

27 **Abstract**

28

29 The CreBC (carbon source responsive) two-component regulation system of *Escherichia coli*
30 affects a number of functions, including intermediary carbon catabolism. The impact of different
31 *creC* mutations (a $\Delta creC$ mutant and a mutant carrying the constitutive *creC510* allele) on
32 bacterial physiology was analyzed in glucose cultures under three oxygen availability conditions.
33 Differences in the amounts of extracellular metabolites produced were observed in the null
34 mutant compared to the wild-type strain and the mutant carrying *creC510*, and shown to be
35 affected by oxygen availability. The $\Delta creC$ strain secreted more formate, succinate, and acetate,
36 but less lactate in low aeration. These metabolic changes were associated to differences in AckA
37 and LdhA activities, both of which were affected by CreC. Measurement of the
38 NAD(P)H/NAD(P)⁺ ratios showed that the *creC510* strain had a more reduced intracellular
39 redox state, while the opposite was observed for the $\Delta creC$ mutant, particularly at intermediate
40 oxygen availability conditions, indicating that CreC affects redox balance. The null mutant
41 formed more succinate than the wild-type strain in both low aeration and no aeration. Over-
42 expression of the genes encoding phosphoenolpyruvate carboxylase from *E. coli* and a NADH-
43 forming formate dehydrogenase from *Candida boidinii* in the $\Delta creC$ mutant further increased the
44 yield of succinate on glucose. Interestingly, the elimination of *ackA* and *adhE* did not improve
45 significantly the production of succinate. The diverse metabolic effects of this regulator on the
46 central biochemical network of *E. coli* make it a good candidate for metabolic engineering
47 manipulations to enhance the formation of bioproducts such as succinate.

48

49 Introduction

50

51 The survival of an organism depends, at least in part, on its ability to sense and respond to
52 changes in the environment. In bacteria, global regulators control the transcription of genes in
53 response to specific external stimuli and metabolic signals, finely tuning different aspects of their
54 physiology to overcome environmental challenges. In *Escherichia coli*, seven global regulators
55 (ArcA, Crp, Fis, Fnr, Ihf, Lrp, and NarL) directly modulate the expression of about one-half of
56 all genes (1). This facultative aerobe is able to adapt its metabolism to different oxygen
57 availability conditions through the concerted action of a network of regulators, including the
58 global regulators ArcAB and Fnr (2-4). These regulators affect many metabolic pathways,
59 allowing the cells to reach an adequate redox balance in any given condition. There is a very
60 close association between carbon and electron flows, and even small differences in oxygen
61 availability have been observed to elicit profound effects in the distribution of carbon fluxes (5).

62

63 CreBC (for *carbon source responsive*) is a global sensing and regulation system affecting genes
64 involved in a variety of functions, including enzymes of the intermediary catabolism (6).
65 Previous studies have shown that the *creABCD* operon is activated during growth in minimal
66 media when (i) glycolytic carbon sources are being fermented or (ii) during aerobic growth when
67 low-molecular-weight fermentation products are used as carbon sources (6). While *creB* and
68 *creC* encode the two component system (i.e., a cytoplasmic response regulator and a membrane-
69 associated sensor kinase, respectively), *creA* is a hypothetical open reading frame and *creD*
70 encodes an inner-membrane protein of unknown function (7). CreC, originally designated PhoM,
71 was first described as a phosphate donor for the PhoB protein, a response regulator that controls
72 the expression of the *pho* regulon. This regulon includes genes involved in cytoplasmic inorganic
73 phosphate homeostasis, such as *phoA*, encoding an alkaline phosphatase, and is controlled by
74 PhoBR (8, 9). PhoR autophosphorylates when the concentration of inorganic phosphate falls
75 below a critical threshold. In *phoR* null mutants, activation of the *pho* regulon depends on CreC
76 (10).

77 The genes that are known to be under the control of CreBC (i.e., the *cre* regulon) are (i) the
78 *ackA/pta* operon, the products of which catalyze the conversion of acetyl-coenzyme A (CoA)
79 into acetate and ATP, (ii) *talA*, that encodes one of the two transaldolases of the pentose
80 phosphate pathway, (iii) *radC*, that encodes a RecG-like DNA recombination/repair function,
81 (iv) *malE*, the first gene in the *malEFG* maltose transporter operon, (v) *trgB*, that encodes an
82 ADP-ribose pyrophosphorylase; and (vi) three other genes (*creD*, *cbrA*, and *cbrB*), potentially
83 related to resistance to colicin and other antimicrobials, that have not yet been assigned a specific
84 function (6, 7, 11). A direct repeat consensus DNA sequence, termed *cre* tag (5'-
85 TCAC n TTTTTTCAC-3', where n represents any nucleotide), was defined based on the analysis
86 of the region upstream from the genes known to form the *cre* regulon, and observed to be
87 required for the control of gene expression *in vivo* (7). Genome-wide expression profiling with
88 DNA microarrays has revealed that CreBC also affects the expression of *cbrC*, responsible for
89 colicine E2 resistance; *mokB*, an overlapping regulatory peptide which enables *hokB* expression;
90 and the uncharacterized genes *mppA*, *ynaI*, *yafU*, and *yafE* (12).

91
92 The capability of global regulators to affect multiple metabolic pathways makes them useful
93 tools for metabolic engineering, as they can be used to change the flow of carbon and reducing
94 power simultaneously. This strategy has been used to manipulate bacterial metabolism to
95 enhance the synthesis of different bioproducts, specially reduced metabolites (13). On the other
96 hand, concern about the costs of energy used for aeration in bioprocesses has renewed attention
97 on the regulation of aerobic and anaerobic bacterial metabolism, as a means to achieve the
98 sustainable synthesis of a variety of bioproducts under these conditions (14). Among the
99 different regulatory systems of *E. coli*, ArcAB has attracted significant attention in the last years,
100 as manipulations in this sensor/regulator pair offer the possibility of directing carbon flow
101 towards the synthesis of reduced bioproducts in low aeration conditions (15-17). In our
102 laboratory we have analyzed the effect of *arcA* mutants on the central carbon catabolism of *E.*
103 *coli* using glucose and glycerol in microaerobiosis and anaerobiosis, and observed that mutations
104 in *arcA* resulted in significant increases in the synthesis of polyhydroxyalkanoates (18-21) and
105 ethanol (22, 23). In these studies, the constitutive allele *creC510* was observed to affect the

106 intracellular redox state, enhancing carbon catabolism in an *arcA* genetic background so that part
107 of the excess reducing power generated by the *arcA* mutants was consumed by the augmented
108 amount of carbon intermediates due to *creC510*, further increasing the synthesis of reduced
109 products (18).

110

111 The results obtained with the *creC510 arcA* double mutants suggested that the CreBC system
112 could also be an interesting target for metabolic manipulations in *E. coli*, and prompted us to
113 further investigate its metabolic effects. The aim of this work was to evaluate the potential of
114 CreC as a new tool for the design of bacterial strains suitable for the synthesis of different
115 bioproducts. For this purpose, we characterized the effects of this regulator on central carbon
116 metabolism, analyzing physiological traits, carbon flow, and redox balance, focusing on low
117 oxygen availability conditions in the presence of excess carbon source. Succinate was selected as
118 a model metabolite, and several metabolic manipulations were implemented in a $\Delta creC$ mutant
119 to evaluate the potential of this genetic background for the synthesis of this carboxylic acid.

120

121 **Materials and methods**

122

123 **Bacterial strains, mutant construction, plasmids and oligonucleotides.** All *E. coli* strains
124 were derivatives of K1060, a K-12 strain, and are listed in Table 1, along with the plasmids used
125 in this study. All null mutants reported in this work were constructed by allelic replacement (24).
126 Briefly, a kanamycin resistance cassette (FRT-*kan*-FRT) was amplified by PCR from template
127 plasmid pKD4 with the corresponding primers (Table 1). The purified PCR product was
128 electroporated into *E. coli* K1060 carrying pKD46 (a helper plasmid that expresses the λ -Red
129 functions). Insertion of the FRT-*kan*-FRT cassette into the correct locus was confirmed by
130 colony PCR of kanamycin resistant recombinants. For the construction of mutant K1060C,
131 bearing the constitutive *creC510* allele, a previous step was needed. *E. coli* strain 58-161, F-,SR
132 (*creC510*) carrying pKD46 was transformed with the FRT-*kan*-FRT cassette, in order to add a
133 selection marker near the *creABCD* operon in the intergenic region limited by the convergent
134 open reading frames *yjjX* and *trpR*, located 2.5 kb upstream from *creABCD*. The resulting

135 mutant, *E. coli* 58KF, was used as the donor strain in P1 transduction, in which K1060 was the
136 recipient strain. Kanamycin resistant clones were selected and the presence of the *creC510* allele
137 was confirmed by PCR and DNA sequencing. The *kan* cassette was removed in all the strains by
138 transforming them with the thermosensitive plasmid pCP20 (25), encoding the *Saccharomyces*
139 *cerevisiae* FLP recombinase. The excision of *kan*, as well as gene deletion, was confirmed by
140 PCR. Plasmid pCP20 was removed by two consecutive passages at 42°C. Inactivation of *ackA*
141 in *E. coli* CEA1060 ($\Delta creC \Delta adhE \Delta ackA$) was further corroborated by determining the amount
142 of acetate present in the supernatant of cultures grown in M9 minimal medium supplemented
143 with glucose at 30 g liter⁻¹ as described below (the CEA1060 mutant produced 50% less acetate
144 than its parental strain CE1060).

145

146 **Growth media and culture conditions.** The medium used for shaken-flask experiments was M9
147 minimal medium containing (per liter of deionized H₂O): 6.0 g of Na₂HPO₄, 3.0 g of KH₂PO₄,
148 0.5 g of NaCl, 1.0 g of NH₄Cl, 0.4 g of MgSO₄, 0.01 g of CaCl₂, and 0.06 g of ammonium
149 iron(III) citrate. MgSO₄, CaCl₂, and ammonium iron(III) citrate were added to the medium after
150 autoclaving and cooling. Glucose was used at 30 g liter⁻¹ as the sole carbon source in all
151 experiments. The aeration level was adjusted by the combination of rotational agitation and by
152 the relation between the volume of culture medium (V_m) and the volume of the flask (V_f) as
153 follows: for high aeration, 250 rpm and $V_m/V_f = 1/10$; for low aeration, 125 rpm and $V_m/V_f = 1/2$.
154 For high aeration 250 ml erlenmeyer flasks were used, while 10 or 50 ml cylindric bottles
155 (penicillin bottles) with cotton plugs were used for low aeration. Non-aerated cultures were
156 implemented in sealed tubes filled with culture medium (10 ml) and agitated at 4 rpm to keep
157 cells in suspension. Working cultures were inoculated from overnight pre-cultures (started from
158 single colonies grown overnight on LB agar), in the same culture media and growth conditions to
159 be used in the experiment (i.e., with the same V_m/V_f ratio). Non-aerated pre-cultures were
160 inoculated with a low aerated pre-preculture to ensure adaptation of the cells to this condition.
161 Isopropil- β -D-1-thiogalactopyranoside (IPTG, 0.1 or 1 mM), NaHCO₃ (100 mM) and antibiotics
162 (kanamycin and ampicillin at 50 and 100 μ g ml⁻¹, respectively) were added whenever needed.

163 **Analytical determinations.** The cell dry weight (CDW) was determined in cell pellets of 10-ml
164 culture samples that were centrifuged for 10 min at 4°C and 10,000×g. The supernatant was
165 separately stored for metabolite analysis. The cells were washed twice with the same volume of
166 150 mM NaCl, and finally dried in an oven at 65°C until constant weight. The supernatant was
167 filtered through a 0.22-µm pore-size syringe filter (Chrom Tech Inc., Apple Valley, MN) and
168 stored at 4°C for high-pressure liquid chromatography analysis (LC-20AT Prominence;
169 Shimadzu Corp., Kyoto, Japan), equipped with an Aminex column HPX-87-H (Cat no. 125-
170 0140; Bio Rad Laboratories Inc., Hercules, CA) at 50°C. An UV detector (SPD-20AV; Shimadzu
171 Corp.) set to 215 nm was used for the quantification of organic acids. The mobile phase
172 consisted of 5 mM H₂SO₄, run at a flow rate of 0.6 ml min⁻¹. Peaks were identified by their
173 characteristic retention times against a set of standards of known organic acids (Sigma-Aldrich
174 Co., St. Louis, MO). Ethanol concentration was measured by using an enzymatic kit based on
175 alcohol dehydrogenase (Sigma-Aldrich Co.). Glucose was measured in supernatants with the
176 glucose oxidase-peroxidase method utilizing a commercial kit (Wiener Laboratorios SAIC,
177 Rosario, Argentina).

178

179 The NADH/NAD⁺ and NADPH/NADP⁺ ratios were obtained from the content of each
180 nucleotide, quantified in the pellet fraction of 1-ml culture samples. Samples were transferred to
181 pre-cooled plastic tubes and the metabolic activity was quenched by immersion of the tubes in
182 liquid N₂. Thawed samples were treated with 300 µl of either 0.2 M HCl [NAD(P)H extraction]
183 or 0.2 M NaOH [NAD(P)⁺ extraction]. Acid/alkaline extraction was carried out at 50°C for 10
184 min, and samples were rapidly placed on ice to cool them at 0°C afterwards. Suspensions were
185 neutralized by dropwise addition of 1 M HCl or NaOH, and cellular debris was removed by
186 centrifuging at 14,000×g for 5 min. Supernatants were then transferred to new tubes and
187 immediately used for cofactor measurements. Nucleotide determination was performed as
188 described by Bernofsky and Swan (26), using 3-(4,5-dimethylthiazol-2-yl)-2,5-
189 diphenyltetrazolium bromide as the final electron acceptor, as modified by Nikel et al. (22, 27,
190 28). The dinucleotide content was normalized to the CDW as indicated by Nikel and Chavarría
191 (29). All reagents were purchased from Sigma-Aldrich Co.

192

193 ***In vitro* enzyme activity measurements.** The acetate kinase (AckA) assay is based on the
194 formation of acetyl-hydroxamate (30, 31). The assay mixture (1 ml final volume) consisted of 50
195 mM Tris-HCl (pH = 7.4), 10 mM MgCl₂, 10 mM ATP, 800 mM CH₃CO₂K, and 700 mM of
196 freshly neutralized NH₂OH. Working solutions of neutralized NH₂OH were freshly prepared by
197 dropwise addition of 3.5 M KOH to an equal volume of 4.0 M NH₂OH·HCl. The cell-free
198 extract was added to the reaction mixture, and incubated for 5 min at room temperature, after
199 which 1 ml of 10% (w/v) trichloroacetic acid was added, immediately followed by 1 ml of
200 freshly-prepared 1.25% (w/v) FeCl₃ in 1 N HCl. After another 5-min incubation period at room
201 temperature, the mixture was centrifuged at maximal velocity during 1 min, and the absorbance
202 the supernatant was read of at 540 nm. For D-lactate dehydrogenase (LdhA), the activity was
203 assayed by measuring the pyruvate-dependent reduction of NADH (32). The assay mixture
204 consisted of 50 mM sodium phosphate buffer (pH = 7.5), 25 mM pyruvate, and 7.5 mM NADH.
205 The cell-free extract was added to the reaction mixture to initiate the assay, and the rate of
206 change in the absorption at 340 nm was recorded at 30°C using a microtiter plate reader.
207 Calibration curves were performed using lithium-potassium acetyl-phosphate and NADH for
208 AckA and LdhA, respectively. One unit of AckA or LdhA activity was defined as the quantity of
209 enzyme that catalyzes the formation of 1 μmol product in 1 min at 30°C. All reagents were
210 purchased from Sigma-Aldrich Co.

211

212 **Statistical analysis.** The reported experiments were independently repeated at least twice (as
213 indicated in the corresponding figure legend or table), and the mean value of the corresponding
214 parameter ± standard deviation is presented. The statistical significance between multiple
215 comparisons was obtained by a two-tailed Student's *t* test. Data were considered statistically
216 significant when $P < 0.05$.

217

218 **Results**

219

220 **CreC affects growth and the fermentation profile of *E. coli* under low oxygen availability**

221 **conditions.** Previous reports suggested that the CreC dependent regulation is affected by
222 aeration. To analyze this effect further, an *E. coli* strain carrying the wild-type *creC* (K1060), a
223 *creC* deletion derivative (DC1060), and another carrying the constitutive *creC510* allele
224 (K1060C) (Table 1), were grown in three levels of aeration (high, low, and no aeration). Growth
225 and production of different metabolites were determined to characterize the metabolic responses
226 of each strain to oxygen availability in M9 minimal medium cultures supplemented with 30 g
227 liter⁻¹ glucose.

228

229 Clear differences were observed in growth and in metabolite secretion, mainly between *E. coli*
230 DC1060 and the other two strains, both of which showed a similar behavior in the three culture
231 conditions (Table 2). Biomass formation at 24 h was similar in high and low aeration for all
232 strains, but surprisingly strain DC1060 grew twice as much as the other strains with no aeration
233 (see Fig. S1 in the Supplemental material). Metabolite distribution in highly aerated cultures was
234 similar for all strains, with acetate as the main secreted product. In low aeration, *E. coli* K1060
235 and K1060C showed similar trends, while strain DC1060 secreted more formate (2-fold),
236 succinate (13-fold), and acetate (1.5-fold), but approximately half the lactate than the other
237 strains ($P < 0.05$). Differences in succinate production in non-aerated cultures were even more
238 marked, as the null mutant produced 36% more of this metabolite than in low aeration, while the
239 other strains (K1060 and K1060C) had very low accumulation levels (Table 2). Succinate
240 formation in non-aerated cultures of the $\Delta creC$ strain was around 50-fold higher than in cultures
241 of *E. coli* K1060 grown in these conditions (9.4 *versus* 0.2 mmol g_{CDW}⁻¹). Interestingly, and in
242 contrast to what was observed in low aeration, in 24 h non-aerated cultures the null mutant
243 accumulated more lactate (+60%), and less acetate (-60%) than the other two strains, while all
244 strains accumulated similar amounts of formate (Table 2). While all strains produced higher
245 amounts of ethanol when no aeration was supplied than in the other growth conditions, K1060C

246 was the strain that accumulated the highest levels, followed by wild-type *E. coli* K1060, and
247 strain DC1060 had the lowest ethanol formation.

248

249 **The effect of CreC over the fermentation profile and enzyme activities is mediated by its**
250 **partner response regulator CreB.** As CreC is the sensor protein of a two-component signal
251 transduction system (the partner response regulator is encoded by *creB*), the possibility that the
252 changes observed could be due to cross talk with other regulators was considered. To investigate
253 this possibility, the fermentation profile of a $\Delta creB$ derivative of strain K1060 (*E. coli* DB1060)
254 was analyzed in low aeration and no aeration, conditions in which the most significant
255 differences between *E. coli* DC1060 and the parental strain had been observed. The fermentation
256 profile was also analyzed in a double $\Delta creB \Delta creC$ mutant (*E. coli* DBC1060).

257

258 While in low aeration the final biomass was the same for all the strains, 24 h non-aerated cultures
259 of the three null mutants produced 2-fold more biomass than the wild-type strain in this
260 condition (Table 2). When comparing the specific production of organic acids in both low
261 aeration and no aeration, all the deletion mutant strains produced comparable amounts of all
262 metabolites, with similar differences when compared to the wild-type strain. The same metabolic
263 profile was obtained in strains in which either component or both were inactivated, clearly
264 indicating that the effects observed are due to the regulation exerted by the two component
265 system CreBC.

266

267 **CreC influences the NADH/NAD⁺ ratio.** When no aeration was supplied, ethanol levels were
268 lower in cultures of the $\Delta creC$ strain, while the *creC510* mutant accumulated the highest
269 amounts. These results suggested that CreC affects the redox state of the cells, as the formation
270 of this metabolite is associated to a high availability of reducing equivalents. To have an accurate
271 measurement of the intracellular redox state of each strain, the levels of cofactors
272 NAD(P)H/NAD(P)⁺ were quantified *in vitro*, and their ratios determined.

273

274 While in high aeration the NADPH/NADP⁺ ratios were higher than the NADH/NAD⁺ ratios for
275 all strains, the opposite was observed in non-aerated cultures. The ratios of both types of
276 cofactors were similar in low aeration, except for the null mutant, in which this relationship was
277 approximately the same in high and low aeration. Interestingly, the NADPH/NADP⁺ ratios were
278 observed to vary on a much wider range under the conditions tested, while variations in
279 NADH/NAD⁺ were more discreet. However, when the values obtained for each of the strains
280 were compared, no significant variations were detected among the NADPH/NADP⁺ ratios, while
281 differences in the NADH/NAD⁺ ratio were observed in the three aeration conditions. In all cases,
282 the highest NADH/NAD⁺ ratio was observed for the mutant carrying the constitutive allele
283 *creC510* (*E. coli* K1060C), and in low aeration the deletion mutant (*E. coli* DC1060) had the
284 lowest redox ratio values (Fig. 1). In high aeration, a condition in which CreC is believed to be
285 inactive, the NADH/NAD⁺ ratios differed slightly, and the highest value was observed for *E. coli*
286 K1060C that has the constitutively active regulator. In low aeration, in which CreC is expected
287 to be active, the differences were more conspicuous (Fig. 1). In this condition *E. coli* DC1060
288 showed clear differences with both the wild-type strain and the *creC510* mutant, with a
289 NADH/NAD⁺ ratio 47% lower than the value for *E. coli* K1060, and 66% lower than that of *E.*
290 *coli* K1060C. These results indicate that, under oxygen limitation, CreC affects the availability of
291 redox cofactors, promoting a more reduced intracellular environment. When no aeration was
292 supplied, the wild-type strain and the null mutant behaved similarly, but strain K1060C showed a
293 significantly higher NADH/NAD⁺ ratio compared to the two other *E. coli* strains ($P < 0.05$).

294

295 **AckA and LdhA activities are subjected to CreC regulation.** The metabolic profiles of the
296 strains showed differences in metabolite distribution, affecting several organic acids, mainly
297 succinate, formate, lactate, and acetate. CreC has been reported to activate the *pta-ackA* operon
298 (7), so the increase in acetate observed in the absence of CreC led us to investigate whether the
299 variations on acetate synthesis could be attributed to differences in AckA (acetate kinase)
300 activity. On the other hand, variations in the amount of lactate could be attributed to metabolic
301 regulation or to differences in LdhA (lactate dehydrogenase) activity. However, LdhA is not
302 considered to be regulated by CreBC (at least at the transcriptional level) since the *ldhA*

303 promoter region does not present a *cre* tag. In an attempt to elucidate these interrogants, strains
304 K1060 and DC1060 were grown in the three aeration conditions indicated previously, and the
305 specific activity of AckA and LdhA were determined *in vitro* during exponential growth (Fig. 2).
306 In high oxygen availability, no significant differences were observed in the specific AckA or
307 LdhA activities between both strains, while in low aeration the enzyme activities reflected the
308 metabolic profile shown in Table 2. That is, the $\Delta creC$ strain had a higher AckA activity and a
309 lower LdhA activity compared to the wild-type strain, in agreement with the higher acetate and
310 lower lactate production in this condition. The specific activities of AckA and LdhA in the $\Delta creB$
311 mutants were also analyzed in low aeration to compare them with the corresponding metabolic
312 profiles and, as expected, both strains behaved identically (data not shown). From these
313 experiments it can be concluded that either directly or indirectly, CreC affects the activities of
314 both AckA and LdhA.

315

316 In non-aerated cultures, however, there was no clear correspondence between enzyme activity
317 and metabolite levels. Although *E. coli* DC1060 produced more lactate and less acetate than the
318 parental strain in 24 h cultures, the LdhA and AckA activities (measured during the exponential
319 phase) in the non-aerated cultures were approximately 50% lower and 200% higher, respectively
320 ($P < 0.05$). The discrepancy observed in non-aerated cultures led us to make additional
321 measurements in order to consider possible variations due to culture age: (i) metabolite levels
322 were determined in the exponential cultures used for the initial enzyme determinations, and (ii)
323 enzyme activities were also measured at the onset of the stationary phase of growth. Similar
324 amounts of acetate were detected in the supernatants of exponential non-aerated cultures of both
325 strains (15.6 mM in K1060 *versus* 12.6 mM in DC1060), but K1060 produced more lactate than
326 DC1060 (0.7 mM *versus* undetectable levels, respectively) in this condition, in accordance with
327 the differences observed in LdhA activity.

328

329 It must be noted that, in the absence of aeration, *E. coli* DC1060 produced more biomass and
330 grew faster than the wild-type strain, making it difficult to choose the best moment along the
331 growth curve for comparisons. For this reason, the cultures used for enzyme determinations were

332 harvested in what could be considered as exponential and early stationary phase in each case
333 (sampling times are shown in Fig. S1 in the Supplemental material).

334 Although in early stationary non-aerated cultures the AckA activity did not match the observed
335 trend in acetate formation, differences between these two parameters were more moderate than in
336 the exponential phase (Fig. 2A). In contrast, the results obtained for LdhA in the early stationary
337 phase of non-aerated cultures were quite different to those obtained in the exponential phase. The
338 early stationary phase cultures of strain DC1060 displayed a higher LdhA activity compared to
339 the wild-type K1060 (Fig. 2B), and produced more lactate (6.3 mM in DC1060 *versus*
340 undetectable levels in K1060), in accordance with the larger amounts of lactate measured in 24 h
341 non-aerated cultures (Table 2).

342

343 To further investigate this we measured pH in the cultures, as this parameter is known to affect
344 LdhA activity (33). In high and low aeration the pH was observed to decrease throughout the
345 exponential phase for all strains, and to remain stable upon entry into the stationary phase. No
346 differences in pH evolution were observed among the strains except in the un-aerated cultures. In
347 this condition, the wild type (K1060) grew very little, and the pH decreased only slightly, while
348 the null mutant (DC1060) grew more, and had a much greater drop in pH (Fig. S1 in the
349 Supplemental material).

350

351 Given that (i) the $\Delta creC$ mutant exhibited lower LdhA activity and lactate levels than the wild-
352 type strain in all conditions except in stationary non-aerated cultures and (ii) appreciable
353 differences in pH were only observed in this condition, it is apparent that the low pH could be at
354 least partially responsible for the increase in LdhA activity observed in DC1060, that in turn
355 correlates with higher lactate levels.

356

357 **Manipulation of CreC as a genetic tool for the improvement of succinate formation.** One of
358 the most interesting properties of the *creC* null mutant was the augmented production of
359 succinate, since this organic acid is a biotechnologically interesting compound (34). Many
360 strategies have been performed so far to optimize the production of this metabolite (35), such as

361 over-expressing carboxylating enzymes (36-39) and supplying carbon dioxide by the addition of
362 sodium bicarbonate to the culture medium (39-41). Since the *creC* mutant produced higher
363 amounts of succinate compared to the parental strain, additional strategies were tested to increase
364 the titer of this metabolite in *E. coli* DC1060. Two plasmids were introduced in the strains:
365 plasmid pEcPpc, carrying the carboxylating enzyme phosphoenolpyruvate carboxylase (Ppc^{Ec})
366 from *E. coli* (42), and plasmid pSBF2, carrying a NAD⁺-dependent formate dehydrogenase from
367 the methylotrophic yeast *Candida boidinii* (FDH1^{Cb}) (43-45). In both plasmids, the
368 corresponding genes are under the control of the *lac* promoter, and their expression can be
369 induced by IPTG. Taking into account that the $\Delta creC$ mutant produces high amounts of formate
370 in low aeration, expression of FDH1^{Cb} could help convert the excess formate to CO₂ and NADH,
371 further increasing the supply of precursors necessary for succinate synthesis. This surplus of
372 CO₂, together with that provided by NaHCO₃ (added to the culture medium), could be funneled
373 to the pyruvate-oxaloacetate node by means of the over-expressed Ppc^{Ec}, which fixes carbon
374 dioxide to pyruvate forming oxaloacetate, that, in turn, could be converted to succinate (Fig. 3).

375

376 To test this hypothesis, strains K1060 and DC1060, co-transformed with plasmids pSBF2 and
377 pEcPpc, were grown for 48h in low aeration with the addition of 100 mM NaHCO₃. Two
378 different concentrations of IPTG (0.1 mM and 1.0 mM) were used to get a better estimation of
379 the relative weight of the conversion catalyzed by Ppc^{Ec} and FDH1^{Cb} on succinate production.
380 With the lowest concentration of IPTG, the *creC* mutant produced 2.7-fold more succinate than
381 the parental strain (Fig. 4A). The yield of succinate on glucose followed this trend, being 0.04
382 and 0.12 mol mol⁻¹ for K1060 and DC1060, respectively (Fig. 4B). When IPTG was supplied at
383 1.0 mM, succinate production was triggered, with marked increases in both titer and yield in both
384 strains. The succinate yield of the $\Delta creC$ mutant strain over-expressing *ppc*^{Ec} and FDH1^{Cb} was
385 around 40% higher than that of the wild type (K1060/pSBF2-pEcPpc) in the same culture
386 conditions.

387

388 The production of other metabolites in the strains carrying the plasmids (Fig. S2 in the
389 Supplemental material) showed some modifications when compared to the strains without

390 plasmids (Table 2). For example, formate synthesis was lower in the strains containing plasmids
391 than in the plasmid-less counterparts, in accordance with the results expected from *FDHI*^{Cb}
392 overexpression.

393

394 In order to eliminate side products and further increase reducing power availability to stimulate
395 succinate production, additional modifications in the genetic background of strain DC1060 were
396 tested. The ethanol pathway was deleted to increase NADH availability, and *ackA* was also
397 eliminated to save carbon atoms in the form of acetyl-CoA, a substrate for succinate formation
398 via the glyoxylate pathway (46). The double mutant CE1060 ($\Delta creC \Delta adhE$) and the triple
399 mutant CEA1060 ($\Delta creC \Delta adhE \Delta ackA$) were co-transformed with plasmids pEcPpc and pSBF2,
400 and succinate was measured in cultures of these strains grown in the same conditions described
401 above. In contrast to what was expected, these strains did not present much higher amounts or
402 yields of succinate when compared to *E. coli* DC1060/pSBF2-pEcPpc (Fig. 4). In all cases, a
403 sharp increase in succinate production was observed with higher amounts of IPTG. These results
404 indicate that over-expression of *ppc*^{Ec} and *FDHI*^{Cb} had a marked effect on succinate production,
405 while the mutations in *adhE* and *ackA* did not.

406

407 Acetate, formate, lactate, and pyruvate were measured in the cultures of the different strains (Fig.
408 S2 in the Supplemental data). Acetate formation in the *ackA* mutant was reduced compared to the
409 parental strain but not completely abolished, probably due to the activity of alternative pathways
410 for acetate formation (e.g., PoxB). In all cases, higher expression of the heterologous enzymes
411 (1.0 mM IPTG) tended to reduce the differences in the metabolic profile among the mutants, and
412 caused a decrease in the amounts of formate when compared to the low induction level (0.1 mM
413 IPTG).

414

415 **Discussion**

416

417 While analyzing different *arcA* mutants, a constitutive *creC* allele was discovered to be
418 responsible for the peculiar phenotypic traits of one of the mutants that grew better than the
419 others (18). In that study, the constitutive *creC510* allele was observed to ameliorate some of the
420 phenotypic characteristics of $\Delta arcA$ mutants, and the effect was attributed to increased substrate
421 consumption. Further work analyzed the carbon fluxes of strains harboring *arcA* and *creB*
422 mutations in microaerobic glucose-limited chemostat cultures (47), revealing that these
423 regulators share the control of carbon catabolism in these conditions. These results opened
424 questions concerning the contribution of the CreBC system to central carbon metabolism under
425 different oxygen availability conditions when the carbon source does not limit bacterial growth.
426 This question was of special interest, since it was reported that CreC does not respond only to the
427 composition of the media (i.e., whether it is rich or mineral medium) as it was initially believed
428 (7), but its activation is also dependent on the aeration level and the carbon source (6).

429

430 To further analyze the metabolic effects of CreBC, three different aeration levels (high, low, and
431 no aeration) were tested to determine the behavior of *creC* mutants considering different aspects
432 of cell metabolism, such as the metabolite profile, enzyme activities, and redox state. These
433 experiments showed that CreC has a clear effect over carbon distribution that varies in different
434 oxygen availability conditions. CreC was observed to affect the accumulation of formate, lactate,
435 acetate, ethanol and succinate. These effects were shown to be mediated by CreB, ruling out the
436 possibility that they could be due to interaction of CreC with other non-cognate response
437 regulators. This observation is not trivial, since CreC was originally associated with the *pho*
438 regulon (48) interacting with the response regulator PhoB. This affirmation does not exclude,
439 however, the possibility that other regulators could affect the expression of genes in common.

440

441 Differences in the distribution of metabolites in the aeration conditions analyzed in this work
442 were also associated to the intracellular redox state. In previous studies that analyzed $\Delta arcA$
443 *creC510* double mutants, the constitutive *creC510* allele was observed to contribute to a high

444 ethanol/acetate ratio (18), while the opposite was observed in *creB* mutants (47). These
445 differences were also observed in this study. For example, ethanol accumulation, that normally
446 correlates with a reduced intracellular state (i.e., increased NADH/NAD⁺ ratio), was lower in the
447 Δcre mutants, and higher in the mutant carrying the constitutive *creC510* allele when aeration
448 was suppressed as compared to the parental strain.

449

450 The effects of CreC on the redox state of the cells were further analyzed by measuring the ratios
451 between reduced and oxidized cofactors. In all oxygen conditions, the strain carrying *creC510*
452 had a higher NADH/NAD⁺ ratio than the wild-type strain and the $\Delta creC$ mutant, showing that
453 CreC contributes to a more reduced intracellular redox state. While the null mutant had
454 significant ($P < 0.05$) differences in NADH/NAD⁺ ratios with both the parental strain and the
455 *creC510* mutant in low aeration, this was not observed in high aeration or no aeration, indicating
456 that the effect of CreC on redox potential was stronger in intermediate oxygen availability
457 conditions.

458

459 The determination of reduced and oxidized cofactors in the three aeration conditions tested
460 revealed additional interesting data. While in high aeration the NADPH/NADP⁺ ratios were
461 higher than the NADH/NAD⁺ ratios for all strains, the opposite was observed in un-aerated
462 cultures. This observation could have two different implications. The first is that the degree of
463 oxidation of the two cofactors in high aeration and no aeration reflects differences in cofactor
464 usage. NADP(H) is considered the preferred cofactor in anabolism and stress resistance
465 mechanisms, whereas NAD(H) is mainly related to catabolism and fermentation processes (49).
466 Therefore, the lower NADPH/NADP⁺ ratio compared to the NADH/NAD⁺ ratio in non-aerated
467 cultures is possibly related to a decrease in the NADPH content, which in turn is reflected in the
468 low biomass formation under these conditions. Another possible explanation could be that cells
469 regulate NADH/NAD⁺ ratios more tightly than NADPH/NADP⁺ ratios, as the latter were
470 observed to vary on a much wider range with respect to oxygen availability. In this scenario, the
471 ratio between reduced and oxidized NAD(H) is maintained with modest variations through

472 different oxygen availability conditions, while the pool of NADP(H) is mostly reduced in highly
473 aerated cultures and mostly oxidized in non-aerated ones.

474

475 In this work, no significant variations were detected when the NADPH/NADP⁺ ratios obtained
476 for the different strains were compared within each condition, while differences in the
477 NADH/NAD⁺ ratios were observed in all aeration conditions. The oxidation state of NAD(H)
478 has previously been reported to vary greatly in different genetic backgrounds (49-52), such as in
479 redox regulatory mutants (53-55), so the differences in NADH/NAD⁺ ratios between the strains
480 carrying the *cre* variants support the hypothesis that CreC affects the intracellular redox state.
481 Additionally, the results obtained in this work could reflect that the content of NADP⁺ responds
482 more strongly to variations in oxygen availability than to different genetic or metabolic
483 backgrounds.

484

485 At least 120 proteins are shown to change their expression in response to a shift from aerobic to
486 anaerobic conditions (2). The expression of over 30 operons (more than 70 genes) are under
487 control of the Fnr regulator (56) and at least 40 operons are regulated by the two-component
488 regulatory ArcAB system (57, 58), including 16 genes that encode proteins playing a role in
489 carbon metabolism (16, 59). The ArcAB and Fnr global regulation systems are major controlling
490 factors of gene expression, and in most cases they operate coordinately to fine-tune catabolism in
491 response to oxygen availability (60). The metabolic changes observed in this work suggest that
492 CreBC might also contribute to the regulation of the intracellular redox state, although it is likely
493 that its role does not involve direct sensing of the redox state of the cells, but rather, as it has
494 been suggested in previous work, a metabolic signal, such as a carbon catabolism intermediary
495 (6).

496

497 The CreBC system was first reported to respond to growth in minimal media (7), and later shown
498 to be active when cells are grown in gluconeogenic C sources or fermenting glucose (6). The
499 results presented in this study, in which all experiments were performed using glucose as the C
500 source, clearly indicate that the regulation mediated by CreBC is affected by oxygen availability.

501 In a mineral culture medium with excess glucose, high aeration did not offer a propitious
502 environment for CreC activation, as shown by similar metabolic profiles (characterized by high
503 acetate formation) and enzyme activities among the three strains. Mutations in CreC gave more
504 conspicuous phenotypes when low or no aeration was supplied. This result is in accordance with
505 the observations by Cariss et al. (6), that indicated that CreC was activated when cells were
506 cultured in sealed flasks, limiting the oxygen supply. However, there was not a clear trend across
507 the different aeration levels. The *in vitro* enzyme activity levels as well as the formation of some
508 metabolites were not affected in the same manner by the absence of CreC at different aeration
509 levels. Except for succinate, that had higher yields in the absence of CreC in both low or no
510 aeration, the relative values of other organic acids differed in the two conditions. For example, in
511 low aeration the null mutant accumulated more formate and acetate, but less lactate than the
512 parental strain, while in the absence of aeration the opposite relationship was observed for
513 acetate and lactate, with similar amounts of formate.

514

515 In an attempt to further characterize this effect, the activities of AckA and LdhA were
516 determined, and both were observed to be affected by CreC. While *ackA* was previously shown
517 to be under transcriptional regulation exerted by CreBC (7), *ldhA* was not thought to be affected
518 by this regulatory system. This gene has also been observed to be negatively regulated by Mlc
519 (which affects the expression of *pts* genes) (61), CsrA (a regulator of carbohydrate metabolism
520 that influences glycogen biosynthesis, gluconeogenesis, glycolysis, and glycogen degradation)
521 (62, 63), and positively regulated by ArcAB and CsrB (which antagonizes CsrA) (64). The
522 differences in lactate production, together with those observed in LdhA activity, suggest that
523 CreC could also have a regulatory effect over *ldhA*, in spite of the lack of a consensus *cre* tag in
524 the region upstream from this gene. Further experiments need to be performed to assess whether
525 these differences are directly mediated by CreBC regulation at the transcriptional level. For all
526 cultures grown in low aeration, a correlation between enzyme activities and metabolite levels
527 was observed, as higher acetate and lower lactate values in the $\Delta creC$ mutant compared to the
528 parental strain corresponded to higher AckA and lower LdhA activities, but this correlation was
529 not observed in non-aerated cultures.

530 Although enzymatic studies are typically performed in exponential cultures, enzyme activity was
531 also determined in early stationary non-aerated cultures to investigate whether there was a
532 correlation between *in vitro* enzyme activities in this growth phase and metabolite levels
533 (measured in 24 h cultures). When analyzing this point of the growth phase, lactate production in
534 strains K1060 and DC1060 correlated with LdhA activity, as a higher enzymatic activity was
535 observed for *E. coli* DC1060, that accumulated more lactate in non-aerated exponential cultures.
536 The conditions in which *ldhA* is normally expressed are anaerobiosis and acidic pH (33). Given
537 that DC1060 grew more than K1060 and that cultures of DC1060 had a marked reduction in pH,
538 it is possible that in the stationary phase the effects of the lower pH contributed to the higher
539 LdhA activity observed. This, in turn, could result in higher lactate accumulation. In the
540 exponential phase of non-aerated cultures, which had only slight differences in pH, both LdhA
541 activity and lactate formation were lower for the null mutant, as observed in the cultures grown
542 with high and low aeration. The only condition in which the $\Delta creC$ mutant had higher LdhA
543 activity and produced more lactate than the parental strain was when these cultures had a much
544 lower pH, so it is possible that this parameter affected LdhA activity, leading to increased lactate
545 levels. In all other conditions, in which no differences in culture pH were observed between the
546 strains, the $\Delta creC$ mutant produced less lactate and had lower LdhA activity, so it can be
547 proposed that CreC has a positive regulatory effect on *ldhA*, the nature of which remains to be
548 elucidated.

549

550 Concerning acetate, while the DC1060 mutant produced higher amounts of this metabolite than
551 the wild-type strain in low aeration but not when aeration was suppressed, the *in vitro* AckA
552 activity was higher for the mutant strain in both conditions, indicating that there was not a direct
553 correlation between AckA activity and acetate accumulation. This suggests there must be
554 additional factors that affect acetate levels in the cultures, which could be due to
555 postranscriptional, allosteric, or even metabolic regulation. It cannot be ruled out that other
556 enzymes might contribute to the effect observed, such as pyruvate oxidase (PoxB), that has been
557 reported to be the main pathway for acetate production in stationary phase (30), although this
558 enzyme is supposedly repressed in anaerobiosis (65).

559 In previous studies, it was seen that *ackA* is transcriptionally activated by CreC (7), and that in
560 the absence of CreB there is a reduced acetate flux when the cells are grown in continuous
561 cultures under carbon-limited conditions and restricted oxygen supply (47). However,
562 differences in aeration conditions have been shown to have dramatic effects over CreC
563 regulation (6). In our work, performed in the presence of an excess carbon source, the *creC* null
564 mutants displayed higher AckA activities compared to the wild-type strain, while the absence of
565 CreC had different effects over acetate accumulation according to the aeration level (low or no
566 aeration). Furthermore, the relative weight of the effects of Cre on other physiological
567 parameters analyzed in this study also depended highly on oxygen availability. While the
568 strongest effects on redox levels were observed in low aeration, a notable influence was seen
569 over growth in non-aerated cultures, and differences in metabolite distribution when compared to
570 the other strains also varied appreciably in the conditions analyzed. These results suggest that the
571 metabolic effects of the *cre* variants are highly susceptible to changes in culture conditions,
572 including oxygen availability.

573

574 The $\Delta creC$ mutation proved useful to increase the production of succinate. This compound is an
575 intermediary metabolite in the tricarboxylic acid cycle, and, under anaerobic conditions, can be
576 produced via the reductive arm of the cycle and the glyoxylate shunt, although the latter route
577 contributes less than the former. As NADH is required for its formation, different strategies have
578 been used to increase cofactor availability and to improve succinate production (66). It has been
579 proposed that the manipulation of the CreBC system (as well as ArcAB) could provide a relevant
580 tool for the modulation of central metabolism and reducing power availability aimed towards
581 biotechnological purposes (16), such as succinate production. Although the positive effect of the
582 *creC* deletion on succinate formation may not seem obvious from the point of view of the redox
583 balance, the increase in succinate production in *E. coli* DC1060 compared to the parental strain
584 was observed in all aeration conditions, and also when plasmids over-expressing
585 decarboxylating enzymes were added to both strains. The complementary strategy of enhancing
586 CO₂ fixation (through Ppc^{Ec}) and increasing the pool of NADH (through FDH1^{Cb}) at the expense
587 of excess formate worked satisfactorily. Additional mutations in structural genes did not produce

588 significant increases in the titer of succinate, even though they affected the secretion of other
589 acids. In general, the differences in succinate production between the mutants became less
590 relevant when other modifications were introduced, such as the expression of FDH1^{Cb} and Ppc^{Ec},
591 indicating that the relative weight of the mutations affecting the different metabolic steps was
592 lower than the over-expression of these two heterologous genes. However, the *creC* mutants
593 continued to accumulate more succinate than the strain carrying the wild-type allele even when
594 the enzymes were over-produced in all strains, evidencing the role of the *creC* mutation in
595 boosting succinate synthesis. A hitherto unknown regulation exerted by CreBC on the enzymes
596 involved in the synthesis and/or consumption of succinate in *E. coli*, as observed in this work for
597 LdhA, cannot be ruled out. The relatively simple strategy of modifying the global regulation
598 exerted by CreBC offers an appealing alternative to the traditional, gene-by-gene metabolic
599 engineering strategies for the production of succinate (40, 46, 67, 68).

600

601 In conclusion, this work shows that CreC affects both carbon catabolism and the intracellular
602 redox state. The *creC* mutants were shown to exhibit different behaviors according to the degree
603 of oxygen limitation when grown in a mineral medium, with diverse effects over growth,
604 metabolites secretion, and redox balance. These results also reveal that, like other global
605 regulators, Cre influences many different aspects of bacterial physiology, although more research
606 is needed to elucidate the mechanisms by which CreC exerts its regulation. The diverse
607 metabolic effects of this regulator over the central biochemical network of *E. coli* make it a good
608 candidate for genetic manipulations to improve the formation of compounds of commercial
609 interest, such as succinate.

610

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612

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623

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625

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855

FIGURE LEGENDS

856

857

858 **FIG. 1.** Determination of the NADH/NAD⁺ and NADPH/NADP⁺ ratios in *E. coli* K1060 (wild-
859 type strain), K1060C (carrying the constitutive *creC510* allele), and DC1060 ($\Delta creC$). Cells were
860 grown in M9 minimal medium containing 30 g liter⁻¹ glucose under high aeration (A), low
861 aeration (B), and no aeration (C). Cells were harvested at mid-exponential phase. Results
862 represent the average \pm standard deviation from duplicated measurements from at least two
863 independent cultures.

864

865 **FIG. 2.** Specific (Sp) acetate kinase (A) and lactate dehydrogenase (B) *in vitro* activities (act) of
866 cells grown in M9 minimal medium containing 30 g liter⁻¹ glucose under high aeration, low
867 aeration, and no aeration. The samples were harvested at mid-exponential phase, except under
868 anaerobic conditions, in which the activity was measured both in exponential (E) and in the early
869 stationary phase (S) (see Fig. S1 in the Supplemental material for detailed information on
870 sampling times). Results represent the average \pm standard deviation from duplicated
871 measurements from at least two independent cultures.

872

873 **FIG. 3.** Diagram of the main metabolic pathways leading to succinate formation from glucose in
874 *E. coli*. The genes encoding Ppc^{Ec} (phosphoenolpyruvate carboxylase) from *E. coli* and FDH1^{Cb}
875 (NADH-forming formate dehydrogenase) from *Candida boidinii* were over-expressed in plasmids
876 pEcPpc and pSBF2, respectively (the corresponding reactions are highlighted in green). The
877 genes encoding AdhE (alcohol dehydrogenase) and AckA (acetate kinase) were knocked-out in
878 an attempt to enhance succinate accumulation (indicated by slanted red arrowheads). Note that
879 several reactions within the biochemical network were grouped for the sake of simplicity. EMP
880 pathway, Embden-Meyerhof-Parnas pathway; PEP, phosphoenolpyruvate; Acetyl-CoA, acetyl-
881 coenzyme A.

882

883 **FIG. 4.** Profile of succinate formation in the *E. coli* strains under study. Cells were grown in M9
884 minimal medium containing 30 g liter⁻¹ glucose and 100 mM NaHCO₃ under low aeration for

885 48h. The *E. coli* strains tested were K1060 (wild-type strain), DC1060 ($\Delta creC$), CE1060 ($\Delta creC$
886 $\Delta adhE$), and CEA1060 ($\Delta creC \Delta adhE \Delta ackA$). All bacteria were transformed with plasmids
887 pSBF2 (carrying *FDHI*^{Cb}, a NADH-forming formate dehydrogenase from *Candida boidinii*) and
888 pEcPpc (carrying *ppc*^{Ec}, the endogenous phosphoenolpyruvate carboxylase from *E. coli*). The
889 expression of the genes in these plasmids was induced by addition of isopropyl- β -D-1-
890 thiogalactopyranoside (IPTG) at two concentrations (0.1 mM and 1 mM). Succinate was assayed
891 in culture supernatants and the results are reported as final concentration (concn.) (A) and yield
892 of succinate on glucose ($Y_{Succ/Glc}$) (B). Results represent the average \pm standard deviation from
893 duplicated measurements from at least two independent cultures.
894

895

TABLES

896

897 **Table 1.** Bacterial strains, plasmids, and oligonucleotides used in this study.

898

Strain, plasmid or oligonucleotide	Relevant characteristics ^a	Reference or source
<i>E. coli</i> strains		
K1060 ^b	F ⁻ <i>fadE62 lacI60 tyrT58(AS) fabB5 mel-1</i>	(69)
K1060C	Same as K1060, but <i>creC510</i> by K1060×P1(58-161,F-,SR)	This work
DB1060	Same as K1060, but $\Delta creB$ by allelic replacement	This work
DC1060	Same as K1060, but $\Delta creC$ by allelic replacement	This work
DBC1060	Same as K1060, but $\Delta creB \Delta creC$ by allelic replacement	This work
CE1060	Same as DC1060, but $\Delta adhE$ by allelic replacement	This work
CEA1060	Same as CE1060, but $\Delta ackA$ by allelic replacement	This work
58-161,F-,SR ^b	F ⁻ <i>relA1 rpsL100(Str^R) spoT1 metB1 creC510</i>	(70)
58KF	Same as 58-161,F-,SR but Km ^R by insertion of <i>kan</i> in an intergenic region between <i>yjjX</i> and <i>trpR</i>	This work
Plasmids		
pCP20	Helper plasmid used for <i>kan</i> excision; <i>Saccharomyces cerevisiae</i> <i>FLP</i> λ <i>cI857</i> λ PR <i>repA</i> (Ts), Ap ^R Cm ^R	(25)
pKD4	Template plasmid carrying the FRT- <i>kan</i> -FRT cassette, Km ^R	(24)
pKD46	Helper plasmid expressing the λ -Red functions, Ap ^R	(24)
pSBF2	Plasmid pDHK30 (71) carrying <i>FDH1</i> from <i>Candida boidinii</i> under control of the <i>lac</i> promoter, Km ^R	(43)
pEcPpc	Plasmid pTrc99A (72) carrying <i>ppc</i> from <i>E. coli</i> under control of the <i>lac</i> promoter, Ap ^R	(39)
Oligonucleotides ^c (5' → 3')		
<i>cre</i> /F	TAG GCC TGA TAA GAC GTG GCG CAT CAG GCA	This work

	TCG TGC ACC GAA TGC CGG ATG TGT AGG CTG GAG CTG CTT C (<i>KI060C construction</i>)	
<i>cre/R</i>	GCC GCG TCT TAT CAT GCC TAC CAA ACA TAT TGA AAT TAC GGG TAT TTG TAC ATA TGA ATA TCC TCC TTA G (<i>KI060C construction</i>)	This work
<i>creB-KF/F</i>	TTA GCG CGG TTC CTG TCA TGC CGT GGC GGC AAT AAC AGA GGC GAT TTA TGG TGT AGG CTG GAG CTG CTT C (<i>DB1060 and DBC1060 construction</i>)	This work
<i>creB-KF/R</i>	GCC CAG CAA CAA CCG CAT GCC GAT ACG CAT TAC AGG CCC CTC AGG CTA TAC ATA TGA ATA TCC TCC TTA (<i>DB1060 construction</i>)	This work
<i>creC-KF/F</i>	GTC AAA GAA GTT AAA CCG GGC GTG CGA AGA GCA ACG GAG GGG ACG TTG ATC GTG TAG GCT GGA GCT GCT TC (<i>DC1060 construction</i>)	This work
<i>creC-KF/R</i>	GAC GTG TTC CTG ATC CAC TTC GGC GCT TAG CGT GAT GCA ACC GCT CTC GGG CAT ATG AAT ATC CTC CTT AG (<i>DC1060 and DBC1060 construction</i>)	This work
DW- <i>ack/F</i>	AAC TCA GCG GGA CAA CG (<i>CEA1060 construction</i>)	This work
DW- <i>ack/R</i>	GAA AGC AGA CCT TCA ACG (<i>CEA1060 construction</i>) AGA CGC GCT GAC AAT ACG (<i>CE1060 and CEA1060</i>	This work This work
DW- <i>adh/F</i>	<i>construction</i>)	
DW- <i>adh/R</i>	GCC ACC AGA CGC ATA ACC (<i>CE1060 and CEA1060</i> <i>construction</i>)	This work

899

900 ^a Antibiotic markers: Ap, ampicillin; Cm, chloramphenicol; Km, kanamycin; and Str,
901 streptomycin.

902 ^b Strain obtained through the *E. coli* Genetic Stock Center, University of Yale, CT.

903 ^c The use of each oligonucleotide in the construction of mutant strains is indicated in
904 parentheses.

905

906 **Table 2.** Growth and fermentation profile of the strains under study under different conditions of
 907 oxygen availability.

Aeration condition	<i>E. coli</i> strain	Biomass (g _{CDW} liter ⁻¹)	Yield of fermentation metabolites on biomass (mmol g _{CDW} ⁻¹)				
			Succinate	Lactate	Formate	Acetate	Ethanol
High aeration	K1060	1.29 ± 0.02	0.4 ± 0.1	1.0 ± 0.1	0	14 ± 2	9.0 ± 1.0
	K1060C	1.25 ± 0.01	1.0 ± 1.0	1.0 ± 0.1	0	12 ± 1	9.0 ± 0.1
	DC1060	1.25 ± 0.01	0.3 ± 0.1	1.0 ± 0.1	0	11 ± 1	6.0 ± 0.1
Low aeration	K1060	0.38 ± 0.02	0.5 ± 0.2	46 ± 8	17 ± 3	89 ± 13	18 ± 5
	K1060C	0.39 ± 0.02	0.4 ± 0.1	48 ± 7	22 ± 3	91 ± 9	17 ± 2
	DC1060	0.36 ± 0.01	6.9 ± 0.3	22 ± 2	44 ± 3	127 ± 10	19 ± 3
	DB1060	0.39 ± 0.01	6.3 ± 0.2	19 ± 1	48 ± 1	120 ± 4	N.D.
	DBC1060	0.36 ± 0.01	6.7 ± 0.3	20 ± 1	50 ± 5	125 ± 9	N.D.
No aeration	K1060	0.14 ± 0.01	0.2 ± 0.1	27 ± 8	61 ± 2	118 ± 4	68 ± 10
	K1060C	0.11 ± 0.04	0.1 ± 0.1	19 ± 8	41 ± 11	150 ± 7	120 ± 23
	DC1060	0.26 ± 0.01	9.4 ± 0.9	45 ± 5	58 ± 5	46 ± 9	29 ± 12
	DB1060	0.23 ± 0.02	11.0 ± 1.0	46 ± 4	56 ± 4	53 ± 10	N.D.
	DBC1060	0.24 ± 0.02	11.6 ± 0.2	51 ± 6	61 ± 5	55 ± 3	N.D.

908

909 Cells were grown in M9 minimal medium supplemented with 30 g liter⁻¹ glucose for 24 h. CDW,
 910 cell dry weight; N.D., not determined. Results represent the mean value ± standard deviation of
 911 three determinations from at least four independent cultures. The *E. coli* strains used are K1060
 912 (wild type), K1060C (*creC510*, constitutive *creC* allele), DC1060 ($\Delta creC$), DB1060 ($\Delta creB$),
 913 and DBC160 ($\Delta creB \Delta creC$). Results represent the average ± standard deviation from triplicate
 914 measurements from at least three independent cultures.







