli* strains in MOPS/glucose medium with different phosphate concentrations to define a phosphate concentration that affected the growth of *E. coli* strains without severely limiting it. In MOPS/glucose medium with 0.4 mM phosphate, slight growth inhibition was observed when compared to non-limiting phosphate concentrations of standard MOPS/glucose medium (2.6 mM). Phosphate concentrations below 0.4 mM severely affected the growth of *E. coli* strains. Therefore, a phosphate concentration of 0.4 mM was chosen for further experiments. In this set of experiments, we included the host strains, the host strains with the empty plasmid, and the host strains expressing the synthetic operon. We determined the effect of the presence of OLs-OH on

198 biomass production of *E. coli* grown under phosphate-limiting (0.4 mM) and
199 phosphate-replete (2.6 mM) concentrations. In MOPS/glucose medium with 2.6
200 mM phosphate, no difference in biomass formation was observed between the
201 different strains analyzed (Fig. S1A). In the phosphate-limited MOPS/glucose
202 medium, the biomass production in the strains that synthesize OLs increased by
203 23 to 39 % compared to the strains with the empty plasmid (Fig. 2A). In all cases,
204 the biomass formation of the OL-forming strains was higher than the wild-type
205 strains lacking a plasmid. These results suggest that the synthesis of OLs
206 provides *E. coli* an advantage when growing under growth-limiting phosphate
207 concentrations.

208 **OLs-OH production improves growth phenotype at low pH**

209 Bacteria are frequently exposed to acid stress[22]. To survive these unfavorable
210 conditions, they have evolved various resistance mechanisms, which include the
211 pumping of protons out of the cytoplasm, the production of ammonia, proton-
212 consuming decarboxylation reactions, and modifications of the membrane lipid
213 composition [21]. The presence of hydroxylated OLs has been related to increased
214 resistance to acid growth conditions, for example, the growth of a *R. tropici*
215 mutant deficient in the OL hydroxylase OlsC and lacking the hydroxylation of the
216 C2 of the secondary fatty acid of OLs is strongly affected in its growth at pH 4.5.
217 We wondered if the presence of OLs and hydroxylated OLs would improve growth
218 in MES/glucose medium without phosphate limitation at pH 5.8 in our engineered
219 *E. coli* strains. Again, we included the four host strains, the host strains with the
220 empty plasmid, and the host strains expressing the synthetic operon. During the
221 initial 6 hours, all strains (with or without OLs) exhibited similar growth kinetics.

222 However, a noticeable difference emerged when comparing the plasmid-
223 harboring strains in the subsequent hours. The presence of OLs in the *E. coli*
224 strains (triangles) significantly enhanced the maximum growth rate of the bacteria
225 at pH 5.8, resulting in approximately a 40% increase compared to three out of the
226 four genetic backgrounds examined (Fig. 2B to E). Furthermore, all four OL-
227 forming strains accumulated more biomass than the vector control strains (Fig.
228 2F). The *E. coli* strains harboring the empty vector controls reached the lowest
229 final optical density of all groups of strains. The OL-forming strains showed a
230 shortened lag phase compared to the four wild-type strains lacking plasmids. As
231 a control, we compared the maximum growth rate of the strains grown without
232 phosphate limitation at pH 7.4. No statistically significant differences were
233 observed between the strains at pH 7.4 (Fig S2).

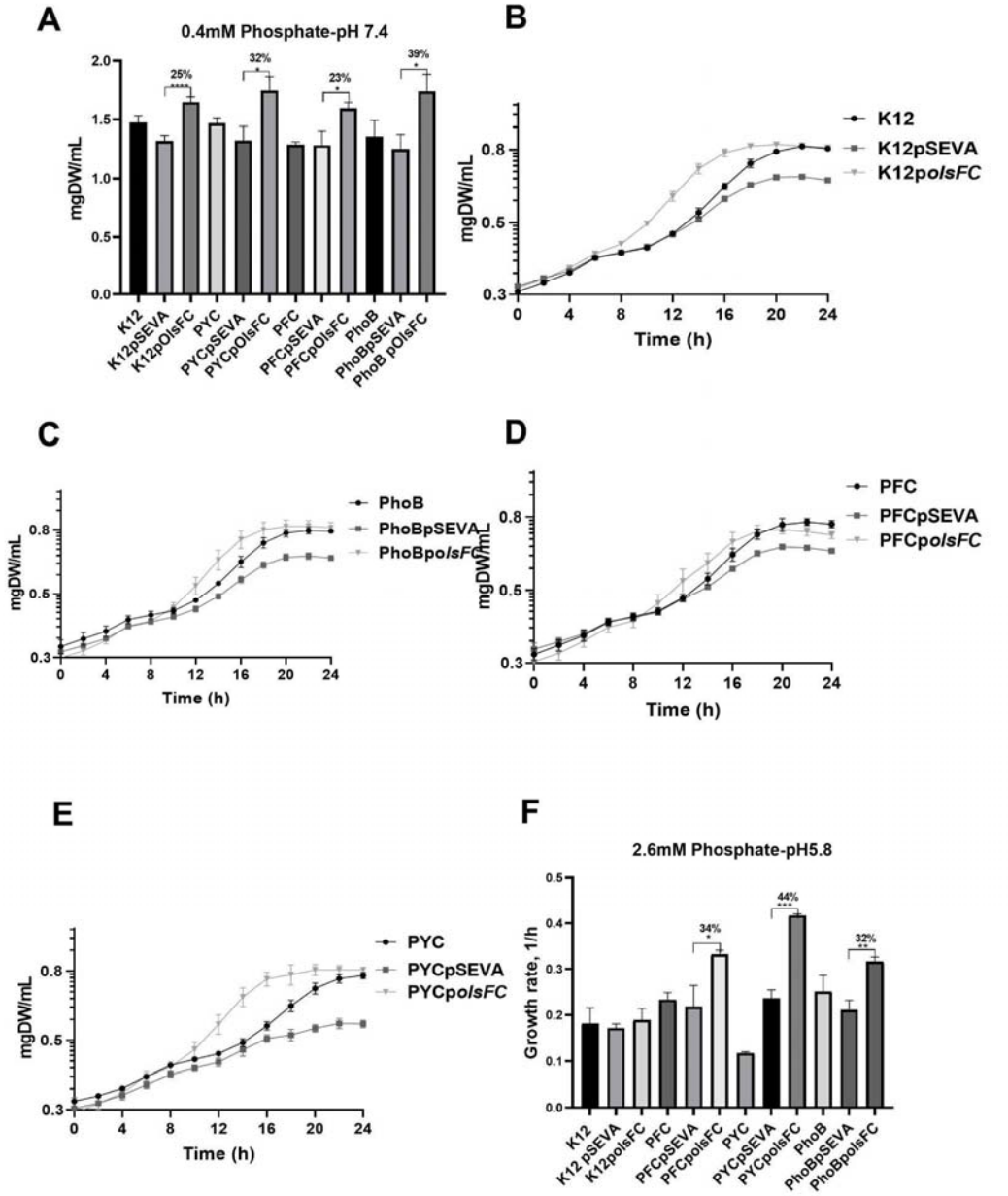


Figure 2. The presence of ornithine lipids in *E. coli* strains improves their growth characteristics under phosphate limitation and at pH 5.8 (A) Dry weight concentration (mgDW/mL) of cultures after 24-hour growth in MOPS/glucose medium with low phosphate concentration (0.4 mM). Growth curves of *E. coli* K12 (B), *E. coli* triple mutant PFC (C), *E. coli* triple mutant PYC (D), and *E. coli* mutant *phoB* (E) in MES/glucose medium with 2.6 mM phosphate at pH 5.8. (F) Growth rates of the different *E. coli* strains were calculated from the growth curves in MES/glucose medium with 2.6

242 mM phosphate at pH 5.8. The data represent the average of three independent
243 experiments. The error bars indicate the standard deviation using a two-tailed unpaired
244 Student's t-test. P-values *P ≤ 0.05, ****P ≤0.0001.
245

246 **4.- Transcriptional response to OLs expression at pH 7.4 and pH 5.8**

247 We wondered if part of the positive phenotypic effects of the presence of OLs-
248 OH could be explained by transcriptional changes. To evaluate this, we compared
249 transcriptomes of *E. coli* K12.pSEVA and *E. coli* K12.p*olsFC* strains grown at pH
250 7.4 and pH 5.8 without phosphate limitation. Total RNA was extracted from
251 exponential growing cultures (11h). At this time point, a clear difference in growth
252 rate could be observed between the strains forming OLs and the strains lacking
253 them (Fig. 2B). We conducted RNA-Seq analysis on two independent biological
254 samples and sequenced the libraries to approximately 10 million read depth. We
255 then compared the expression profiles of these samples based on their response
256 to different perturbations: Low pH with and without OLs, response to OLs at low
257 pH, and response to OLs at neutral pH (Table 1).

258

259 **Table 1. Transcriptional response to OLs expression at neutral and low pH.**

260 **Perturbations and descriptions**

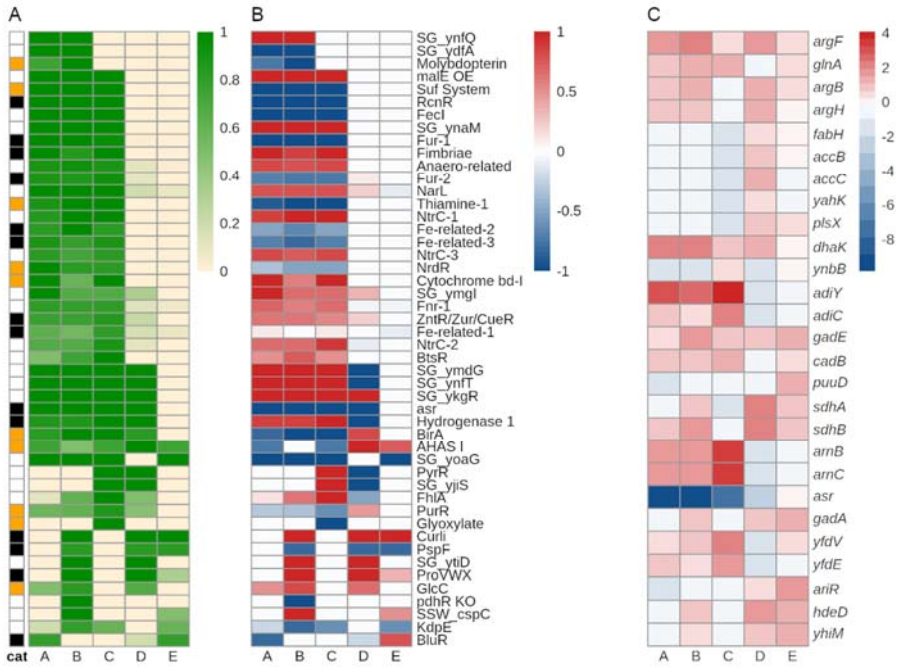
PERTURBATION	DESCRIPTION
A	Response of wild-type strain to low pH. Comparison between K12pSEVA pH 7.4 and K12pSEVA pH 5.8.
B	Differential response to low pH challenge and OLs formation. Comparison between K12pSEVA pH 7.4 and K12pOlsFC pH 5.8.
C	Response of OL-forming strain to low pH. Comparison between K12pOlsFC pH 7.4 and K12pOlsFC pH 5.8.
D	Response to the presence of OLs at pH 7.4. Comparison between K12pSEVA pH 7.4 and K12pOlsFC pH 7.4.
E	Response to the presence of OLs at pH 5.8. Comparison between K12pSEVA pH 5.8 and K12pOlsFC pH 5.8.

261

262 Overall, the results show that there is a large transcriptional response to low pH
263 conditions (A, B, C), and a mild transcriptional response to OLs expression (B, D,
264 E). We used the iModulon classification of the DEGs (differentially expressed
265 genes) data, which consists of the clustering of genes sharing an independently
266 modulated signal. The iModulons were obtained by independent component
267 analysis (ICA) of 815 sample transcriptional profiles from 422 conditions for *E.*
268 *coli* [23]. We considered only those iModulons that covered 80% or more of the
269 DEGs for further analysis (fig. 3A). We observed changes in iModulons related to
270 metabolism, stress response, two-component systems, energy production, and
271 transcription/translation. Of special interest to us were the specific transcriptional
272 changes observed in OLs expressing strains (fig. 3B, perturbations B, D, E) which
273 occurred in the iModulons *pspF*, the *kdpE*, *proW* and *curli*. The iModulon *pspF*
274 was of special interest because its involvement in membrane and proton motive

force maintenance. It is composed of genes encoding the so-called Phage Shock Proteins, which are membrane proteins that play a critical role in regulating the permeability of the membrane to protons. Recent research by Wang et al. (2021) indicated that *pspA* mutants had a decreased ability to maintain the Proton Motive Force (PMF) during periods of starvation [24]. Recently, also its participation in long-term stationary phase survival was shown[25]. Expression of the potassium two-component system *kdpE* was reduced in the presence of OLs, genes from efflux pumps such as *kdpE* were recently shown to be overrepresented in bacterial taxa with low pH preferences [26]. On the contrary, the expression of the glycine betaine *proVWX* and the *curli* iModulon were consistently overexpressed in OLs-producing strains. Furthermore, we analyzed individual genes, not classified by the iModulon analysis but related to ornithine synthesis, synthesis and turnover of membrane lipids, and response to acid stress as shown in Figure 3C. Of particular interest are the downregulation of *asr* (Fig 3C, perturbations A, B, and C), a highly charged, stress-induced, intrinsically disordered protein that contributes to the maintenance of outer membrane integrity under moderate acid stress [27]. Again, of special interest to us were the specific transcriptional changes observed in OLs expressing strains (fig. 3C, pertubations B, D, E). The genes *adiY/adiC* encoding an arginine/agmatine antiporter, of the arginine-dependent extreme acid resistance (XAR) were induced in the transcriptional response to low pH. AdiY/AdiC is one of several, decarboxylase-antiporter systems that protect *E. coli* from exposure to the acid environment [28,29]. Ornithine is an intermediate of arginine synthesis in *E. coli* and we hypothesized that the expression of OlsFC which uses ornithine as a precursor for OL synthesis might cause arginine limitation. This perceived low concentration of intracellular

arginine might explain the induction of arginine synthesis genes in pertubations B and D. A lower amount of free arginine would also reduce the efficacy of the XAR system [28]. Interestingly, some genes known to be involved in the acid stress response are induced by the formation of OLs, such as *gadA*, *hdeD* and *yhiM*. This differential expression might possibly contribute to the shortening of the lag phase at pH 5.8 in OL-forming strains. By performing a comprehensive transcriptional profiling experiment, we showed that modifications of the membrane composition by OLs change the regulatory response to low pH.



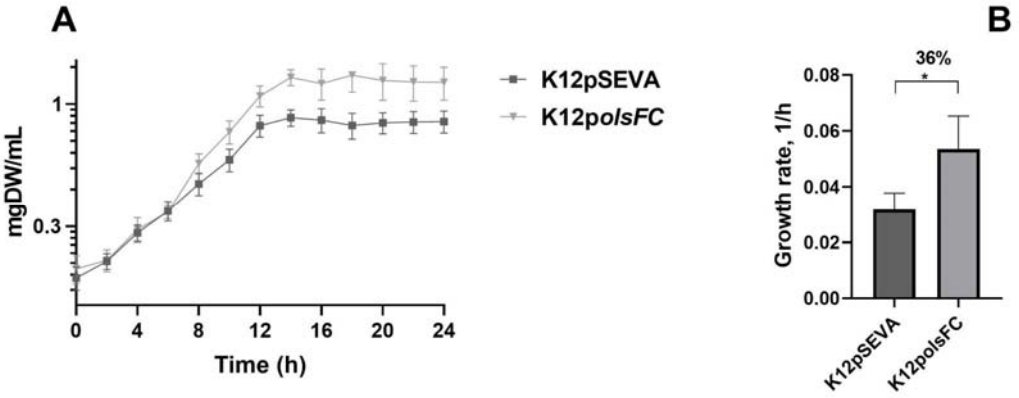
312 **Figure 3. Low pH conditions cause a large transcriptional response whereas OL**
313 **formation causes a mild transcriptional response.** (A) 48 iModulons with coverage
314 greater than 80% in at least one perturbation. The functional categories are denoted
315 according to color, yellow: metabolism, black: stress response, and white: other
316 functions. (B) Differential expression of each analyzed iModulon. (C) Expression
317 changes of individual genes involved in arginine metabolism, membrane and lipid
318 synthesis, and stress response to low pH not classified by iModulons. (value= DEGup -
319 DEGdown), blue color, reduced expression, and red color, induced expression.
320

321 **OLs-OH producing strains are more tolerant to the proton motive force**
322 **uncoupler CCCP**

323 The consistently reduced expression of the *pspF* iModulon in strains producing
324 OLs suggests that changes in membrane composition could affect how
325 engineered strains manage proton membrane permeability, potentially providing
326 a mechanism for their increased tolerance to low pH. To investigate this possible
327 mechanism, we performed growth experiments using the ionophore carbonyl
328 cyanide m-chlorophenyl hydrazine (CCCP), a compound that dissipates the
329 proton gradient across the inner membrane. Our results show that strains
330 producing OLs can grow 36% faster than strains harboring the empty plasmid
331 (Fig. 4). The growth profiles of the strains are similar to those observed in the low
332 pH experiments where the OLs-OH producing strains start growing at the same
333 rate as the control but then they separate around the 5th hour, and they ultimately
334 have an overall faster growth rate and higher biomass production. This result
335 indicates that the presence of OLs-OHs in the *E. coli* membranes makes the cells

336 less susceptible to the ionophore CCCP providing a mechanism for the low pH
337 resistance of the engineered strains.

338



339

340 **Figure 4. The presence of OLs in *E. coli* increases resistance to the ionophore**
341 **CCCP.** *E. coli* strains harboring the empty control plasmid or the plasmid containing
342 *olsFC* were grown at pH 7.4 in MOPS/glucose medium without phosphate limitation. The
343 growth curves are shown in (A), and the maximum specific growth rates in (B). The data
344 represent the average of three independent experiments. The error bars indicate the
345 standard deviation using a two-tailed unpaired Student's t-test. P-values *P ≤ 0.05

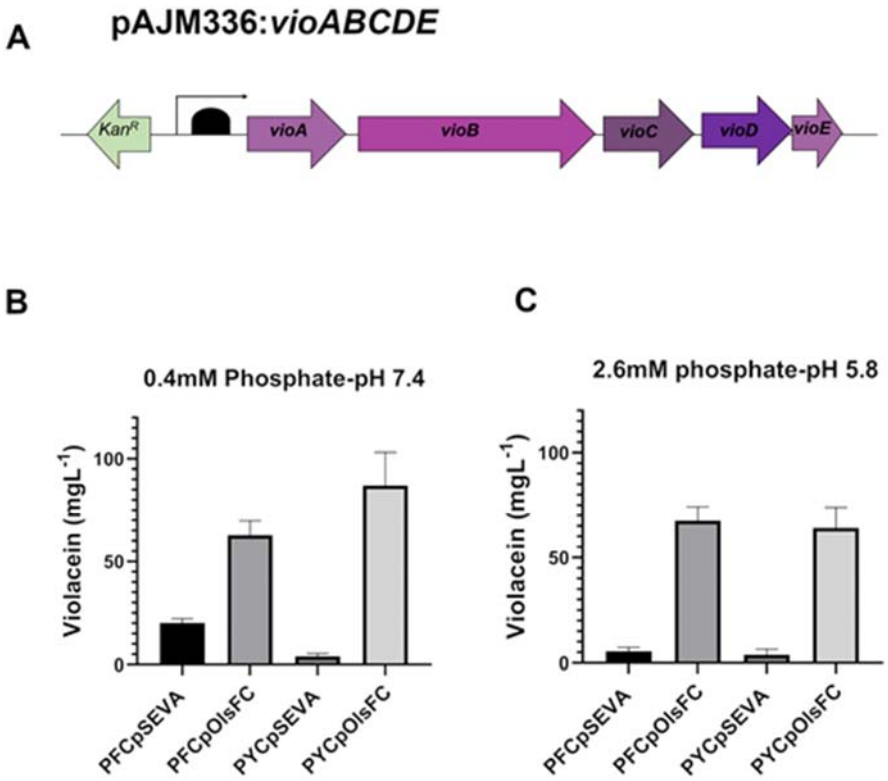
346

347 **6.- OLs-OH expression increases heterologous violacein production**

348

349 We have shown here that the presence of OLs in *E. coli* benefits its growth under
350 both phosphate-limiting and acidic pH growth conditions. These improved
351 properties could enhance the behavior of *E. coli* strains during heterologous
352 expression, for example during an industrial application. As a proof-of-concept
353 experiment, we tested the ability of our engineered strains to synthesize violacein.
354 Violacein is a pigment naturally synthesized by *Chromobacterium violaceum* with

355 antiviral, antiparasitic, and antibacterial activities, and it has several applications
356 at the industrial level. This compound is synthesized in a five-step metabolic
357 pathway using tryptophan as a precursor [30]. The biosynthetic operon *vioABCDE*
358 was cloned into the plasmid pAJM336 under the control of an IPTG inducible
359 promoter. The resulting plasmid pAJM336:*vioABCDE* was transformed into OLs-
360 producing derivatives of the triple mutants PFC and PYC which had been
361 obtained by the ReProMin analysis. First, we showed that these strains,
362 transformed with both plasmids, were producing unmodified and hydroxylated
363 OLs (Fig S2). We quantified violacein production in the evaluated strains after
364 24 hours at 30 °C in MOPS/glucose medium (pH 7.4) and MES/glucose medium
365 (pH 5.8) under phosphate-replete conditions (2.6 mM phosphate) and in
366 MOPS/glucose medium (pH 7.4) in phosphate-limiting conditions (0.4 mM
367 phosphate). At pH 7.4 in the absence of OLs, the triple mutant PFC accumulated
368 more violacein than the triple mutant PYC (Fig. 5A). The presence of OLs at pH
369 7.4, increased the amount of violacein formed by the triple mutant PYC (Fig. 5A).
370 At pH 7.4 under phosphate-limiting conditions, more violacein formation can be
371 detected in the OL-forming strains than in the vector control strains (Fig. 5B). At
372 pH 5.8 with 2.6 mM phosphate, again comparing the strains harboring the empty
373 plasmid with the strains expressing *olsFC* violacein formation was more than 10-
374 fold higher in strains forming OLs (Fig. 5C).
375 In both low-phosphate and low-pH conditions, the presence of OLs-OH increased
376 violacein production compared to the control. These results show that our
377 membrane engineering approach is beneficial for producing complicated
378 metabolites via a heterologous pathway.



379

380 **Figure 5. *E. coli* strains forming OLs-OH show an increase in total violacein**
381 **production.** All strains harbored the plasmid pJAM336:*vioABCDE* (A) in addition to
382 pSEVA or p*olsFC*. Strains were grown in MOPS/glucose medium at pH 7.4 with 0.4 mM
383 phosphate (B) and in MES/glucose medium at pH 5.8 with 2.6 mM phosphate (C),
384 supplemented with 2 g/L tryptophan, 800 μM IPTG and 0.01% casamino acids (mean
385 +/- s.d., n=9).

386
387

388 **CONCLUSIONS**

389

390 Our study provides a solid basis to demonstrate the applicability of engineering
391 of the membrane composition to reduce phosphate need, to increase tolerance
392 to low pH stress and we demonstrate the advantage of the approach by
393 expressing a heterologous metabolite from a costly pathway in proteome-
394 reduced strains of *E. coli.*

395 We show for the first time that the presence of OLs-OH seems to reduce the
396 permeability of the internal membrane to protons, and we provide a possible
397 mechanism for the enhanced tolerance to acid stress by the modification of the
398 membrane composition.

399 To be able to design specific membrane properties in the future, further
400 experiments are needed to describe and understand the changes in membrane
401 characteristics such as permeability and fluidity, etc. caused by the presence of
402 OLs. Another area of opportunity is how the engineering of the bacterial
403 membrane affects the resistance to other kinds of stresses, such as osmotic
404 pressure, shear stress, or temperature changes that can be present in large-scale
405 fermentations [31]. The abiotic stress response involves cellular mechanisms
406 beyond the change in membrane composition and the combination of our
407 approach with others should aid in the generation of highly tolerant microbial cell
408 factories.

409

410

411 # MATERIALS AND METHODS

412 ## Strain, plasmids and growth conditions

413 Bacterial strains and plasmids used are listed in supplementary table 1 and the
414 oligonucleotides used are listed in supplementary table 2.

415 Plasmid pSEVA63 confers ampicillin resistance and has the replication system
416 of the plasmid pBBR1 which is regarded as a "medium copy" plasmid. It was used
417 to construct pSEVA*OlsFC* containing the *olsF* gene (1722 bp) from *Serratia*
418 *proteamaculans* and the *olsC* gene (846 bp) from *Rhizobium tropici.* The
419 *synthetic olsFC* operon has a ribosome binding site of 12 bp between both genes.
420 The transcription of this operon is regulated by C6-.AHL (acyl homoserine
421 lactone) and LuxR, but the system has some basal level expression even without
422 induction. Therefore, we decided to use the empty plasmid pSEVA63 as negative
423 control for our studies.

424 The plasmid pAJM.336Vio confers kanamycin resistance and contains the genes
425 *vioA* (1257 bp), *vioB* (2994 bp), *vioC* (1290 bp), *vioD* (1122 bp), *vioE* (576 bp)
426 from *Chromobacterium violaceum* encoding the enzymes required for violacein
427 synthesis using tryptophan as a precursor.

428 Gibson assembly was used for plasmid construction. 10 µL of 2X NEBuilder HiFi
429 DNA Assembly Master Mix were incubated with one or more DNA fragments,
430 0.05 pmol each, in a final volume of 20 µL at 50°C for 60 minutes. TOP10
431 chemically competent *E. coli* were transformed with 2 µL of the assembled
432 products and incubated overnight at 37°C after transformation.

433 LB medium (Sigma), LB medium supplemented with 1 g/L glucose and MOPS
434 minimal media supplemented with 0.01% casamino acids (CAA) (Sigma), and 4
435 g/L glucose and K_2HPO_4 (0.4 or 2.6 mM) were used[32]. For low pH medium MOPS
436 buffer was replaced with the same concentration of MES buffer (MES/glucose
437 medium). Antibiotics were used in the following concentrations: gentamicin 25
438 µg/mL, spectinomycin (Spc) 50 µg/mL, ampicillin 100 µg/mL, and kanamycin 50
439 µg/mL.

440

441 **Bacterial growth experiments**

442 For the evaluation of bacterial growth at different phosphate concentrations, we
443 used MOPS/glucose medium. For growth experiments at pH 5.8 MES/glucose
444 medium was used. Cells were cultured overnight in MOPS/glucose medium
445 without phosphate limitation at 37 °C and 250 rpm. The next day cells were
446 washed three times with the receptor medium (phosphate limitation or low pH)
447 and cells were again cultured overnight. Then, strains were diluted to an OD_{600}
448 of 0.05 in fresh medium and 150 µL of the fresh culture were transferred to a
449 transparent 96-well plate (Corning) and incubated at 30 °C with fast linear shaking
450 in a microplate reader (Synergy 2.0, BioTek) for 24 hours, taking measurements
451 of the OD_{600} every 20 min. In all growth experiments, three replicates were
452 included, and the experiment was repeated independently on three different
453 days.

454 For the evaluation of bacterial tolerance to the proton motive force uncoupler
455 CCCP, we grew the strains in MOPS/glucose medium with 10µM CCCP in
456 transparent 96-well plates as described above.

457

458 The analysis of the growth kinetics of the plate experiments was conducted using
459 the algorithm developed by Swain *et al*., with the default parameters running on
460 Python [33]. From this analysis, the magnitude and time of the maximum growth
461 rate and the maximum OD_{600} were obtained. The p-value was determined using
462 a two-tailed Student t-test as the statistical significance reliability index [34].

463

464 **Lipid analysis**

465 The lipid compositions of bacterial strains were determined following labeling with
466 [^{14}C]acetate (Amersham Biosciences). Cultures (1 mL) of wild-type and mutant
467 strains were inoculated from pre-cultures grown in the same medium. After the
468 addition of 0.5 mCi of [^{14}C]acetate (60 mCi $mmol^{-1}$) to each culture, the cultures
469 were incubated for 4 h. The cells were harvested by centrifugation, washed with
470 500 µL of water, and resuspended in 100 µL of water. Lipid extracts were
471 obtained according to Bligh and Dyer (1959). Aliquots of the lipid extracts were
472 spotted on high-performance TLC silica gel 60 (Merck, Poole, UK) plates and
473 were separated in one dimension using chloroform/ methanol/water (150:30:8,
474 v/v) as mobile phase. To visualize the membrane lipids, developed one-
475 dimensional TLC plates were exposed to a PhosphorImager screen (Amersham
476 Biosciences). The individual lipids were quantified using ImageQuant software
477 (Amersham Biosciences). Primary amine-containing lipids (PE, OL, OL-OH) were
478 visualized by spraying the plates with a solution of 0.2% (w/v) ninhydrin in
479 acetone and subsequent treatment at 120°C for 10 minutes [12].

480

481 **Characterization of violacein-producing strains.**

482 Strains were inoculated into MOPS/glucose medium supplemented with
483 gentamicin (20 µL/mL) and grown overnight. The next day cells were washed
484 three times with the receptor medium (MOPS/glucose, phosphate limitation or
485 MES/glucose) and then inoculated at an OD_{600} of 0.05 in fresh receptor medium
486 supplemented with tryptophan (2.0 g/L). Then 150 µL of the fresh culture were
487 transferred to a 96-well plate and incubated as described above. After 24 h the
488 plates were centrifuged (13,000×g, 10 min), and the supernatant of each well was
489 discarded. Violacein was extracted by suspending the pellet in each well in 200
490 µL absolute ethanol and incubating the plate at 95 °C for 10 min followed by
491 pelleting cell debris (13,000×g, 10 min). The violacein present in the extract was
492 determined spectrophotometrically at 575 nm in a microplate reader (Synergy
493 2.0, BioTek) [6].
494

495 **RNA extraction for transcriptome analysis**

496 *E. coli* K12pSEVA, and K12pOlsFC cells were pre-grown at 37°C in 25 mL of
497 MOPS minimal medium with 4 g/L glucose overnight. The cells were washed
498 three-time and cultured in MES/glucose medium. During exponential growth
499 (11h), 3 mL were transferred to a tube, and 3 mL of RNA protect reagent were
500 added and mixed. Cells were harvested by centrifugation and the pellets were
501 immediately frozen in liquid nitrogen. Total RNA was prepared using the RNeasy
502 mini kit (QIAGEN, Hildesheim, Germany). Pellets were resuspended in RLT
503 buffer supplemented with lysozyme (20 mg per mL; QIAGEN, Hildesheim,
504 Germany) and containing Zirconium Oxide Beads (0.5 mm, Next Advance). Cells

505 were disrupted using the Bullet Blender Tissue Homogenizer (Next-Advance,
506 Troy, NY, USA) in impact-resistant 2mL tubes. Genomic DNA was eliminated by
507 digestion with RNase-free DNase (QIAGEN, Hildesheim, Germany) for 20 min at
508 room temperature. Final RNA concentrations were determined using a NanoDrop
509 (Thermo Scientific). The typical OD_{260} to OD_{280} ratio of RNA samples was
510 approximately 2.0. The integrity of RNA samples was verified by determining the
511 RNA integrity number (RINe) in a TapeStation 2200 instrument (Agilent
512 Technologies). Two independent total RNA extractions were obtained for each
513 condition and each one was analyzed separately [35] [28].

514

515 **RNA-Seq**

516 The samples of RNA-seq were analyzed by Novogene (Sacramento, CA, USA).
517 A total amount of 1 µg RNA per sample was used as input material for the RNA
518 simple preparations. Sequencing libraries were generated using NEBNext®
519 UltraTM RNA Library Prep Kit for Illumina® (NEB, USA) following the
520 manufacturer's recommendations and index codes were added to attribute
521 sequences to each sample. After rRNA removal, fragmentation was carried out
522 using divalent cations under elevated temperature in NEBNext First Strand
523 Synthesis Reaction Buffer (5X). First strand cDNA was synthesized using random
524 hexamer primer and M-MuLV Reverse Transcriptase (RNase H-). Second-strand
525 cDNA synthesis was subsequently performed using DNA Polymerase I and
526 RNase H. Remaining overhangs were converted into blunt ends via
527 exonuclease/polymerase activities. After adenylation of 3' ends of DNA
528 fragments, NEBNext Adaptor with hairpin loop structure were ligated to prepare

529 for hybridization. In order to select cDNA fragments of preferentially 150~200 bp
530 in length, the library fragments were purified with AMPure XP system (Beckman
531 Coulter, Beverly, USA). Then 3 μL USER Enzyme (NEB, USA) was used with
532 size-selected, adaptor- ligated cDNA at 37 °C for 15 min followed by 5 min at 95
533 °C before PCR. Then PCR was performed with Phusion High-Fidelity DNA
534 polymerase, Universal PCR primers and Index (X) Primer. At last, PCR products
535 were purified (AMPure XP system) and library quality was assessed on the
536 Agilent Bioanalyzer 2100 system. The clustering of the index-coded samples was
537 performed on an Illumina Novaseq sequencer according to the manufacturer's
538 instructions. After cluster generation, the libraries were sequenced on the same
539 machine and paired-end reads were generated.
540

541 **Data processing and differential expression**

542 The raw reads were processed for adapter trimming and quality control filtering
543 using Trim Galore (v.0.6.6). The reads were aligned using R package Rsubread
544 (v.2.2.6) and mapped to the reference genome (GCF_000005845.2). For gene
545 expression quantification the function featureCounts from Rsubread package
546 were used, generating the read counts matrix. We used the batch correction
547 ARSyseq and TMM for normalization. The differential expression analysis was
548 performed using DESeq2 (v.1.28.1). The filters used for the DEGs were: absolute
549 log2FoldChange >= 1 and p-value adjust <= 0.05. The accession number for the
550 RNAseq data reported in this paper is GEO: GSE233657
551 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE233657).

552 The iModulon analysis consists of adding the differential expression data to each
553 iModulon and the order within the graphs was made based on the "system
554 category" field [23,36].

555

556 **Acknowledgment**

557 We thank Mishael Perez for initial RNAseq processing and Miguel Angel Vences-
558 Guzmán for technical support. LPBP was a recipient of the Postdoctoral
559 Scholarship Program in the UNAM-DGAPA. This work was supported by grants
560 from PAPIIT-UNAM IN214923 to JU and by grants from CONACyT (237713 and
561 425886) and PAPIIT UNAM (IN208116 and IN208319) to CS.

562

563

REFERENCES

(1) Cordell, W. T.; Avolio, G.; Takors, R.; Pfleger, B. F. Milligrams to Kilograms: Making Microbes Work at Scale. *Trends Biotechnol* **2023**. https://doi.org/10.1016/J.TIBTECH.2023.05.002.

(2) Calero, P.; Nikel, P. I. Chasing Bacterial *Chassis* for Metabolic Engineering: A Perspective Review from Classical to Non-Traditional Microorganisms. *Microb Biotechnol* **2019**, *12* (1), 98–124. https://doi.org/10.1111/1751-7915.13292.

(3) Beites, T.; Mendes, M. V. Chassis Optimization as a Cornerstone for the Application of Synthetic Biology Based Strategies in Microbial Secondary Metabolism. *Front Microbiol* **2015**, *6*. https://doi.org/10.3389/fmicb.2015.00906.

(4) Marquez-Zavala, E.; Utrilla, J. Engineering Resource Allocation in Artificially Minimized Cells: Is Genome Reduction the Best Strategy? *Microb Biotechnol* **2023**, *16* (5), 990–999. https://doi.org/10.1111/1751-7915.14233.

(5) Chi, H.; Wang, X.; Shao, Y.; Qin, Y.; Deng, Z.; Wang, L.; Chen, S. Engineering and Modification of Microbial Chassis for Systems and Synthetic Biology. *Synthetic and Systems Biotechnology*. KeAi Communications Co. March 1, 2019, pp 25–33. https://doi.org/10.1016/j.synbio.2018.12.001.

(6) Lastiri-Pancardo, G.; Mercado-Hernández, J. S.; Kim, J.; Jiménez, J. I.; Utrilla, J. A Quantitative Method for Proteome Reallocation Using Minimal Regulatory Interventions. *Nat Chem Biol* **2020**, *16* (9), 1026–1033. https://doi.org/10.1038/s41589-020-0593-y.

(7) Marzan, L.; Shimizu, K. Metabolic Regulation of Escherichia Coli and Its PhoB and PhoR Genes Knockout Mutants under Phosphate and Nitrogen Limitations as Well as at Acidic Condition. *Microb Cell Fact* **2011**, *10* (1), 39. https://doi.org/10.1186/1475-2859-10-39.

(8) Bachhawat, P.; Swapna, G. V. T.; Montelione, G. T.; Stock, A. M. Mechanism of Activation for Transcription Factor PhoB Suggested by Different Modes of Dimerization in the Inactive and Active States. *Structure* **2005**, *13* (9), 1353–1363. https://doi.org/10.1016/j.str.2005.06.006.

(9) Sohlenkamp, C. Membrane Homeostasis in Bacteria upon PH Challenge. In *Biogenesis of Fatty Acids, Lipids and Membranes*; Springer International Publishing, 2017; pp 1–13. https://doi.org/10.1007/978-3-319-43676-0_57-1.

(10) Sohlenkamp, C.; Geiger, O. Bacterial Membrane Lipids: Diversity in Structures and Pathways. *FEMS Microbiology Reviews*. Oxford University Press September 5, 2015, pp 133–159. https://doi.org/10.1093/femsre/fuv008.

(11) Kim, S. K.; Park, S. J.; Li, X. H.; Choi, Y. S.; Im, D. S.; Lee, J. H. Bacterial Ornithine Lipid, a Surrogate Membrane Lipid under Phosphate-Limiting Conditions, Plays Important Roles in Bacterial Persistence and Interaction with Host. *Environ Microbiol* **2018**, *20* (11), 3992–4008. https://doi.org/10.1111/1462-2920.14430.

603 (12) Vences-Guzmán, M. Á.; Guan, Z.; Ormeño-Orrillo, E.; González-Silva, N.; López-Lara, I.
604 M.; Martínez-Romero, E.; Geiger, O.; Sohlenkamp, C. Hydroxylated Ornithine Lipids
605 Increase Stress Tolerance in Rhizobium Tropici CIAT899. *Mol Microbiol* **2011**, *79* (6),
606 1496–1514. https://doi.org/10.1111/j.1365-2958.2011.07535.x.

607 (13) Vences-Guzmán, M. Á.; Guan, Z.; Escobedo-Hinojosa, W. I.; Bermúdez-Barrientos, J. R.;
608 Geiger, O.; Sohlenkamp, C. Discovery of a Bifunctional Acyltransferase Responsible for
609 Ornithine Lipid Synthesis in Serratia Proteamaculans. *Environ Microbiol* **2015**, *17* (5),
610 1487–1496. https://doi.org/10.1111/1462-2920.12562.

611 (14) Córdoba-Castro, L. A.; Salgado-Morales, R.; Torres, M.; Martínez-Aguilar, L.; Lozano, L.;
612 Vences-Guzmán, M. Á.; Guan, Z.; Dantán-González, E.; Serrano, M.; Sohlenkamp, C.
613 Ornithine Lipids in Burkholderia Spp. Pathogenicity. *Front Mol Biosci* **2021**, *7*.
614 https://doi.org/10.3389/fmolb.2020.610932.

615 (15) Nikaido, H. Molecular Basis of Bacterial Outer Membrane Permeability Revisited.
616 *Microbiology and Molecular Biology Reviews* **2003**, *67* (4), 593–656.
617 https://doi.org/10.1128/MMBR.67.4.593-656.2003.

618 (16) Escobedo-Hinojosa, W. I.; Vences-Guzmán, M. Á.; Schubotz, F.; Sandoval-Calderón, M.;
619 Summons, R. E.; López-Lara, I. M.; Geiger, O.; Sohlenkamp, C. OlsG (Sinac_1600) Is an
620 Ornithine Lipid N-Methyltransferase from the Planctomycete Singulisphaera Acidiphila.
621 *Journal of Biological Chemistry* **2015**, *290* (24), 15102–15111.
622 https://doi.org/10.1074/jbc.M115.639575.

623 (17) Rojas-Jiménez, K.; Sohlenkamp, C.; Geiger, O.; Martínez-Romero, E.; Werner, D.;
624 Vinuesa, P. A ClC Chloride Channel Homolog and Ornithine-Containing Membrane
625 Lipids of *Rhizobium Tropici* CIAT899 Are Involved in Symbiotic Efficiency and Acid
626 Tolerance. *Molecular Plant-Microbe Interactions®* **2005**, *18* (11), 1175–1185.
627 https://doi.org/10.1094/MPMI-18-1175.

628 (18) Silva-Rocha, R.; Martínez-García, E.; Calles, B.; Chavarría, M.; Arce-Rodríguez, A.; de las
629 Heras, A.; Páez-Espino, A. D.; Durante-Rodríguez, G.; Kim, J.; Nikel, P. I.; Platero, R.; de
630 Lorenzo, V. The Standard European Vector Architecture (SEVA): A Coherent Platform for
631 the Analysis and Deployment of Complex Prokaryotic Phenotypes. *Nucleic Acids Res*
632 **2013**, *41* (D1), D666–D675. https://doi.org/10.1093/nar/gks1119.

633 (19) Baba, T.; Ara, T.; Hasegawa, M.; Takai, Y.; Okumura, Y.; Baba, M.; Datsenko, K. A.;
634 Tomita, M.; Wanner, B. L.; Mori, H. Construction of *Escherichia Coli* K-12 In-frame,
635 Single-gene Knockout Mutants: The Keio Collection. *Mol Syst Biol* **2006**, *2* (1).
636 https://doi.org/10.1038/msb4100050.

637 (20) Barbosa, L. C.; Goulart, C. L.; Avellar, M. M.; Bisch, P. M.; von Kruger, W. M. A.
638 Accumulation of Ornithine Lipids in Vibrio Cholerae under Phosphate Deprivation Is
639 Dependent on VC0489 (OlsF) and PhoBR System. *Microbiology (United Kingdom)* **2018**,
640 *164* (3), 395–399. https://doi.org/10.1099/mic.0.000607.

641 (21) Foster, J. W. Escherichia Coli Acid Resistance: Tales of an Amateur Acidophile. *Nat Rev*
642 *Microbiol* **2004**, *2* (11), 898–907. https://doi.org/10.1038/nrmicro1021.

643 (22) Guan, N.; Liu, L. Microbial Response to Acid Stress: Mechanisms and Applications. *Appl*
644 *Microbiol Biotechnol* **2020**, *104* (1), 51–65. https://doi.org/10.1007/s00253-019-10226-
645 1.

646 (23) Rychel, K.; Decker, K.; Sastry, A. V; Phaneuf, P. V; Poudel, S.; Palsson, B. O. IModulonDB:
647 A Knowledgebase of Microbial Transcriptional Regulation Derived from Machine
648 Learning. *Nucleic Acids Res* **2021**, *49* (D1), D112–D120.
649 https://doi.org/10.1093/nar/gkaa810.

650 (24) Wang, M.; Chan, E. W. C.; Wan, Y.; Wong, M. H. yin; Chen, S. Active Maintenance of
651 Proton Motive Force Mediates Starvation-Induced Bacterial Antibiotic Tolerance in
652 Escherichia Coli. *Commun Biol* **2021**, *4* (1). https://doi.org/10.1038/s42003-021-02612-
653 1.

654 (25) Takano, S.; Takahashi, H.; Yama, Y.; Miyazaki, R.; Furusawa, C.; Tsuru, S. Inference of
655 Transcriptome Signatures of Escherichia Coli in Long-Term Stationary Phase. *Sci Rep*
656 **2023**, *13* (1), 5647. https://doi.org/10.1038/s41598-023-32525-4.

657 (26) Ramoneda, J.; Stallard-Olivera, E.; Hoffert, M.; Winfrey, C. C.; Stadler, M.; Niño-García,
658 J. P.; Fierer, N. Building a Genome-Based Understanding of Bacterial PH Preferences. *Sci*
659 *Adv* **2023**, *9* (17). https://doi.org/10.1126/sciadv.adf8998.

660 (27) Ren, C.; Zheng, Y.; Liu, C.; Mencius, J.; Wu, Z.; Quan, S. Molecular Characterization of an
661 Intrinsically Disordered Chaperone Reveals Net-Charge Regulation in Chaperone Action.
662 *J Mol Biol* **2022**, *434* (5), 167405. https://doi.org/10.1016/j.jmb.2021.167405.

663 (28) Iyer, R.; Williams, C.; Miller, C. Arginine-Agmatine Antiporter in Extreme Acid Resistance
664 in *Escherichia Coli*. *J Bacteriol* **2003**, *185* (22), 6556–6561.
665 https://doi.org/10.1128/JB.185.22.6556-6561.2003.

666 (29) Tsai, M.-F.; Fang, Y.; Miller, C. Sided Functions of an Arginine–Agmatine Antiporter
667 Oriented in Liposomes. *Biochemistry* **2012**, *51* (8), 1577–1585.
668 https://doi.org/10.1021/bi201897t.

669 (30) Durán, N.; Justo, G. Z.; Durán, M.; Brocchi, M.; Cordi, L.; Tasic, L.; Castro, G. R.;
670 Nakazato, G. Advances in Chromobacterium Violaceum and Properties of Violacein-Its
671 Main Secondary Metabolite: A Review. *Biotechnology Advances*. Elsevier Inc.
672 September 1, 2016, pp 1030–1045. https://doi.org/10.1016/j.biotechadv.2016.06.003.

673 (31) Wehrs, M.; Tanjore, D.; Eng, T.; Lievense, J.; Pray, T. R.; Mukhopadhyay, A. Engineering
674 Robust Production Microbes for Large-Scale Cultivation. *Trends Microbiol* **2019**, *27* (6),
675 524–537. https://doi.org/10.1016/j.tim.2019.01.006.

676 (32) Neidhardt, F. C.; Bloch, P. L.; Smith, D. F. Culture Medium for Enterobacteria. *J Bacteriol*
677 **1974**, *119* (3), 736–747. https://doi.org/10.1128/jb.119.3.736-747.1974.

678 (33) Swain, P. S.; Stevenson, K.; Leary, A.; Montano-Gutierrez, L. F.; Clark, I. B. N.; Vogel, J.;
679 Pilizota, T. Inferring Time Derivatives Including Cell Growth Rates Using Gaussian
680 Processes. *Nat Commun* **2016**, *7* (1), 13766. https://doi.org/10.1038/ncomms13766.

681 (34) Hidalgo, D.; Martínez-Ortiz, C. A.; Palsson, B. O.; Jiménez, J. I.; Utrilla, J. Regulatory
682 Perturbations of Ribosome Allocation in Bacteria Reshape the Growth Proteome with a

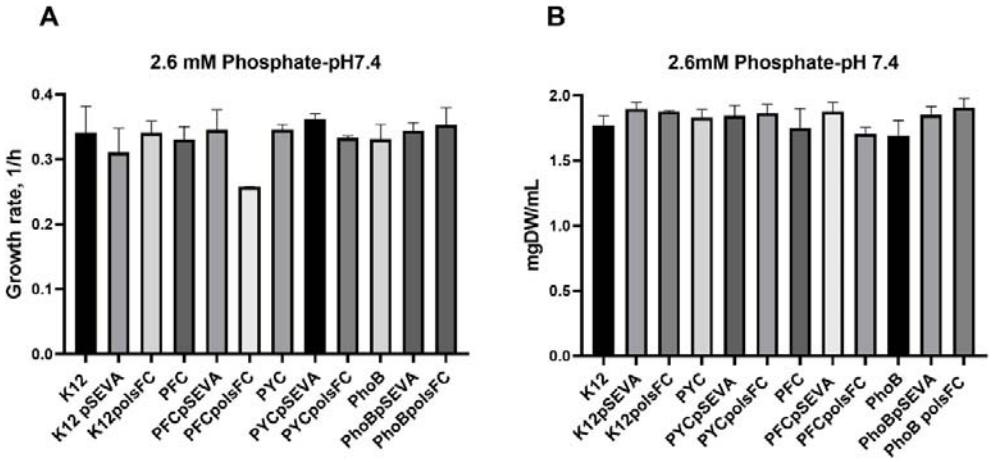
683 Trade-off in Adaptation Capacity. *iScience* **2022**, *25* (3).
684 https://doi.org/10.1016/j.isci.2022.103879.

685 (35) Guerrero-Castro, J.; Lozano, L.; Sohlenkamp, C. Dissecting the Acid Stress Response of
686 Rhizobium Tropici CIAT 899. *Front Microbiol* **2018**, *9*.
687 https://doi.org/10.3389/fmicb.2018.00846.

688 (36) Lamoureux, C. R.; Decker, K. T.; Sastry, A. V; Rychel, K.; Gao, Y.; Mcconn, J. L.; Zielinski,
689 D. C.; Palsson, B. O. A Multi-Scale Transcriptional Regulatory Network Knowledge Base
690 for Escherichia Coli. https://doi.org/10.1101/2021.04.08.439047.

691

692 ## Supplementary material



693

694 **Figure S1. Synthesis of ornithine lipids (OLs) in *E. coli* increases biomass**
695 **production under phosphate-limiting conditions**. (A) Maximum specific growth rate
696 and (B) dry weight concentration (mgDW/mL) are shown. Cells were grown in 96-well
697 plates for 24 hours at pH 7.4 in MOPS/glucose medium without phosphate limitation (2.6
698 mM). Each strain was grown without plasmid, harboring the empty control plasmid or the
699 plasmid containing *olsFC*. The data represent the average of three independent
700 experiments. The error bars indicate the standard deviation using a two-tailed unpaired
701 student's t-test.

702

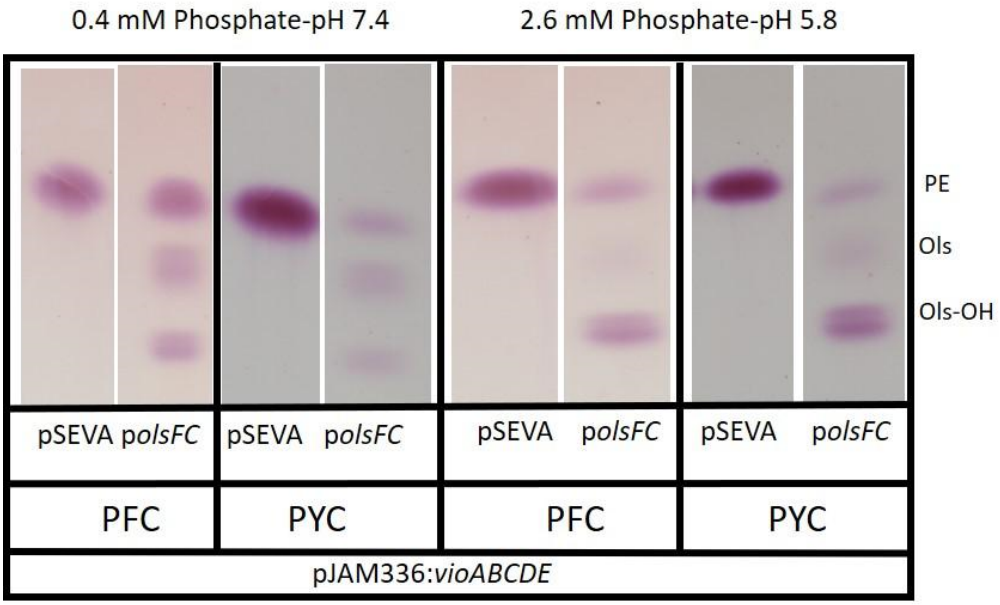


Figure S2. *E. coli* strains forming OL and synthesizing violacein. Thin layer chromatography (TLC) analysis of total lipids from *E. coli* strains forming OLs and harboring the violacein synthesis pathway. The TLC plate was stained with ninhydrin to detect primary amine-containing lipids which are in this case PE, OL, and OLs-OH. All strains analyzed for their lipid composition harbored the plasmid pJAM336:*vioABCDE* in addition to a second plasmid. Strains PFC and PYC with pSEVA (empty) and p*olsFC* with pJAM336:*vioABCDE* grown in MOPS medium with 0.4 mM phosphate and pH 7.4 (left) and MES 2.6 mM and pH 5.8 (right). PE: phosphatidylethanolamine; OL: unmodified ornithine lipid; OLs-OH:hydroxylated ornithine lipid.

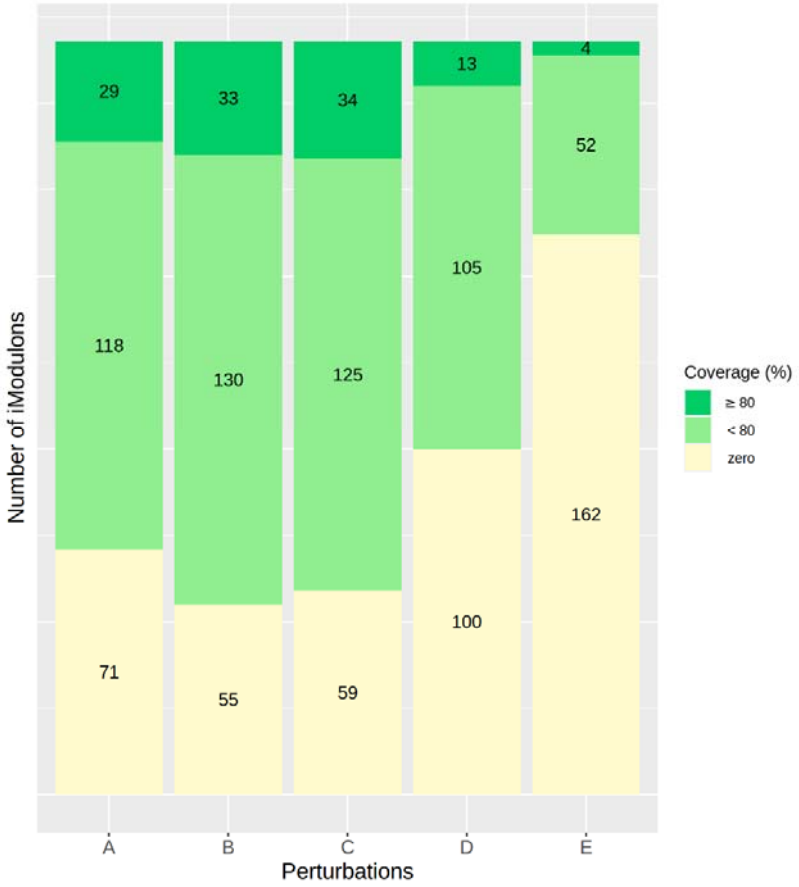


Figure S3. iModulon classification of pertubations. This classification consists in the clustering of genes that share an independent modulated signal. IModulons coverage is represented in dark green when is ≥80% of genes, light green <80% and beige when no changes in expression were observed.

729 **Table S1. Strains and plasmids used in this study**

STRAINS	GENOTYPE OR DESCRIPTIONS	REFERENCE
PFC	*E. coli* K12BW25113 with mutations in *phoB, yedW, cusR*	Lastiri-Pancardo *et al.*, 2020
PYC	*E. coli* K12BW25113 with mutations in *phoB, fhlC y cueR*	Lastiri-Pancardo *et al.*, 2020
PhoB	*E. coli* K12BW25113 with mutation in *phoB*	KEIO collection
E. coli K12 BW25113	*E. coli* K12BW25113 wild-type strain	Laboratory stock
E. coli K12 BW25113pSEVA	*E. coli* K12BW25113 wild-type strain harboring pSEVA631 Gm^r	This study
phoBpSEVA	*phoB* strain harboring pSEVA631 Gm^r	This study
PYCpSEVA	PYC strain harboring pSEVA631 Gm^r	This study
PFCpSEVA	PFC strain harboring pSEVA631 Gm^r	This study
PFCp*olsFC*	PFC strain harboring pSEVA:*olsFC* Amp^r	This study
PYCp*olsFC*	PYC strain harboring pSEVA:*olsFC* Amp^r	This study
PhoBp*olsFC*	*pho* strain harboring pSEVA:*olsFC* Amp^r	This study
K12p*olsFC*	*E. coli* K12BW25113 wild-type strain harboring pSEVA:*olsFC* Amp^r	This study
PYCpSEVA:*vioABCDE*	PYC strain harboring pSEVA Amp^r and pAJM.336*pvioABCDE* Km^r	This study
PFCpSEVA:*vioABCDE*	PFC strain harboring pSEVA Amp^r and pAJM.336*pvioABCDE* Km^r	This study
PYC*polsFC*:*vioABCDE*	PYC strain harboring pSEVA:*olsFC* Amp^r and pAJM.336*pvioABCDE* Km^r	This study
PFC*polsFC*:*vioABCDE*	PFC strain harboring pSEVA:*olsFC* Amp^r and pAJM.336*pvioABCDE* Km^r	This study

730

731

732

733

734 **Table S2. Oligonucleotides used in this study.** All sequences are written in 5' to 3'
735 direction.

pSEVA*olsF*	
pluxpR-T7/pTU1-A-lacZ REV	AGAGAGTCCAAGCTAAACATCTAGTATTTCTCCTCTTTCTCTAGTATTTAT
pluxpR-T7/pTU1-A-lacZ FWD	AGGACACCTGTATAGAGTAATACTAGAGCCAGGCATCGCA
Spro_2569olsF FWD	AGAAAGAGGAGAAATACTAGATGTTTAGCTTGGACTCTCT
Spro_2569olsF REV	TGCGATGCCTGGCTCTAGTATTACTCTATACAGGTGTCCTGT
pSEVA*olsFC*	
OlsF/Cind.WizGA FWD (1)	GAAACCGCCCGAAGCCCTGACGCAAAAAACCCCGCTTCGG
OlsF/Cind.WizGA REV (1)	TCCGTCATTCCAAACCTCCTATGCCTGGCTCTAGTATTACTCTATAC
OlsF/Cind.WizGA FWD (2)	GTAATACTAGAGCCAGGCATAGGAGGTTTGGAATGACGGAGA
OlsF/Cind.WizGA REV (2)	CCGAAGCGGGGTTTTTTGCGTCAGGGCTTCGGGCGGTTTC
pAJM.336:*vioABCDE*	
pAJM.336 FWD	TTTTTTCTGCCTCGTGATACGCCTACTCGGTACCAAATTCCAGAAAAGAG
pAJM.336 REV	TGCAGATATCGGAAGAATGCTTCATCTAGTATTTCCCCTCTTTCTCTAGTATTAAAC
pSEVA63-viogene FWD (1)	ACTAGAGAAAGAGGGGAAATACTAGATGAAGCATTCTTCCGATATCTGCATTGTCG
pSEVA63-viogene REV (1)	TAGTATTTCTCCTCTTTCTCTAGTATAGAGGATCCCCGGGTACCGAGCTC
pSEVA63-viogene FWD (2)	GAGCTCGGTACCCGGGGATCCTCTATACTAGAGAAAGAGGAGAAATACTAGATGAG
pSEVA63-viogene REV (2)	CTCTTTTCTGGAATTTGGTACCGAGTAGGCGTATCACGAGGCAGAAAAAA
revBvio(2)	TTTCTCTAGTAGGATCCTTAGGCCTCTCTAGA
fwCvio(2)	TACTAGAGAAAGAGGAGAAATACTAGATGAAAAGAGCAATC

736

737

738