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4 Comprehensive analysis of central carbon metabolism reveals multiple connections
5 between nutrients, biosynthetic capacity, and cell morphology in *Escherichia coli*
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7 Short title: Central carbon metabolism, cell growth, and cell morphology
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Abstract

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27   Bacterial morphology is a complex trait that is highly sensitive to changes in the

28   environment. For heterotrophic organisms, such as *Escherichia coli*, increases in

29   nutrient levels are frequently accompanied by several-fold increases in both size

30   and growth rate. Despite the dramatic nature of these changes, how alterations in

31   nutrient availability translate into changes in growth and morphology remains a

32   largely open question. To understand the signaling networks coupling nutrient

33   availability with size and shape, we examined the impact of deletions in the entirety

34   of non-essential central carbon metabolic genes on *E. coli* growth rate and cell size.

35   Our data reveal the presence of multiple metabolic nodes that play important yet

36   distinctive roles in shaping the cell. Consistent with recent work from our lab and

37   others, although both are sensitive to nutrient availability, size and growth rate vary

38   independently. Cell width and length also appear to be independent phenomena,

39   influenced by different aspects of central carbon metabolism. These findings

40   highlight the diversity of factors that can impact cell morphology and provide a

41   foundation for further studies.

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Author summary

43

44   Often taken for granted, the shape of bacterial cells is a complex trait that is highly

45   sensitive to environmental perturbations. Nutrients in particular, strongly impact

46   bacterial morphology together with growth rate. The ubiquitous, rod-shaped

47   bacteria *Escherichia coli* increases both length and width several fold upon a shift

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48   from nutrient poor to nutrient rich medium, a change accompanied by an equally

49   dramatic increase in growth rate. Central carbon metabolism is an obvious site for

50   the integration of nutrient dependent signals that dictate cell size and shape. To

51   develop a clearer picture of the molecular mechanisms coupling nutrient

52   assimilation with cell growth and morphology, we screened the entirety of non-

53   essential carbon metabolic genes for their contribution to growth rate and cell

54   shape. Our data reveal the presence of multiple regulatory circuits coordinating

55   different metabolic pathways with specific aspects of cell growth and morphology.

56   Together, these data firmly establish a role for central carbon metabolism as an

57   environmentally sensitive sculptor of bacterial cells.

58

59   Introduction

60   The behavior and physiology of single celled organisms is at the mercy of

61   their environment. Nutrients, in particular, dramatically impact the growth rate, cell

62   cycle, and morphology of bacteria. *Salmonella typhimurium, Escherichia coli,* and

63   *Bacillus subtilis* cells cultured in nutrient rich conditions exhibit mass doubling

64   times up to 6-fold faster than their counterparts cultured in nutrient poor medium

65   [1-3]. These increases in growth rate are accompanied by similarly dramatic

66   increases in cell size. *E. coli* increases length (2-fold) and width (1.5-fold) for a ~3-

67   fold increase in total square area between nutrient poor and nutrient rich

68   conditions [3]. *B. subtilis* increases length ~3-fold in nutrient rich conditions while

69   width remains more or less constant [2,4].

70   The positive relationship between nutrient availability, growth rate and cell

71   size is generally referred to as the "Growth Law". Besides cell size, DNA, RNA, and

72   protein content of the cell also show a positive relationship with growth rate, with

73   faster growing cells containing more of these macromolecules [1]. Although there is

74   more total DNA/RNA/protein in larger, faster growing cells, their concentration

75   remains relatively constant due to the concomitant increase in cell size [1,5].

76   Despite its name, the relationship between growth rate and cell size is

77   complicated and there are several, well known exceptions to the Growth Law.

78   Reducing growth rate by lowering culture temperature does not impact cell length

79   or width, suggesting that it is nutrients rather than growth rate that determine cell

80   size [6]. Conversely, mutations that lead to modest delays in division frequently

81   result in increases in cell length without detectably impacting mass doubling time

82   [7-9].

83

84   **Central carbon metabolism: a hub connecting nutrient availability with**

85   **growth rate and cell size**

86   As the entry and processing point for the raw materials required for cell

87   growth and proliferation, central carbon metabolism (CCM) plays a key role in

88   coordinating nutrient availability with cell growth and size. Recent work indicates

89   that CCM impacts bacterial morphology in several ways: by its impact on cell cycle

90   progression [10-12]; by its impact on lipid synthesis [6]; and through its impact on

91   cell wall biogenesis [13].

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92   CCM-mediated changes in cell cycle progression primarily affect cell length,

93   while changes in lipid synthesis and cell wall biogenesis impact both length and

94   width. In an example of the former, UDP-glucose, produced in two steps from

95   glucose-6-phosphate at the beginning of glycolysis, stimulates interactions between

96   the moonlighting glucosyltransferase OpgH in *E. coli* and the unrelated enzyme UgtP

97   in *B. subtilis* and the essential tubulin-like cell division protein FtsZ, delaying

98   division and increasing cell length during growth in carbon rich medium [4,12]. In

99   contrast, defects in fatty acid synthesis (FAS) lead to reductions in both length and

100   width, effectively recapitulating the impact of nutrient limitation on cell morphology

101   [6,14].

102   CCM can also effect morphology via its impact on the availability of cellular

103   building blocks, particularly those required for synthesis of the cell wall. In a recent

104   study, the Young laboratory determined that defects in *wecE*, a gene encoding an

105   aminotransferase important for making the *E. coli* outer membrane molecule

106   enterobacterial common antigen (ECA), led to the formation of swollen, filamentous

107   cells. ECA is transported to the cell wall via undecaprenyl-phosphate, the lipid

108   carrier responsible for shuttling precursors for peptidoglycan and other

109   components of the bacterial cell envelope across the plasma membrane. Careful

110   genetics revealed the morphological defects associated with defects in *wecE* were

111   due to sequestration of undecaprenyl-phosphate conjugated to an ECA intermediate,

112   interfering with peptidoglycan synthesis [15].

113   More generically, nutrient starvation leads to an accumulation of the small

114   molecule guanosine tetraphosphate (ppGpp) that interacts directly with RNA

115   polymerase, to inhibit transcription of a wide array of genes, effectively curtailing

116   flux through multiple biosynthetic pathways [16]. ppGpp concentration is inversely

117   proportional to both size and growth rate: ectopic expression of the ppGpp

118   synthetase RelA during growth in nutrient rich medium reduces both growth rate

119   and cell size [6,17]. The reduction in size is indirect and dependent on ppGpp-

120   mediated inhibition of fatty acid synthesis, while the reduction in growth rate is

121   almost certainly a consequence of ppGpp-dependent inhibition of a host of essential

122   processes [6].

123   While these examples highlight several ways in which CCM might impact the

124   positive relationship between cell growth and cell morphology, they represent only

125   a small fraction of the pathways that constitute CCM. To identify additional

126   connections between nutrient levels, metabolism, and cell size in *E. coli*, we

127   screened non-essential (CCM) gene deletions for defects in cell size and growth rate.

128   Our data highlight three CCM nodes—the first branch-point of glycolysis, the

129   pentose-phosphate pathway, and acetyl-CoA metabolism—as critical links between

130   nutrient availability, cell growth and cell size. While some aspects of CCM

131   bottlenecks appear to directly impact the relationship between nutrient availability

132   and biosynthetic flux, other aspects of CCM effect growth and morphology in more

133   nuanced ways.

134

135   **Results**

136   **Multiple connections exist between CCM, growth rate, and cell size**

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137   To identify key steps of CCM that impact the growth and morphology of *E.*

138   *coli*, we phenotypically analyzed cells defective in 44 non-essential CCM genes (S1

139   Table) to. These experiments took advantage of the Keio Collection, an ordered

140   insertion-deletion library of all non-essential genes in *E. coli* [18] and Coli-Inspector,

141   an Image J plug in designed for high throughput analysis of bacterial morphology

142   [19]. Prior to analysis, the Keio insertion-deletions were transduced into the

143   laboratory wild type strain MG1655. Briefly, single colonies of each mutant were

144   picked and cultured under nutrient rich conditions (Luria-Bertani (LB) broth

145   supplemented with 0.2% glucose) to an OD_{600} of ~0.2. Cultures were then back-

146   diluted to OD_{600} of 0.01 and tracked for 4 generations until they reached a maximum

147   OD_{600} of 0.2 (the low OD_{600} ensures cells are all actively growing and at

148   approximately the same growth phase prior to analysis). Cells were sampled, fixed,

149   and imaged by phase contrast microscopy. Cell size (length and width) was

150   determined using Coli-Inspector in ImageJ.

151   Based on size and mass doubling time, we assigned the 44 mutants into six

152   classes (Table 1). Represented by two mutations, Class I cells were >10% smaller

153   than wild type cells with mild to undetectable changes in growth rate (within two

154   SD of wild type mass doubling time). Eight mutations fell into Class II: small (more

155   than 10% decrease in square area than wild type) with a significant decrease in

156   doubling time (>20% wild type). Class III was heterogenous, consisting of a

157   population dominated by smaller than normal cells and punctuated by the presence

158   of a few (5-10%) very long cells. Three mutants fell into Class IV long (>10% longer

159   than WT) and two into Class V, heterogenous with a wide distribution of cell lengths

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160   and variable cell widths (Fig 1A). Surprisingly, the majority of mutants (26) were

161   essentially wild type with regard to growth rate and cell size (Class VI). This finding

162   suggests the presence of alternative pathways that permit *E. coli* to bypass

163   particular CCM enzymes and/or that nutrients that allow for bypass are present in

164   LB. We elected not to investigate the impact of deletions in either *ppsA*, the first step

165   of gluconeogenesis, or *fumC*, one of the three isoforms involved in the conversion of

166   fumarate to malate in the TCA cycle, on cell size as we were unable to obtain stable

167   MG1655 transductants of either mutant. Representative images of each mutant are

168   shown (Fig 2).

169   **Table 1: CCM deletion mutants exhibiting significant alterations in growth rate**

170   **and/or morphology.**

Gene Name	Doubling time (per minute)	Area (microns squared)	Pathway
Wild Type	22	5.1	
Class I (Small with doubling times <20% from WT)			
sucC	25	4.6	TCA Cycle
gnd	21	4.6	Pentose-Phosphate
Class II (Small and doubling times >20% from WT)			
crr	27	4.4	Glycolysis
aceE	34	2.9	Glycolysis/Acetate/Fatty acid/TCA
tktA	33	3.7	Pentose-Phosphate
pgl	26	4.5	Pentose-Phosphate
rpiA	28	3.8	Pentose-Phosphate
rpe	37	3.1	Pentose-Phosphate
pta	33	3	Acetate Metabolism
ackA	36	2.7	Acetate Metabolism
Class III (Short and Long)			
acnB	25	5.6	TCA Cycle
zwf	30	4.4	Pentose Phosphate
Class IV (Long)			
yggF	19	6.7	Putative Gluconeogensis
talB	23	5.8	Pentose-Phosphate
eda	22	6	Entner-Douderoff
Class V (heterogenous)			
tpiA	30	ND	Glycolysis
gpmM	21	ND	Glycolysis

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173   Of the different classes, only the eight Class II mutants "obeyed" the Growth

174   Law, with reductions in growth rate accompanied by predictable reductions in cell

175   size (Fig 1B). The close correlation between growth rate and size for Class II

176   mutants suggests that the loss of individual genes and/or their product's catalytic

177   function, leads to a biosynthetic bottleneck that recapitulates the impact of nutrient

178   starvation on cellular physiology. In contrast, despite their heterogeneity with

179   regard to cell length, both Class I and Class IV mutants exhibited more or less

180   normal growth rates, suggesting a potential defect in the signal transduction

181   pathways coordinating CCM with cell growth and cell size. The heterogeneity of

182   Class III and Class V indicate a loss of proper regulation of cell cycle progression

183   and/or width control, leading to stochastic variations in cell morphology.

184

185   **Growth in carbon poor medium largely ameliorates CCM mutant**

186   **morphological defects**

187   To clarify the impact of nutrient flux on individual CCM mutant phenotypes,

188   we assessed the growth rate and size of Class I-V mutants in three different media:

189   LB + 0.2% glucose, the minimal media AB + 0.2% glucose, and AB + 0.2% succinate

190   representing high nutrient conditions, low nutrient glycolytic conditions, and low

191   nutrient gluconeogenic conditions respectively. Cells were measured using the

192   Matlab software package SuperSegger, which facilitates the rapid analysis of large

193   numbers of clumped cells [20]. In general, SuperSegger results corresponded almost

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194   exactly with the Coli-Inspector data reported above, although cell areas were

195   smaller over all most likely due to a difference in how the two programs define cell

196   boundaries and at what point of the cell cycle that they "divide" the parent cell into

197   two daughter cells (see wild type area in Table 1 and S3 Table).

198   In light of data indicating that cells defective in *crr*, encoding the

199   phosphotransferase system subunit EIIA^{glc}, exhibited significant reductions in size

200   (Table 1), we also examined the nutrient dependent phenotype of cells defective for

201   adenylate cyclase or the cAMP receptor (Crp) production. Crr is part of a

202   phosphodependent transferase system that is essential for the import of glucose and

203   other sugars into *E. coli* [21]. Crr facilitates glucose import via transfer of a

204   phosphate group from Hpr to EIIB^{glc} and finally to glucose, generating glucose-6-

205   phosphate at the beginning of glycolysis (reviewed in [22]). During growth on a

206   non-glycolytic substrate, phosphorylated EIIA^{glc} accumulates and activates the

207   adenylate cyclase, CyaA, leading to synthesis of cyclic-AMP (cAMP) [23,24]. A co-

208   activator, cAMP binds to Crp facilitating DNA binding and stimulating transcription

209   of a broad swathe of genes including those involved in the transport and utilization

210   of non-glycolytic carbon sources [25,26]. If *crr* impacts cell size via its impact on

211   cAMP production and Crp activation, defects in both *cyaA* and *crp* should be

212   phenotypically identical to *crr* mutants with regard to cell size and growth rate. We

213   found that this is indeed true as deletions in *crr*, *cyaA*, or *crp* led to similar growth

214   rates (29, 29, and 28 minutes) and cell size (2.1 square micron area for all three

215   mutants). This implies that the growth rate and size phenotypes of *crr::kan* are

216   caused not by a lack of glucose import but instead by blocking cAMP synthesis.

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217   Generally speaking, most of the CCM mutants retained a positive relationship

218   between nutrient availability and cell size with the exception of the three cAMP

219   mutants, *crr*::*kan*, *cyaA*::*kan*, and *crp*::*kan* (Fig 3). During growth in carbon poor

220   and/or gluconeogenic conditions, the lengths of Class I and Class II mutants

221   approached that of wild type, particularly in AB-succinate medium, and the

222   heterogeneous phenotype exhibited by mutants in Class III and Class IV was largely

223   relieved. Width also decreased, although more modestly. The average width of the

224   mutants ranged from 0.68-0.86 microns during growth in LB-glu and 0.48-0.60

225   microns during growth in AB-suc. Consistent with catabolite repression serving to

226   inhibit division during growth on a gluconeogenic carbon source, the *crp*◻*kan*

227   mutants (dotted line in Fig 3B, 3C, and 3D) did not show any change in size when

228   switched from LB-glu to AB-glu. Both *crr*::*kan* and *cyaA*::*kan* (magenta and gray in

229   Fig 3) were similar to *crp*::*kan*, showing only minor, insignificant changes in length,

230   with no change in width. Unfortunately, *crr*, *cyaA*, and *crp* are essential under

231   gluconeogenic conditions, preventing analysis of these mutants in AB-succinate.

232   Although we were able to obtain cell size data for *rpiA*, *crr*, *rpe*, *aceE*, and

233   *sucA* in carbon rich (LB-glu) and carbon poor (AB-glu) conditions, all five mutants

234   were unable to grow in the gluconeogenic condition, AB + 0.2% succinate. At first

235   glance this finding appears to be in conflict with data indicating that *rpiA*, *rpe*, *aceE*,

236   and *sucA* are capable of growth in another carbon poor gluconeogenic condition: M9

237   + 0.1% glycerol [27]. However, this discrepancy likely reflects the ability of glycerol

238   to enter the CCM partway through glycolysis—as dihydroxyacetone phosphate—

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239   while succinate enters through the TCA cycle, leading to dependence on different

240   pathways through CCM to synthesize all needed carbon metabolites.

241

242   **Defects in CCM differentially impact assembly of the cell division machinery**

243   To gain insight into the relationship between growth rate, cell cycle

244   progression and CCM during growth in LB-glucose, we assessed the ability of

245   different mutants to suppress the heat sensitivity associated with the GTP binding

246   defective FtsZ mutant, *ftsZ84* (G105S) [28]. While *ftsZ84* supports both FtsZ

247   assembly and division under permissive conditions (LB at 30°C), under non-

248   permissive conditions (LB with no salt at 42°C), FtsZ assembly and division are

249   blocked, leading to filamentation and cell death [7,29]. Based on extensive work

250   from our group and others, conditions or mutations that enhance FtsZ assembly are

251   expected to suppress the heat sensitivity of temperature sensitive *ftsZ* alleles

252   [4,17,30,31]. Changes in the availability of CCM products or components that

253   normally inhibit FtsZ assembly should restore growth and colony formation to

254   *ftsZ84* mutants under non-permissive conditions while defects in CCM-related

255   factors that normally promote FtsZ assembly will enhance *ftsZ84* heat sensitivity.

256   Double mutants in CCM deletions and *ftsZ84* were generated to examine the impact

257   of these mutants on FtsZ assembly.

258   Consistent with a positive impact on FtsZ assembly, the majority of Class I-V

259   mutants promoted growth of *ftsZ84* cells under mildly restrictive conditions (37°C,

260   LB no salt) (Fig 4A). The strongest suppressors were loss-of-function mutations in

261   *aceE* or *ackA* both of which increased *ftsZ84* cell viability 10,000-fold during growth

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262   at 37°C on salt-free LB medium. Mild suppression was observed in loss of function

263   mutations in *crr*, *tktA*, and *pta* which increased *ftsZ84* cell viability 1,000-fold, 100-

264   fold, and 100-fold respectively. The *zwf* deletion was alone in leading to

265   enhancement of *ftsZ84*, i.e. lack of growth at 30° on LB-no salt (Fig 4B). Combined

266   with the short and long phenotype of *zwf☐kan*, it appears that loss of *zwf* leads to

267   impairment of FtsZ activity. Significantly, we did not observe a consistent

268   correlation between reduced growth rate and suppression of *ftsZ84* heat-sensitivity

269   among the various CCM mutants.

270   As part of these experiments we also revisited the impact of a loss of function

271   mutation in *pgm*, encoding phosphoglucomutase—the enzyme that converts

272   glucose-6-phosphate to glucose-1-phosphate. In previous work, we had reported

273   that deletions in *pgm* suppress *ftsZ84* heat sensitivity [12]. However, we were

274   unable to detect any suppression of *ftsZ84* by *pgm* in this instance. Resequencing

275   BH173, the strain employed in the original study revealed acquisition of a reversion

276   mutation that restored *ftsZ84* sequence to wild type.

277   To gain a better understanding of the mechanisms coordinating central

278   carbon metabolism with cell morphology, we undertook an in depth analysis of two

279   distinctive phenotypes: the reduction in length and width observed in cells defective

280   in catabolite repression and the link between acetate metabolism, cell growth and

281   cell cycle progression revealed by defects in *aceE, ptaA, and ackA* (Fig 7). The

282   results of these efforts are described below.

12

283   **Catabolite-dependent regulation of FtsZ assembly and function**

284   Reasoning that if the product of *crr,* EIIA^{glc}, was impacting division indirectly

285   via its role in stimulation of adenylate cyclase and subsequent activation of the Crp

286   transcription factor, we hypothesized that 1) defects in either the adenylate cyclase

287   (encoded by *cya*) or *crp* should restore growth to *ftsZ84* cells under non-permissive

288   conditions, and 2) the addition of extracellular cAMP (which is able to diffuse

289   through the membrane [32]) to *crr::kan ftsZ84* cells should bypass *crr::kan* mediated

290   suppression of *ftsZ84* heat sensitivity but only in the presence of Crp. Consistent

291   with this model, deletion of either *cya* or *crp* phenocopied *crr☐kan* mediated *ftsZ84*

292   suppression at 37°C on salt free medium (Fig 5). More directly, 5 mM cAMP was

293   sufficient to restore heat sensitivity to *crr::kan ftsZ84* cells as well as cells defective

294   in adenylate cyclase (*cyaA*) but only in the presence of *crp* (Fig 5). Together, these

295   data strongly argue for the presence of at least one inhibitor of FtsZ assembly within

296   the extensive Crp regulon.

297

298   **cAMP controls cell width through regulation of the morphogene *bolA***

299   In contrast to wild type cells, which decrease in both length and width upon a

300   shift from glycolytic (low cAMP) to gluconeogenic (high cAMP) conditions, the *crp*

301   deletion showed no significant changes in width when changing media from LB-glu

302   to AB-glu, suggesting a potential role for cAMP in cell width regulation (Fig 3). To

303   explore this idea, we examined the impact of extracellular cAMP on wild type

304   (MG1655) cells cultured in both glycolytic and gluconeogenic conditions.

13

305   Consistent with a negative impact on cell width, 5 mM exogenous cAMP was

306   sufficient to decrease wild type cell width in both LB and AB + 0.2% glucose (0.96 to

307   0.81 microns and 0.73 to 0.68 microns respectively) (Fig 6A). The addition of cAMP

308   also significantly decreased cell length (3.8 to 3.2 microns for LB and 2.7 to 2.5

309   microns for AB-Glu) and growth rate (2.7 to 2.3 doubling per hour for LB and 0.98 to

310   0.88 doublings per hour for AB-Glu) (Fig 6B). Significantly, cAMP-dependent

311   reductions in size are independent of growth rate. Reducing levels of exogenous

312   cAMP from 5 mM to 1 mM decreased the growth rate of *cyaA*::*kan* (which is unable

313   to synthesize any cAMP) in gluconeogenic conditions (AB + 0.2% succinate) from

314   0.87 to 0.79 doublings per hour, while cell width increased from 0.65 to 0.75

315   microns (Fig 6C). Although width was independent of growth rate, length was not as

316   the *cyaA*::*kan* mutant length decreased with lower cAMP from 2.6 microns in 5 mM

317   cAMP to 2.2 microns in 1 mM cAMP in a manner similar to the growth rate (Fig 6D).

318   cAMP has been implicated in the Crp dependent activation of the

319   transcription factor OmpR , which inhibits expression of a host of genes, including

320   several whose products have morphogenic activity [33]. Of particular interest is

321   *bolA*, encoding a transcription factor that inhibits transcription of *mreB* [34], which

322   encodes a key component of the elongation machinery [35]. Reductions in MreB

323   activity lead to a block in synthesis of lateral cell wall material and the formation of

324   wide and rounded cells [36,37]. *bolA* transcription has previously been shown to be

325   inhibited by addition of extracellular cAMP [38].

326   Together, genetic interactions between *crp, ompR,* and *bolA,* support a model

327   in which cAMP increases cell width indirectly, via down regulation of OmpR and the

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328   concomitant increase in BolA expression leading to repression of MreB mediated

329   cell wall synthesis. Consistent with this model, cells defective in *ompR* and *bolA*

330   were refractile to the addition of exogenous cAMP with regard to cell width (Figs 6E

331   and 6F).

332

333   **Defects in acetate metabolism support links between fatty acid synthesis and**

334   **assembly of the cell division machinery**

335   Cell cycle progression is tightly correlated with growth rate in wild-type

336   cells. Fast growing cells are not only larger but contain a higher frequency of FtsZ

337   rings (Z-rings) with approximately 90% of *E. coli* cells containing visible Z-rings in

338   LB-glucose. Meanwhile, slow growing cells contain fewer Z-rings (~50%). Z-ring

339   frequency shows a roughly linear relationship to growth rate when grown in

340   different media (Fig 4C dotted line) [12,39]. Taking advantage of a green fluorescent

341   protein (GFP)-FtsZ fusion that allows us to label the FtsZ ring without detectably

342   altering size or growth [6], we assessed the frequency of FtsZ ring formation in the

343   17 mutants in Classes I through V.

344   Although the Z-ring frequency and growth rate were correlated in the

345   majority of CCM mutants, several exhibited a disconnect between these two

346   parameters, specifically those associated with acetate metabolism (black squares in

347   Fig 4C). Cells defective in *aceE*, the E1 protein of pyruvate dehydrogenase that

348   converts pyruvate to acetyl-CoA, exhibited an extremely high (86%) Z-ring

349   frequency relative to wild type cells cultured in medium of the same growth rate

350   (~75%) [12], while both *ackA* and *pta*, involved in converting acetyl-CoA to acetate,

15

351   exhibited lower Z-ring frequencies relative to growth rate (69% and 67%

352   respectively). Assembly of the cell division machinery is a multistep process

353   involving first, recruitment of FtsZ and other so-called early proteins to the nascent

354   division site followed by a brief delay and assembly of the late proteins, which

355   include enzymes required for synthesis of the septal cell wall (reviewed in [40]). In

356   *E. coli*, division is coincident with recruitment of FtsN, the last protein to be

357   incorporated into the division machinery and the putative "trigger" for cross wall

358   synthesis [41,42]. The two-step nature of division machinery assembly suggests two

359   potential explanations for the elevated Z-ring frequency in *aceE*::*kan* mutants: 1)

360   premature recruitment of FtsZ to the septum or 2) delays in late protein

361   recruitment and activation of cytokinesis. Conversely, the lower frequency of FtsZ

362   rings in *ackA*::*kan* and *pta*::*kan* mutants could be a consequence of either 1) delays

363   in FtsZ recruitment or 2) premature late protein recruitment and/or activation of

364   division mediated cell wall synthesis.

365   To clarify the underlying cause of the elevated Z-ring frequency in *aceE*::*kan*

366   cells and depressed Z ring frequency in the *ackA*::*kan* and *pta*::*kan* backgrounds we

367   took advantage of a heterologous, IPTG-inducible FtsN-GFP [43] to assess the

368   efficiency of FtsN recruitment in all three mutants. Growth in LB-glu showed 32% of

369   wild-type cells with FtsN-GFP localization at mid-cell (Fig 7A). In contrast, despite

370   varying Z-ring frequencies, all three mutants exhibit more or less the same

371   frequency of FtsN ring formation (24%, 23%, and 26% for *aceE*::*kan*, *ackA*::*kan*, and

372   *pta*::*kan* respectively) with all three being significantly less than wild type. The

373   consistency of FtsN ring frequency, despite differences in Z-ring frequency between

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374   the three CCM mutants, suggests that it is delayed recruitment of the late division

375   proteins and/or activation of cytokinesis that is responsible for the high Z-ring

376   frequency in *aceE*::*kan* cells while the lower Z-ring frequeny in *ackA*::*kan* and

377   *pta*::*kan* mutants would appear to be a consequence of a delay in FtsZ assembly.

378   Acetyl-CoA metabolism plays a critical role in the ability of cells to synthesize

379   the wide variety of fatty acids and lipids that constitute the cell envelope [44]. *aceE*,

380   in particular, encodes a subunit of the pyruvate dehydrogenase complex responsible

381   for aerobic synthesis of the majority of acetyl-CoA, a necessary precursor in the first

382   step of FAS [45]. Defects in *aceE* are thus expected to reduce flux through FAS, and

383   based on previous work, shift the balance in lipid composition [46]. Although not

384   directly involved in FAS, defects in *pta* and *ackA* may lead to a build-up of acetyl-CoA

385   and potentially also shift flux through FAS.

386   Based on previous studies linking lipid composition to cell morphology [47],

387   we speculated defects in acetate metabolism might result in changes in plasma

388   membrane composition that interfere with assembly and activity of the cell division

389   machinery. To test this idea, we measured the Z-ring frequency of wild type and

390   mutant cells cultured in the presence of sub-inhibitory concentrations of the fatty

391   acid synthesis inhibitor triclosan (50 ng/mL). This concentration is too low to

392   impact growth rate but has a modest positive impact on cell size [6]. Triclosan

393   inhibits FabI, the enzyme that catalyzes the reduction of enoyl-ACP during the

394   elongation cycle of fatty acid synthesis [48]. Although not a perfect recapitulation of

395   a block upstream of acetyl-CoA synthesis, as it blocks a downstream step, both

17

396   triclosan treatment and the *aceE* mutations should similarly curtail flux through

397   FAS.

398   In support of a model in which modest reductions in flux through FAS

399   negatively impact assembly of the cell division machinery, Z-ring frequency was

400   significantly increased in wild type cells as well as in *aceE::kan, pta☐kan*, and

401   *ackA☐kan* cells during growth in sub-MIC (minimum inhibitory concentration), 50

402   ng/mL triclosan. (Z-ring frequency: wild type 88% +T/95% -T; *pta::kan* 56% -T/

403   79% + T; *ackA::kan* 60% -T/74% +T; *aceE☐kan* 79% -T to 87% +T) (Fig 7B). *pta* or

404   *ackA* deletion mutants were slightly more sensitive to triclosan with regard to Z-ring

405   frequency than either *aceE* or wild type cells, likely a reflection of the already high

406   frequency of FtsZ rings in these latter strains.

407   Further supporting a positive connection between FAS and assembly of the

408   cell division machinery, 50 ng/mL triclosan enhanced the heat sensitive phenotype

409   of conditional mutations in several cell division genes including *ftsA* (*ftsA27*-an

410   early, actin-like protein), *ftsK* (*ftsK44*—a late division protein involved in

411   chromosome partitioning), *ftsQ* (*ftsQ1*—a late division protein believed to serve

412   primarily as a scaffold), and *ftsI* (*ftsI23*—a late division protein with a trans-

413   peptidase domain) [49-52]. Somewhat surprisingly given its impact on FtsZ ring

414   frequency (Fig 7B), triclosan had no impact on the heat-sensitivity of *ftsZ84* (Fig 7C).

415   We speculate that the differential impact of triclosan on *ftsZ84* relative to the other

416   conditional mutants is a consequence of their subcellular location (cytoplasmic –

417   FtsZ) versus membrane associated (FtsA) and integrated into the membrane (FtsQ,

418   FtsK, and FtsI) [40].

18

419   **Discussion**

420   Our systematic analysis of the relationship between central carbon

421   metabolism, cell growth, and cell size both reinforces the Growth Law as a general

422   principal (i.e. growth rate and size are positively correlated) but also calls into

423   question many of the fundamental assumptions that underlie it. While the largest

424   class of mutants with observable phenotypes—Class II: small with reduced growth

425   rates, essentially "obeyed" the law so to speak, growth rate and size varied

426   independently in the other classes of CCM mutants (Fig 1B). Class II mutants closely

427   resemble cells cultured in nutrient-poor conditions with regard to growth rate and

428   size, suggesting these cells are experiencing a near global reduction in biosynthetic

429   capacity. In contrast, growth rate and morphology are independent of one another

430   in the other nine CCM mutants (Table 1), consistent with defects in specific

431   regulatory or structural functions that directly impact cell length and/or width.

432   These results are in line with accumulating work indicating that growth rate

433   and cell size are independent phenomena. Modest defects in cell division are well

434   known to increase bacterial cell size—particularly length—without impacting

435   growth rate [4,7,50,51,53], while defects in transcription severely curtail growth but

436   have little detectable impact on morphology [6]. Most importantly, an exhaustive

437   phenotypic analysis of the entirety of non-essential genes in *E. coli* by the Jacobs-

438   Wagner group, failed to identify any evidence supporting a direct connection

439   between growth rate and size [54].

440

441

19

442 **Overflow metabolism and cell size**

443   In a common, yet paradoxical, fermentative mechanism known as "overflow

444   metabolism", *E. coli* convert up to 50% of available glucose into acetate during

445   aerobic growth in nutrient rich conditions [55] . Acetate fermentation is inefficient,

446   resulting in ~14 fewer ATP per glucose than oxidative phosphorylation and, unlike

447   other fermentative pathways, does not regenerate NAD$^+$ from NADH [56]. While it is

448   unclear why bacteria perform overflow metabolism, it is potentially a more

449   proteome-efficient means of moving carbon through CCM, ensuring that glycolysis

450   proceeds at a maximum pace when nutrients are plentiful.

451   Supporting the idea that overflow metabolism is critical for maximizing

452   glycolytic flux, defects in *pta, ackA,* and *aceE*, encoding the first two enzymes in

453   acetate fermentation and a subunit of pyruvate dehydrogenase respectively, are

454   dramatically impaired in both growth rate and cell size. This is consistent with a

455   global reduction in biosynthetic capacity. Whether this reduction is the result of

456   lower ATP production or the consequence of a bottleneck in flux through critical

457   biosynthetic pathways is unclear. Regardless, we suspect that reductions in ATP

458   levels and/or biosynthesis indirectly result in accumulation of the alarmone ppGpp

459   [57], providing an explanation for the observed suppression of *ftsZ84* heat-

460   sensitivity in *pta, ackA,* and *aceE* deletion strains. Increases in ppGpp are well

461   known to suppress the conditional nature of *ftsZ84,* although the precise mechanism

462   has yet to be determined [17,58] .

463

464

20

465 **cAMP is a key player in maintaining the correct cellular aspect ratio**

466   A somewhat surprising finding is the identification of cAMP as a contributor

467 to nutrient dependent reduction in both cell length and width. Accumulating under

468 gluconeogenic conditions, cAMP binds to the transcriptional activator Crp and

469 regulates expression of more the than 280 genes [59]. Although the precise

470 member(s) of the cAMP-Crp regulon responsible remains elusive, our data indicate

471 that cAMP-Crp function together to reduce width under nutrient poor conditions via

472 their impact on the morphogenic factor BolA and cell length via its impact on the

473 assembly of FtsZ (Figs 5 and 6). In this regard, cAMP differs from metabolic

474 signaling molecule, ppGpp, which genetics indicate is a positive regulator of division

475 (Fig 8).

476

477 **CCM is robust but not necessarily efficient**

478   Perhaps the biggest surprise of this study is our finding that the defects in the

479 majority of non-essential CCM genes had little to no detectable impact on either cell

480 size or growth rate. While the lack of phenotype in many cases is likely a

481 consequence of our screening conditions, nutrient-rich, complex medium

482 supplemented with glucose, in certain cases the robust growth we observed is

483 difficult to explain. For example, we anticipated that defects in phosphoglucose

484 isomerase (encoded by *pgi*), the first dedicated step in glycolysis would dramatically

485 impact growth rate and size by virtue of its impact on not only glycolysis, but also

486 other associated pathways leading to reductions in ATP synthesis and overall

487 biosynthetic capacity [60]. Instead, we were unable to distinguish *pgi* mutants from

21

488   wild type cells during growth LB-glucose (Table S2). Based on our identification of

489   seven mutants in the pentose-phosphate pathway, we hypothesize that despite the

490   ubiquitous nature of glycolysis in nutrient- rich medium, glucose is predominantly

491   driven through the pentose-phosphate pathway to generate essential building

492   blocks.

493   Similarly, we observed little in the way of a detectable phenotype associated

494   with defects in the gene encoding citrate synthase *gltA*. Essential for the complete

495   oxidation of carbon, GltA synthesizes citrate from oxaloacetate and acetyl-CoA at the

496   entry point into the TCA cycle [61]. Despite its seeming importance, previous work

497   indicates that *E. coli* represses *gltA* transcription in the presence of glucose,

498   suggesting that it can forgo complete carbon oxidation under nutrient rich

499   conditions [59,62]. It may be that under nutrient rich condition, *E. coli* maximizes

500   rapid growth over the efficient use of available carbon, a proposition that is in line

501   with our data indicating that defects in acetate metabolism significantly curtail

502   growth in nutrient rich medium (Table 1).

503

504   **Concluding thoughts**

505   While this screen demonstrates the ability of systematic phenotypic analysis

506   to identify new connections between CCM, cell growth and cell size, many questions

507   remain unanswered. In addition to the large number of insertion mutants with no

508   observable phenotype, defects in 17 genes resulted in dramatic changes in growth

509   rate and cell morphology. Of these, we were able to identify the proximal cause in

510   24% (*crr*, *aceE*, *pta*, *ackA*; Figs 5-7). A better understanding of the remaining 13

22

511   mutations will likely require a combination of genetics and metabolomics to tease

512   apart the root cause of their morphological and growth related defects.

513   Additionally, although this effort focused specifically on carbon metabolism,

514   the availability of other nutrients—particularly nitrogen, phosphorous, sulfur and

515   iron— have also been implicated in growth and morphology. Assessing the

516   phenotype of the CCM insertion collection under conditions in which these factors

517   are limiting is thus also worthwhile. Finally, *E. coli* is believed to spend much of its

518   time under microaerobic or anaerobic conditions. Understanding how oxygen levels

519   impact growth, cell cycle progression, and cell morphology thus remains a

520   tantalizingly open question.

521

522   **Materials and Methods**

523   **Strains and media**

524   All chemicals, media components, and antibiotics were purchased from

525   Sigma-Aldrich (St. Louis, MO). *E. coli* strains were constructed through standard P1

526   vir transductions [63] moving kanamycin marked insertion deletions from Keio

527   collection strains [18] purchased from the Coli Genomic Stock Center into MG1655

528   (F⁻ λ⁻ *ilvG*- *rfb*-50 *rph*-1), our "wild type" parental strain. Cell were cultured in Luria-

529   Bertani (LB) broth supplemented with 0.2% glucose or AB defined media [64]

530   supplemented with either 0.2% glucose or 0.2% succinate as the sole carbon source.

531   Unless otherwise noted (e.g. for heat sensitive conditional alleles of cell division

532   genes including *ftsZ84*, *ftsA27*, *ftsK44*, *ftsQ1*, and *ftsI23*), cells were cultured at 37° C

533   and sampled for analysis at OD_{600} between 0.1 and 0.2. For the primary analysis cells

23

534   were cultured in 5 mL LB + 0.2% glucose and aerated at 200 rpm. For measuring the

535   effect of different media, cells were cultured in 200 µL of specified media in a 96-

536   well plate and aerated at 567 rpm in a BioTek© Eon© plate reader (Fig 3).

537

538   **Microscopy**

539   Cells were sampled directly from cultures and fixed in 16%

540   paraformaldehyde (Electron Microscopy Sciences) as described [6]. Fixed cells were

541   mounted on agarose pads and visualized using a 100x phase-fluor objective on a

542   Nikon© Ti-E inverted microscope. To facilitate visualization of FtsZ rings, 1 mM IPTG

543   was added to cultures of cells encoding a chromosomal P_{IPTG}-*ftsZ-GFP* [65].

544   Expression of a chromosomal P_{IPTG}-*ftsN-GFP* was induced with 50 µM IPTG [43]. GFP

545   fluorescence was detected with a C-FL GFP filter cube with 470/40nm excitation

546   and 525/50nm emission (Chroma Technology Corporation) Nikon Elements

547   software (Nikon Instruments, Inc.) was used for image capture and analysis. >200

548   cells were counted per experimental replicate and at least three independent

549   replicates were used for each data point.

550

551   **Cell Size Measurements**

552   Cell size from phase-contrast images was determined with one of two freely

553   available segmentation software packages: the ImageJ© plug-in Coli Inspector [19]

554   (Fig 1, Tables 1 and S2) or SuperSegger, a MatLab© based application [20] (Figs 3-7,

555   Table S3). As Coli Inspector does not determine area directly, we estimated it as

556   length x width. SuperSegger directly determines the area of individual cells. We

24

557   determined the area of ≥100 cells for each experiment and/or condition. Unless

558   otherwise noted, average size was determined from 3 independent experiments.

559

560   **Dot plating efficiency assays**

561   Heat-sensitive cell division alleles, *ftsZ84*, *ftsA27*, *ftsK44*, *ftsQ1*, and *ftsI23*

562   were cultured in LB at 30°C degrees to an OD_{600} of ~0.5. 1 mL of cells were then

563   pelleted, washed once with LB-no salt, and re-suspended to an OD_{600} of 0.1 in LB-no

564   salt. 10 µL of serial dilutions between 10^{-1} to 10^{-6} were plated under permissive

565   conditions (LB at 30°C) or non-permissive conditions (LB-no salt at 37°C). Due to

566   the strains extreme sensitivity, LB-no salt at 30°C was non-permissive for *zwf::kan*

567   *ftsZ84* double mutants. For cAMP or triclosan addition experiments, 5 mM cAMP or

568   50 ng/mL triclosan was added directly to LB agar prior to pouring the plates. Each

569   plating efficiency experiment was repeated three times with representative images

570   shown.

571

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579

25

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791

792   **Figure Legends**

793   **Figure 1: The effect of central carbon metabolic gene deletions on cell growth**

794   **and size.** (A) Mutant phenotypes mapped onto central carbon metabolic pathways.

795   Arrows are colored based on the phenotype as follows: black= normal growth and

796   size; green = normal growth and small size (<10% smaller than WT); red = slow

797   growth (>20% increase in doubling time) and small size; orange = normal growth

798   and long cells (>10% larger than WT); blue = short and long cells; purple = highly

799   variable size; and dotted lines = essential genes. Brown arrows correspond to

800   deletions that obtained suppressor mutations rapidly and were not analyzed. (B)

801   Relationship between cell area and growth rate, as doublings per hour. Icons are

802   colored as in panel (A).

803

804   **Figure 2: Representative images of all CCM mutants exhibiting morphology**

805   **distinct from the wild type parental strain.** Merged images of morphologically

806   distinct CCM mutant strains expressing an *ftsZ-GFP* fusion to permit visualization of

807   the cytokinetic ring. Scale bar = 5 microns and is the same for all images.

808

809   **Figure 3: CCM deletions exhibit medium-dependent effects on growth and**

810   **morphology.**   (A) Cell size versus growth rate in LB-glucose (black diamonds), AB-

811   glucose (orange triangles), and AB-succinate (blue circles). Length (B), width (C),

812   and area (D) are plotted for each mutant in the three different media. Dotted line =

813   *crp*::*kan* in LB-glucose and AB-glucose.

814

32

815   **Figure 4: CCM mutations differentially impact FtsZ localization and activity.**

816   (A) Dot-plating efficiencies of the CCM deletion *ftsZ84* double mutants cultured

817   under permissive (LB, 30°C) and non-permissive (LB-no salt, 37°C) conditions. (B)

818   Dot-plating efficiency of *zwf*::*kan ftsZ84* double mutant. Note the reduction in

819   *zwf*::*kan ftsZ84* viability under intermediate growth conditions (30°C on LB-no salt).

820   (C) FtsZ ring frequency versus growth rate for the CCM mutants. For reference we

821   have included previously reported data on the relationship between nutrient

822   availability and FtsZ ring frequency (dotted black line) [12]. Mutants are grouped

823   by metabolic pathway: black squares = acetyl-CoA production and acetate

824   fermentation; green triangles = pentose-phosphate shunt; purple diamonds = TCA

825   cycle; orange circle = Entner-Doudoroff pathway; blue cross = cAMP synthesis; and

826   red plus = gluconeogenesis.

827

828   **Figure 5: Loss of cAMP signaling suppresses *ftsZ84* heat-sensitivity.**

829   Representative dot-plating efficiencies of *ftsZ84* alone or in combination with

830   insertion deletions in cAMP signaling genes *crr*, *cyaA*, and *crp*. Cells were plated on

831   and grown at permissive (LB, 30°C), non-permissive (LB-no salt, 37°C), and non-

832   permissive containing cAMP (LB-no salt + 5mM cAMP, 37°C) conditions.

833

834   **Figure 6: cAMP signaling reduces cell width via the morphogenic transcription**

835   **factor, *bolA*.** Wild-type MG1655 cell size was measured with and without

836   exogenous 5 mM cAMP in either LB or AB-glucose. Width is shown in (A) and length

837   in (B). Width (C) and length (D) of *cyaA*::*kan* grown in AB-succinate with different

33

838   levels of exogenous cAMP shows that width is not correlated with growth rate but

839   length is. (E) Deletions in *crp*, *ompR*, or *bolA* abolish the cAMP-dependent cell

840   reductions in cell width. (F) Proposed genetic pathway linking cAMP to cell width.

841

842   **Figure 7: Partial inhibition of fatty acid synthesis negatively impacts assembly**

843   **and activity of the late division proteins.** (A). FtsN septal localization was

844   measured in MG1655, *aceE::kan*, *ackA::kan*, and *pta::kan* expressing an *ftsN-GFP*

845   from an IPTG inducible promoter   (B.) FtsZ-ring frequency was measured MG1655,

846   *aceE::kan*, *ackA::kan*, and *pta::kan* cells expressing *ftsZ-GFP* from an IPTG-inducible

847   promoter. Cells were cultured with (gray) or without (black) 50 ng/mL triclosan. (C)

848   Representative plating efficiencies of wild-type cells and the heat-sensitive cell

849   division mutants *ftsZ84*, *ftsA27*, *ftsK44*, and *ftsQ1* cultured on LB at 37°C with or

850   without triclosan. *ftsI23* was plated on LB-no salt at 37°C with or without triclosan.

851

852   **Figure 8: Proposed pathway linking nutrient levels to cell length and width**

853   **through the signaling nucleotides cAMP and ppGpp.**

854

855   **Supporting Information**

856   **Table S1. List of bacterial strains used in this study**

857

858   **Table S2. Raw size and growth rate data for all central carbon metabolic gene**

859   **deletions.**

860

34

861   **Table S3. Raw data for figure 3 (CCM mutant growth in different media).**

862

863   **Table S4. Raw data for figure 4C (Z-ring frequency of CCM mutants).**

864

865

35

A

Glucose

KDG 6-P ← Gluconate 6-P ← Gluconolactone 6-P ← Glucose 6-P

Ribulose 5-P

Glucose 6-P → Fructose 6-P → Fructose 1,6-diP

Ribose 5-P ⇌ Xylulose 5-P

Sedoheptulose 7-P

Fructose 6-P ⇌ Erythrose 4-P

Glyceraldehyde 3-P ↔ Dihydroxyacetone P

1,3-bisphosphoglycerate

3-phosphoglycerate

2-phosphoglycerate

Glyceraldehyde 3-P

PEP

Pyruvate

Acetate ↔ Acetyl-P ↔ Acetyl-CoA

Oxaloacetate → Citrate

Malate ← Glyoxylate ← Isocitrate

Fumarate   2-oxoglutarate

Succinate ← Succinyl-CoA

Legend:
- WT growth/size (black)
- WT growth & long (orange)
- Slow growth & short (red)
- WT growth & short (green)
- Forms suppressors (brown)
- Short and long (blue)
- Heterogenous (purple)
- Essential (dashed black)

B

Plot of Area (μm²) versus Doublings per hour

MG1655

yggF::*kan*

talB::*kan*

eda::*kan*

acnB::*kan*

zwf::*kan*

gpmM::*kan*

tpiA::*kan*

gnd::*kan*

sucC::*kan*

tktA::*kan*

crr::*kan*

ackA::*kan*

pta::*kan*

aceE::*kan*

rpiA::*kan*

rpe::*kan*

pgl::*kan*

A

Area (µm²) vs Doublings per hour

B

Length (µm): LB-Glu, AB-Glu, AB-Suc

C

Width (µm): LB-Glu, AB-Glu, AB-Suc

D

Area (µm²): LB-Glu, AB-Glu, AB-Suc

A

30° on LB | **37° on LB-no salt**

Dilution (10ˣ): 0 -1 -2 -3 -4 -5   0 -1 -2 -3 -4 -5

ftsZ84

tktA::*kan ftsZ84*

ackA::*kan ftsZ84*

pta::*kan ftsZ84*

aceE::*kan ftsZ84*

crr::*kan ftsZ84*

B

30° on LB | **30° on LB-no salt**

Dilution (10ˣ): 0 -1 -2 -3 -4 -5   0 -1 -2 -3 -4 -5

ftsZ84

zwf::*kan ftsZ84*

C

Cells with a FtsZ ring (%) vs Doublings per hour

	30° on LB	37° on LB-no salt	37° on LB-no salt + 5 mM cAMP
Dilution (10ˣ):	0 -1 -2 -3 -4 -5	0 -1 -2 -3 -4 -5	0 -1 -2 -3 -4 -5
MG1655			
ftsZ84			
crr::kan ftsZ84			
cyaA::kan ftsZ84			
crp::kan ftsZ84			
aceE::kan ftsZ84			

A — % N-ring frequency for MG1655, *aceE*::*kan*, *ackA*::*kan*, *pta*::*kan*

B — % Z-ring frequency (-T/+T) for MG1655, *aceE*::*kan*, *pta*::*kan*, *ackA*::*kan*

C — Dilution spot assays

37° on LB / 37° on LB+Tric-50

Dilution (10^x): 0 -1 -2 -3 -4 -5

MG1655, *ftsZ84*, *ftsA27*, *ftsK44*, *ftsQ1*

37° on LB-no salt / 37° on LB-no salt+Tric-50

Dilution (10^x): 0 -1 -2 -3 -4 -5

ftsI23