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1 **Indole modifies the central carbon flux in the anaerobic metabolism of *Escherichia***
2 ***coli*: application to the production of hydrogen and other metabolites**

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4 Antonio De Leon-Rodriguez^{1,2*}, Santiago L. Caño¹, Junyan Liu¹, and David K.
5 Summers^{1*}

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7

8 ¹Department of Genetics, University of Cambridge, Downing Street, Cambridge CB2
9 3EH, U.K.

10

11 ²Division of Molecular Biology, IPICYT, Camino a la Presa San Jose 2055, San Luis
12 Potosi, CP 78216, S.L.P. Mexico.

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14

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18 *Corresponding authors:

19 A De Leon Rodriguez

20 E-mail: aleonr@ipicyt.edu.mx, aleonr@me.com, ad81@cam.ac.uk

21 D.K. Summers

22 E-mail: dks11@cam.ac.uk

23

24 **Abstract**

25 Indole is a bicyclic signaling molecule with effects on both eukaryotic and prokaryotic
26 cells. The majority of studies of indole action have been performed with bacteria
27 cultured under aerobic conditions and little information is available about its effects
28 under anaerobic conditions. Here the effect of the indole on anaerobic metabolism of
29 *Escherichia coli* WDHL was studied. Indole in the range 0.5 to 8 mM was added to the
30 culture medium and cell growth, hydrogen and metabolite production were compared to
31 cultures lacking indole. Results showed that while 8 mM indole abolished growth
32 completely, 4 mM indole had a partial bacteriostatic effect and the maximum optical
33 density of the culture decreased by 44% compared to the control cultures. In addition, 4
34 mM indole had an important effect on anaerobic metabolism. Hydrogen production
35 increased from 650 ± 115 to $1,137 \pm 343$ mL H₂/L, and hydrogen yield increased from
36 0.45 ± 0.1 to 0.94 ± 0.34 mol H₂/mol glucose, compared to the control culture. Carbon
37 flux was also affected and the composition of the final by-products changed. Lactate (41
38 mM) was the main metabolite in the control cultures, whereas ethanol (56.2 mM) and
39 acetate (41.2 mM) were the main metabolites in the cultures with 2 mM indole. We
40 conclude that the supplementation of *E. coli* cultures with exogenous indole is a simple
41 and novel strategy to improve the production of hydrogen as well as other metabolites
42 such as ethanol used as biofuels.

43

44 **Keywords:** bacteriostatic, *Escherichia coli*, hydrogen, ethanol, indole, ionophore,
45 lactate.

46

47

48 **Introduction**

49 Under anaerobic conditions *E. coli* naturally produces a mixture of organic acids,
50 ethanol and hydrogen [1] (Fig. 1). Biotechnology exploits this to produce succinate [2]
51 and lactate [3], and recently ethanol and hydrogen as potential biofuels [4-8]. Molecular
52 hydrogen (H₂) is an attractive energy carrier because it has high energy density and is
53 environmentally friendly, since its combustion produces only water. Hydrogen is also
54 an important feedstock for chemical, food, pharmaceutical and some other industries
55 [9]. Among various production processes, fermentative hydrogen production has the
56 advantages of high production rate and simple operation [7-10]. Hydrogen production
57 by dark fermentation is a promising method since it has a higher production rate, it does
58 not need light and it utilizes a wide range of carbon sources such as glucose and other
59 simple substrates [6, 8, 11, 12]. *E. coli* is currently used as a model for production
60 studies of hydrogen, ethanol and other metabolites, since its anaerobic metabolism is
61 well documented and genetic manipulations are possible. Both genetic modifications
62 and modified fermentation strategies have been implemented to improve the production
63 processes [7, 13, 14]. Since the production of metabolites normally correlates with cell
64 growth, an important fraction of the substrate is inevitably channeled toward unwanted
65 biomass rather than the desired product. Hence an attractive strategy to improve
66 metabolite production would be to induce the producer cells to enter a quiescent state,
67 where they are metabolically active but not growing or dividing.

68

69 Quiescence has been defined as a reversible absence of cell proliferation [15]. In
70 contrast to multi-cellular organisms where quiescence is the most common cellular
71 state, bacteria exist mainly in a proliferative state. Quiescent *E. coli* cells have been
72 obtained through the controlled overexpression of a small RNA called Rcd (regulator of

73 cell division), a ColE1-encoded regulatory transcript [16]. To achieve complete
74 cessation of growth it was necessary to over-express Rcd in an *hns205* mutant of *E. coli*.
75 More recently it has been reported that the addition of approx. 3mM indole to the
76 culture supernatant induces a quiescence state in *E. coli hns205* under aerobic
77 conditions [17]. The bacteriostatic effect of indole has been attributed to its action as a
78 proton ionophore, affecting the membrane polarity and respiratory ATP generation [18-
79 20]. Studies of the effect of indole on the physiology of *E. coli* have been performed
80 under aerobic conditions [18, 21]. However to our knowledge, there are no reports on
81 the effect of indole on the anaerobic metabolism of *E. coli*. Thus the goal of this work
82 was to evaluate the effect of exogenous indole on the anaerobic metabolism of *E. coli*
83 with a particular focus on its effect on the production of hydrogen.

84

85 **Materials and methods**

86 *Strain and culture media*

87 *E. coli* WDHL is a hydrogen over-producer strain obtained by the deletion of *hycA* and
88 *lacI* in *E. coli* W3110. A complete description of WDHL can be found elsewhere [11].
89 The strain was stored routinely on Luria Bertani (LB) agar plates at 4°C. The hydrogen
90 production medium (HP) contains per liter: K₂HPO₄ 0.125 g, Na₂HPO₄ 11.86 g,
91 KH₂PO₄ 4.5 g, MgSO₄ 1 g, tryptone (Difco) 1 g, yeast extract (Difco) 1 g, glucose 15 g
92 and 1 mL of trace elements solution [11]. The initial pH of the HP medium was 7.2.

93

94 *Evaluation of the effect of indole on E. coli metabolism*

95 To evaluate the effect of indole on hydrogen production, pre-inocula were grown
96 aerobically overnight in LB medium at 37°C, shaken at 150 rpm. Anaerobic

97 experiments were performed in 120 mL serological bottles containing 110 mL of HP
98 medium. Bottles were closed with 20 mm butyl septum and an aluminum crimp seal
99 (Supelco) using an E-Z crimper (Wheaton). The cultures were started at pH 7.2 with an
100 initial OD₆₀₀ of 0.3 and incubated at 37°C in a water bath shaking at 175 rpm.
101 Concentrations of indole (Sigma) between 0.5 to 8 mM were assessed, for this the
102 required volume of a 2 M indole stock was added to the serological bottles using a
103 sterile syringe. Control cultures were grown in HP medium plus 400 µL of ethanol
104 (ethanol was used as the solvent for the stock indole solution). Samples of 1 mL taken
105 at various times during fermentation were centrifuged at 13,000 rpm and stored at -20°C
106 until analyzed as described below.

107

108 *Analytical methods*

109 Hydrogen produced was measured by liquid displacement using 2 N NaOH in an
110 inverted burette and a gas chromatograph with a thermal conductivity detector (GC-
111 TCD, Agilent Technologies) as described previously [22]. Optical cell density was
112 measured at 600 nm (OD₆₀₀) in a GeneQuant 1300 (G&E) spectrophotometer. Indole
113 was determined by the Kovacs spectrophotometric method at 540 nm using the *p*-
114 dimethyl amino-benzaldehyde reagent [23]. The concentrations of glucose and the
115 metabolites succinate, lactate, acetate, formate and ethanol were analyzed by High
116 Performance Liquid Chromatography coupled to a Refraction Index Detector (HPLC,
117 Infinity LC 1220, Agilent Technologies, Santa Clara CA, USA), with a column
118 Phenomenex Rezex ROA (Phenomenex, Torrance, CA, USA) at 60°C, and using
119 0.0025 M H₂SO₄ as mobile phase at 0.41 mL/min.

120

121 *Statistics.*

122 The data are presented as the mean \pm standard deviation. We used Student's t-test
123 unpaired to compare each treatment with the control group, and p values < 0.05 were
124 considered significant. The experiments were carried out at least in triplicate.

125 **Results**

126 Before investigating the effect of exogenous indole on *E. coli* WDHL, endogenous
127 indole production by this strain was measured in HP medium under anaerobic
128 conditions. Indole was assayed in culture supernatants using the Kovacs method, and
129 was found to be below 0.18 mM. In subsequent experiments, cultures were
130 supplemented with indole in the range 0.5 to 8 mM at the time of inoculation. As shown
131 in Fig. 2, cultures to which 1 mM indole was added behaved indistinguishably from the
132 control cultures with a specific growth rate of 0.35 h^{-1} , whereas the cultures with 2 and
133 4 mM indole showed slightly reduced specific growth rates of 0.29 and 0.26 h^{-1} ,
134 respectively. Cultures with 8 mM indole did not grow.

135 The effect of the indole supplementation on the maximum optical density and hydrogen
136 production by each culture is shown in Fig. 3. The inhibitory effect of indole on the
137 growth of *E. coli* WDHL is evident and the maximum OD_{600} decreased from 3.13 ± 0.54
138 to 0.18 ± 0.04 as external indole was increased from 0 to 8 mM (Fig. 3A). Indole up to 4
139 mM increased hydrogen production ($1,032 \pm 124 \text{ mLH}_2/\text{L}$ at 4 mM, compared to
140 $650 \pm 115 \text{ mLH}_2/\text{L}$ in the control). However, no hydrogen production was observed in
141 the culture with 8 mM indole (Fig. 3B). Both hydrogen production per unit biomass and
142 hydrogen per unit substrate (Figs. 3C and 3D, respectively) showed the same response
143 to indole addition. When 4 mM indole was added, the yields increased from 23.8 ± 7.9 to
144 $66.8 \pm 14.5 \text{ mLH}_2/\text{OD}$ and from 0.45 ± 0.1 to $0.94 \pm 0.3 \text{ mol H}_2/\text{mol glucose}$, respectively.

145 Metabolite analysis revealed that succinate was found in the cultures with 1 mM indole
146 or less, with a maximum concentration of 6.7 ± 0.5 mM in the control culture. Lactate
147 was the main metabolite in the cultures with 1 mM indole and below, reaching a
148 maximum of 57.1 ± 3.3 mM in the cultures with 0.5 mM indole, whereas in the cultures
149 with 2 and 4 mM indole, ethanol and acetate were the main metabolites, reaching
150 56.2 ± 22.7 and 41.2 ± 6.2 mM respectively in the culture supplemented with 2 mM
151 indole. Formate was not detected in any of the cultures (Fig. 4).

152

153 **Discussion**

154 We have explored the effect of indole on the growth and the production of hydrogen
155 and ethanol by *E. coli* in anaerobic culture, and have found that productivity can be
156 increased significantly by the addition of 2 to 4 mM to the growth medium. Previous
157 studies of indole signaling [24-28] and the use of indole to induce entry of *E. coli* into a
158 quiescent state [17] have been carried out under aerobic culture conditions. Indeed, very
159 little information is available on the effect of indole in the anaerobic metabolism of *E.*
160 *coli*. It has been reported that when *E. coli* is grown anaerobically in medium containing
161 tryptophan and sodium nitrate, it produces toxic 3-nitrosoindole and its derivatives, as a
162 result of the condensation of nitrite with indole [24]. However, in the experiments
163 reported here, nitrate or ammonium salts were not used as nitrogen source because they
164 inhibit the hydrogen production [25, 26]. Since only organic nitrogen sources like yeast
165 extract and tryptone were used, nitroindole compounds were not produced.

166 Typical indole concentrations in aerobic stationary phase *E. coli* cultures are 0.5-1 mM
167 [21, 27]. Even our unsupplemented control cultures contained a low concentration of
168 indole (0.18 mM). This was almost certainly generated from tryptophan, originating

169 from the yeast extract and tryptone in the HP medium. It has been reported that in
170 aerobic culture indole production up to 5 mM is a direct reflection of the amount of free
171 tryptophan in the culture medium [28].

172 Indole-induced quiescence in aerobic *E. coli* cultures has been used to improve the
173 expression of heterologous proteins [17]. The authors reported that the addition of 0-3
174 mM indole had little effect on the growth of wild-type *E. coli*, while 4 or 5 mM indole
175 caused significant growth inhibition. Under anaerobic conditions we found that 1-2 mM
176 indole had little effect while 4 mM indole caused a reduction in both the specific growth
177 rate (25% of wild-type) and the maximum optical density (44.4% of wild-type). The
178 growth-inhibited cultures showed an increased yield of both hydrogen and ethanol.

179 In anaerobic glycolysis, *E. coli* accumulates mainly lactate, but succinate, formate,
180 acetate and a low yield of ethanol are also produced. In the case of glucose fermentation
181 by strain WDHL, formate was not detected as result of the *hycA* deletion, indicating that
182 the WDHL strain is very efficient in the conversion of formate into hydrogen and CO₂.
183 The *hycA* gene codes for the negative regulator of the formate regulon, which contains
184 the formate hydrogen lyase complex (FHL), therefore strains with defective *hycA* gene
185 overproduce hydrogen [29]. The theoretical maximum yield of hydrogen is 2 mol
186 H₂/mol glucose, if there are no other cellular requirements and all pyruvate is converted
187 into formate and acetyl-CoA. However, glucose is also needed for building biomass,
188 anabolism and ATP production for maintaining energy. In the case of strain WDHL, a
189 typical value is 0.3 mol H₂/mol glucose due to catabolic repression by glucose and the
190 conversion of pyruvate into lactate by lactate dehydrogenase (LDH) to recycle the
191 NADH cofactor [30]. However, here we attained up to 0.94±0.3 mol H₂/mol glucose in
192 the cultures with 4 mM indole. Succinate was a minor by-product with 6.7±0.5 mM in
193 the control cultures. Succinate is generated by the fumarate reductase (FRD), an enzyme

194 with high similarity to succinate dehydrogenase, but FRD uses FADH instead of
195 NADH. FRD also has important differences in both regulation and catalytic activity
196 [31]. As expected, lactate was the main by-product in the control culture and at lower
197 indole concentrations (e.g. 57.1 ± 3.3 mM in the cultures supplemented with 0.5 mM
198 indole) because the recycling of NADH by the LDH is the main route to continue the
199 assimilation of glucose [32]. In the cultures supplemented with 2 or 4 mM indole, the
200 hydrogen yield increased to 0.94 ± 0.34 mol H₂/mol glucose, which strongly suggests
201 that a greater proportion of pyruvate was transformed by the pyruvate formate lyase
202 (PFL). This is consistent with the accumulation of C₂ by-products (ethanol and acetate)
203 instead C₃ by-products (lactate) as shown in Fig. 4. Consistent with the results reported
204 here, Chen *et al.* [17] observed that at higher indole concentrations (up to 2.5 mM in
205 aerobic cultures of *E. coli*), the specific yield of GFP increased throughout their
206 experiment. The maximum value (three-fold greater than the no-indole control) was
207 obtained for the culture that entered quiescence in response to treatment with 2.5 mM
208 indole.

209 Acetate is typically generated as by-product in the anaerobic metabolism of *E. coli*. It
210 can be produced from acetyl CoA by acetyl CoA synthetase (ACS) as well by the
211 phosphotransacetylase followed by acetate kinase A. Acetate can be also produced by
212 pyruvate oxidase, and a typical inhibitory concentration for *E. coli* is 5 g/L (83mM)
213 [13]. However, inhibitory acetate concentrations were not reached in in this study where
214 the maximum concentration was 41.2 ± 6.2 mM. Ethanol is produced in *E. coli* by the
215 alcohol dehydrogenase (ADH). In the control cultures ethanol production was 6.5 mM
216 with a yield of 0.11 mol ethanol/mol glucose. Ethanol accumulation increased
217 substantially in the cultures treated with indole even though WDHL is not an
218 alcohologenic strain. In cultures supplemented with 2 mM indole, ethanol production

219 increased approximately ten-fold (56.2 mM with a yield of 1.02 mol ethanol/mol
220 glucose). Thus indole supplementation could be a useful novel strategy to increase
221 ethanol production in processes designed for this purpose [33-35].

222

223 **Conclusions**

224 Our results indicate that indole has a bacteriostatic activity on *E. coli* WDHL in
225 anaerobic culture, as well as a substantial effect on hydrogen production due to
226 increased pyruvate flux through PFL rather than LDH. The bacteriostatic effect and
227 metabolic switching are evident at 2 mM indole, however, the mechanisms
228 underpinning this metabolic change are not clear. They could be related to changes in
229 H⁺ flux through the cytoplasmic membrane mediated by the proton ionophore effect of
230 indole [19] or to regulatory changes in the central carbon metabolism [1, 36]. Whatever
231 the mechanism we conclude that addition of exogenous indole into the anaerobic *E. coli*
232 cultures can be used as a strategy to improve the production of hydrogen and other
233 metabolites such as ethanol.

234

235 **List of abbreviations**

236	ACS	Acetyl CoA synthetase
237	hycA	Negative transcriptional regulator of the formate regulon
238	lacI	Lac I repressor
239	LDH	Lactate dehydrogenase
240	NADH	Nicotinamide adenine dinucleotide reduced

241 FADH₂ Flavin adenine dinucleotide reduced

242 FRD Fumarate reductase

243 PFL Pyruvate formate lyase

244 TnaA Tryptophanase

245

246 **Competing interests**

247 No competing financial interests exist.

248

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252

253 **Authors' contributions**

254 All authors contributed in the development of this manuscript.

255

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259

260 **References**

- 261 1. Kang YS, Weber KD, Yu Q, Kiley PJ, Blattner FR: **Genome-wide expression**
262 **analysis indicates that FNR of Escherichia coli K-12 regulates a large**
263 **number of genes of unknown function.** *Journal of Bacteriology* 2005,
264 **187:1135-1160.**
- 265 2. Thakker C, Martinez I, San KY, Bennett GN: **Succinate production in**
266 **Escherichia coli.** *Biotechnol J* 2012, **7:213-224.**
- 267 3. Niu DD, Tian KM, Prior BA, Wang M, Wang ZX, Lu FP, Singh S: **Highly**
268 **efficient L-lactate production using engineered Escherichia coli with**
269 **dissimilar temperature optima for L-lactate formation and cell growth.**
270 *Microbial Cell Factories* 2014, **13.**
- 271 4. Valle A, Cabrera G, Cantero D, Bolivar J: **Identification of enhanced**
272 **hydrogen and ethanol Escherichia coli producer strains in a glycerol-based**
273 **medium by screening in single-knock out mutant collections.** *Microb Cell*
274 *Fact* 2015, **14:93.**
- 275 5. Maeda T, Sanchez-Torres V, Wood TK: **Hydrogen production by**
276 **recombinant Escherichia coli strains.** *Microb Biotechnol* 2012, **5:214-225.**
- 277 6. Rosales-Colunga LM, Alvarado-Cuevas ZD, Razo-Flores E, Rodriguez AD:
278 **Maximizing Hydrogen Production and Substrate Consumption by**
279 **Escherichia coli WDHL in Cheese Whey Fermentation.** *Applied*
280 *Biochemistry and Biotechnology* 2013, **171:704-715.**
- 281 7. Rosales-Colunga LM, Rodriguez AD: **Escherichia coli and its application to**
282 **biohydrogen production.** *Reviews in Environmental Science and Bio-*
283 *Technology* 2015, **14:123-135.**

- 284 8. Trchounian K, Trchounian A: **Hydrogen production from glycerol by**
285 **Escherichia coli and other bacteria: An overview and perspectives.** *Applied*
286 *Energy* 2015, **156**:174-184.
- 287 9. Ramachandran R, Menon RK: **An overview of industrial uses of hydrogen.**
288 *International Journal of Hydrogen Energy* 1998, **23**:593-598.
- 289 10. Wang JL, Wan W: **Factors influencing fermentative hydrogen production: A**
290 **review.** *International Journal of Hydrogen Energy* 2009, **34**:799-811.
- 291 11. Rosales-Colunga LM, Razo-Flores E, Ordonez LG, Alatraste-Mondragon F, De
292 Leon-Rodriguez A: **Hydrogen production by Escherichia coli Delta hycA**
293 **Delta lacI using cheese whey as substrate.** *International Journal of Hydrogen*
294 *Energy* 2010, **35**:491-499.
- 295 12. Rosales-Colunga LM, Razo-Flores E, Rodriguez ADL: **Fermentation of lactose**
296 **and its constituent sugars by Escherichia coli WDHL: Impact on hydrogen**
297 **production.** *Bioresource Technology* 2012, **111**:180-184.
- 298 13. Fernandez-Sandoval MT, Huerta-Beristain G, Trujillo-Martinez B, Bustos P,
299 Gonzalez V, Bolivar F, Gosset G, Martinez A: **Laboratory metabolic**
300 **evolution improves acetate tolerance and growth on acetate of**
301 **ethanologenic Escherichia coli under non-aerated conditions in glucose-**
302 **mineral medium.** *Applied Microbiology and Biotechnology* 2012, **96**:1291-
303 1300.
- 304 14. Loaces I, Amarelle V, Munoz-Gutierrez I, Fabiano E, Martinez A, Noya F:
305 **Improved ethanol production from biomass by a rumen metagenomic DNA**
306 **fragment expressed in Escherichia coli MS04 during fermentation.** *Applied*
307 *Microbiology and Biotechnology* 2015, **99**:9049-9060.

- 308 15. Daignan-Fornier B, Sagot I: **Proliferation/quiescence: the controversial**
309 **"aller-retour"**. *Cell Div* 2011, **6**:10.
- 310 16. Rowe DC, Summers DK: **The quiescent-cell expression system for protein**
311 **synthesis in Escherichia coli**. *Appl Environ Microbiol* 1999, **65**:2710-2715.
- 312 17. Chen CC, Walia R, Mukherjee KJ, Mahalik S, Summers DK: **Indole generates**
313 **quiescent and metabolically active Escherichia coli cultures**. *Biotechnol J*
314 2015, **10**:636-646.
- 315 18. Han TH, Lee JH, Cho MH, Wood TK, Lee J: **Environmental factors affecting**
316 **indole production in Escherichia coli**. *Research in Microbiology* 2011,
317 **162**:108-116.
- 318 19. Chimere C, Field CM, Pinero-Fernandez S, Keyser UF, Summers DK: **Indole**
319 **prevents Escherichia coli cell division by modulating membrane potential**.
320 *Biochimica Et Biophysica Acta-Biomembranes* 2012, **1818**:1590-1594.
- 321 20. Chimere C, Murray AJ, Oldewurtel ER, Summers DK, Keyser UF: **The effect**
322 **of bacterial signal indole on the electrical properties of lipid membranes**.
323 *Chemphyschem* 2013, **14**:417-423.
- 324 21. Gaimster H, Cama J, Hernandez-Ainsa S, Keyser UF, Summers DK: **The indole**
325 **pulse: a new perspective on indole signalling in Escherichia coli**. *PLoS One*
326 2014, **9**:e93168.
- 327 22. Davila-Vazquez G, Alatraste-Mondragon F, de Leon-Rodriguez A, Razo-Flores
328 E: **Fermentative hydrogen production in batch experiments using lactose,**
329 **cheese whey and glucose: influence of initial substrate concentration and**
330 **pH**. *International Journal of Hydrogen Energy* 2008, **33**:4989-4997.
- 331 23. Kovacs N: **A Micro Method for Detecting Indol Formation**. *Journal of*
332 *Clinical Pathology* 1959, **12**:90-90.

- 333 24. Kwon YM, Weiss B: **Production of 3-nitrosoindole derivatives by**
334 **Escherichia coli during anaerobic growth.** *J Bacteriol* 2009, **191**:5369-5376.
- 335 25. Alvarado-Cuevas ZD, Acevedo LGO, Salas JTO, De Leon-Rodriguez A:
336 **Nitrogen sources impact hydrogen production by Escherichia coli using**
337 **cheese whey as substrate.** *New Biotechnology* 2013, **30**:585-590.
- 338 26. Ferchichi M, Crabbe E, Hintz W, Gil GH, Almadidy A: **Influence of culture**
339 **parameters on biological hydrogen production by Clostridium**
340 **saccharoperbutylaceticum ATCC 27021.** *World Journal of Microbiology &*
341 *Biotechnology* 2005, **21**:855-862.
- 342 27. Gaimster H, Summers D: **Regulation of Indole Signalling during the**
343 **Transition of E. coli from Exponential to Stationary Phase.** *Plos One* 2015,
344 **10.**
- 345 28. Li G, Young KD: **Indole production by the tryptophanase TnaA in**
346 **Escherichia coli is determined by the amount of exogenous tryptophan.**
347 *Microbiology-Sgm* 2013, **159**:402-410.
- 348 29. Yoshida A, Nishimura T, Kawaguchi H, Inui M, Yukawa H: **Enhanced**
349 **hydrogen production from formic acid by formate hydrogen lyase-**
350 **overexpressing Escherichia coli strains (vol 71, pg 6765, 2005).** *Applied and*
351 *Environmental Microbiology* 2006, **72**:1716-1716.
- 352 30. Rosales-Colunga LM, Razo-Flores E, De Leon Rodriguez A: **Fermentation of**
353 **lactose and its constituent sugars by Escherichia coli WDHL: impact on**
354 **hydrogen production.** *Bioresour Technol* 2012, **111**:180-184.
- 355 31. Cecchini G, Schroder I, Gunsalus RP, Maklashina E: **Succinate dehydrogenase**
356 **and fumarate reductase from Escherichia coli.** *Biochimica Et Biophysica*
357 *Acta-Bioenergetics* 2002, **1553**:140-157.

- 358 32. Jiang GRJ, Nikolova S, Clark DP: **Regulation of the ldhA gene, encoding the**
359 **fermentative lactate dehydrogenase of Escherichia coli.** *Microbiology-Sgm*
360 2001, **147**:2437-2446.
- 361 33. Ingram LO, Conway T, Clark DP, Sewell GW, Preston JF: **Genetic-**
362 **Engineering of Ethanol-Production in Escherichia-Coli.** *Applied and*
363 *Environmental Microbiology* 1987, **53**:2420-2425.
- 364 34. Ohta K, Beall DS, Mejia JP, Shanmugam KT, Ingram LO: **Genetic-**
365 **Improvement of Escherichia-Coli for Ethanol-Production - Chromosomal**
366 **Integration of Zymomonas-Mobilis Genes Encoding Pyruvate**
367 **Decarboxylase and Alcohol Dehydrogenase-Ii.** *Applied and Environmental*
368 *Microbiology* 1991, **57**:893-900.
- 369 35. Seol E, Sekar BS, Raj SM, Park S: **Co-production of hydrogen and ethanol**
370 **from glucose by modification of glycolytic pathways in Escherichia coli -**
371 **from Embden-Meyerhof-Parnas pathway to pentose phosphate pathway.**
372 *Biotechnology Journal* 2016, **11**:249-256.
- 373 36. Hardiman T, Lemuth K, Keller MA, Reuss M, Siemann-Herzberg M: **Topology**
374 **of the global regulatory network of carbon limitation in Escherichia coli.**
375 *Journal of Biotechnology* 2007, **132**:359-374.

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379

380 **Figure Caption**

381 Fig. 1. Carbon flux in *E. coli* during anaerobic metabolism. The key enzymes to
382 produce hydrogen are shown in bold. FRD: Fumarate reductase, LDH: Lactate
383 dehydrogenase, ADH: Alcohol dehydrogenase, PFL: Pyruvate formate lyase, FHL:
384 Formate hydrogen lyase, ACS: Acetyl CoA synthetase.

385 Fig. 2. The growth kinetics of cultures of *E. coli* WDHL exposed to indole (0-8 mM)..
386 Data are plotted as the average (n=3) and the bars correspond to the standard deviation.

387 Fig. 3. Effect of the indole concentration on the cell-growth and hydrogen production
388 measured at 48h of culture. Maximum OD₆₀₀ (A), hydrogen production (B), hydrogen
389 yield by biomass (C) and hydrogen yield by substrate (D). Control corresponds to the
390 cultures without addition of indole. Data are presented as mean ± SD. * indicates
391 significant difference compared with the control cultures ($p < 0.05$).

392 Fig. 4. Final concentration of the metabolites in the cultures supplemented with
393 different initial concentration of indole measured at 48h of culture. Control corresponds
394 to the cultures without addition of indole. Data are presented as mean ± SD of three
395 independent experiments (n=3). * indicates significant difference compared with the
396 control cultures ($p < 0.05$).

397