1 **Collective dynamics of *Escherichia coli* growth under near-lethal acid stress**

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Abstract

Many neutralophilic bacteria, including *Escherichia coli*, can withstand acidic conditions due to the action of several protective mechanisms. While the survival of *E. coli* under growth-inhibitory extreme acid stress is well understood, less is known about the physiology of *E. coli* growth under severe but permissive acidity. Here, we observed that growth of *E. coli* MG1655 in a rich medium with pH of 4.1 to 4.4 exhibits a characteristic multi-phasic growth pattern, consisting of an initial exponential elongation in the absence of cell division, followed by growth arrest and subsequent resumption of exponential growth. The duration of the growth arrest phase was strongly dependent on the pH of the medium, but also on the initial cell density of the culture, suggesting the collective nature of this phenomenon. Cell-density dependent multiphasic growth at the near-lethal pH, including the initial increase in cell volume associated with either elongation or widening of the cell body, was also observed for all tested natural *E. coli* isolates and for *Salmonella enterica* serovar Typhimurium. Such transient increase in volume apparently enables bacteria to induce acid resistance systems, including the lysine-dependent Cad system, to subsequently modify pH of the medium in the density-dependent manner. Consistent with the collective recovery, even a minor fraction of acid-tolerant cells could fully cross-protect acid-sensitive cells enabling them to resume growth in coculture. Thus, collective dynamics plays a central role in bacterial growth under near-lethal acid stress.

Importance

Since many *E. coli* and other Enterobacteriaceae isolates are gastrointestinal pathogens for humans, it is important to understand their growth under acidic conditions imposed by the host physiology in the stomach, colon, and inside macrophages. Here we show that *E. coli* growth under near-lethal

43 acidic conditions is a collective phenomenon that critically depends on cell density. This collective

44 behavior is favored by changes in cell morphology as a response to high acidity. The observed

45 density dependence might have implications for pathogen proliferation in the acidic environment

46 of the human gastrointestinal tract and possibly also for their interactions with immune cells.

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48 **Keywords:** *Escherichia coli*, Enterobacteriaceae, acid resistance, collective behavior

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50 **Introduction**

51 Neutralophilic bacteria grow optimally about two units below and above pH 7.0 (1). Some

52 neutralophiles, such as *Escherichia coli*, have evolved multiple protective mechanisms that allow

53 them to grow in acidic environments, such as the mammalian gastrointestinal tract and acidified

54 water, soil, and food (2–8). The acid resistance of *E. coli* and other human pathogens has mostly

55 been studied under growth-inhibiting, lethal acid stress (pH < 3.0) that simulates an infection-

56 related passage through the stomach (9, 10), and to a much lesser extent under growth-permitting,

57 near-lethal acid stress (pH 4.0-5.0) that mimics other common acidic environments (9, 11), such as

58 the human duodenum, duodeno-jejunal junction and proximal colon (7, 8).

59 Among multiple protective mechanisms of *E. coli*, the most prominent are the amino acid-

60 dependent acid resistance (AR) systems, which rely on H^+ consumption during amino acid

61 decarboxylation to raise the cytoplasmic pH (2–5). These include the glutamate-dependent Gad

62 system (GDAR or AR2), the arginine-dependent Adi system (ADAR or AR3) and the lysine-

63 dependent Cad system (LDAR or AR4) (2, 12). Each of these systems consists of a pH-dependent

64 transcriptional regulator (GadE, AdiY or CadC, respectively), an amino acid decarboxylase

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65   (GadA/GadB, AdiA or CadA) and an antiporter (GadC, AdiC or CadB) that imports the respective

66   external amino acid and exports the more alkaline product of amino acid decarboxylation, which

67   is γ-aminobutyric acid (GABA) for the Gad system, agmatine for the Adi system, and cadaverine

68   for the Cad system. These different systems are known to be induced under different conditions,

69   with the Gad and Adi systems being particularly important under extreme acid stress, stationary

70   phase, and/or anaerobic conditions, and the Cad system operating under mild (pH 5.0-5.8) and

71   near-lethal acid stress (13). Moreover, a recent study demonstrated that operation of these systems

72   may be partitioned within the same population of *E. coli* cells, with only fractions of cells

73   expressing the Cad or Adi systems (13, 14). Such heterogeneity in the induction of stress response

74   pathways indicates bet-hedging behavior, but possibly also division of labor and potential for

75   collective behaviors in bacterial populations under acidic pH (13). Population density has been

76   indeed shown to affect *E. coli* survival after exposure to lethal pH 2.0 (10, 15), but the nature of

77   this effect remained unknown.

78   Here we show that, upon rapid exposing to near-lethal pH, strains of *E. coli* as well as *Salmonella*

79   *enterica* serovar Typhimurium (*S. Typhimurium*) exhibits cell-density dependent multiphasic

80   growth. In *E. coli* K-12, it consists of the initial phase of exponential elongation in the absence of

81   cell division, followed by a transient cessation and a subsequent recovery of normal growth. The

82   duration of this transient growth arrest was strongly dependent on cell density, apparently due to

83   the collective deacidification of the medium that was partly dependent on the Cad system. The

84   initial phase of the cell size increase was apparently important to enable the biosynthesis of the

85   acid resistance systems, and it was also observed in other isolates of *E. coli* isolates and *S.*

86   *Typhimurium*. Consistent with the collective deacidification, we observed that even a minor

87   proportion of acid-tolerant *E. coli* cells is sufficient to support growth of the acid-sensitive *E. coli*

88   population at near-lethal pH.

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Results

E. coli exhibits density-dependent multiphasic growth under severe acid stress

92 To investigate the growth dynamics of *E. coli* at different values of acidic pH, we inoculated

93 cultures at different inoculum sizes in LB medium with initial pH ranging from 7.0 to 4.0. We

94 observed that the growth rate of *E. coli* K-12 strain MG1655 decreased progressively at lower pH

95 (Fig. 1A-E and Fig. S1). Although the growth rate was apparently independent of the cell culture

96 density at pH 7.0 or 5.0 (Fig. 1A, B), cell-density dependence of growth was observed starting at

97 pH 4.4. Specifically, bacterial cultures exhibited multiphasic growth, including an initial,

98 apparently exponential phase of increase in optical density (OD_{600}) followed by an intermediate

99 lag (mid-lag) phase and then a second exponential growth phase. At pH 4.4, such multiphasic

100 growth was only observed for the lowest density culture (Fig. 1C), whereas high cell-density

101 cultures showed continuous growth. At decreasing pH, the multiphasic growth became increasingly

102 pronounced also for intermediate- and high-density cultures (Fig. 1D, E and Fig. S1A, B). No

103 growth was observed at the pH 4.0 (Fig. S1C). Importantly, the duration of the growth arrest phase

104 showed strongly pronounced dependence not only on pH but also on the cell density, with higher-

105 density cultures exhibiting proportionally shorter or no lag phase.

106 We further investigated whether the density-dependent multiphasic growth is affected by the

107 buffering capacity of the medium. Indeed, for *E. coli* cultures grown in LB with pH 4.3, buffered

108 using homopiperazine-1,4-bis-2-ethanesulfonic acid (Homo-PIPES), the density dependence of

109 growth shifted to higher-density cultures at intermediate buffering capacity (Fig. 1F), and growth

110 entirely ceased at high buffering capacity (Fig. S2).

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Density-dependent growth is characterized by cell elongation and medium deacidification

Given the observed effects of buffering, medium deacidification may dictate the observed multiphasic growth. Consistently, the final pH of all cultures that reached high OD increased to >8.0, irrespective of their initial pH, while that of non-growing cultures remained low (Fig. 1 and Table S1). To elucidate how deacidification of the medium correlates with the multiphasic growth, OD and medium pH were monitored simultaneously for *E. coli* cultures with different inoculum size growing at an initial pH of 4.2 (Fig. 2 and Fig. S3). We observed that changes in pH were consistent with the cell growth (Fig. 2A-B and Fig. S3A-B). In particular, for low-density cultures where the multiphasic growth was pronounced, the exit from the intermediate lag phase coincided with the increase in pH. This resumption of cell growth occurred at pH ~4.4, and it was preceded by a very slow gradual elevation of pH over the entire duration of the initial phase of OD increase and the mid-lag phase. In contrast, the high-density culture crossed this apparently critical pH value already during the first phase and thus did not enter the intermediate phase.

To understand the nature of the initial phase of largely density-independent OD increase, we investigated changes in cell morphology during growth using microscopy. In all cultures growing at pH 4.2, cells showed pronounced but transient elongation, with its extent and the duration of the elongation phase dependent on the inoculum density (Fig. 2C, D and Fig. S3C). Cell length primarily increased during the first phase of growth, suggesting that this up to ~10-fold increase in OD during the initial, apparently exponential growth phase, reflects the elongation of cells from the initial average cell length of 4.4 µm to ~54 µm in the absence of cell division. The reduction of the cell length subsequently occurred concurrently with the resumption of growth after the mid-lag phase.

Pre-induced non-growing cells are able to deacidify the medium

These results indicate that the density dependence of growth at pH below 4.4 could be explained by gradual, density-dependent deacidification of the medium over the net duration of the initial elongation and subsequent mid-lag phase, until the critical permissive pH above 4.4 is reached and the growth/division arrest is relieved. The ability of non-growing cells to deacidify the medium was further confirmed by monitoring pH in a culture of translation-inhibited cells (Fig. 3). Here we compared *E. coli* cultures that were pre-grown in LB at either neutral or acidic (4.2) pH for 1 hour and subsequently transferred to LB containing chloramphenicol at pH 4.2 for 3 hours to inhibit translation. The density of both cultures was adjusted to $OD_{600} = 0.1$ to reflect the cell density at the mid-lag phase of the 0.0125 inoculum culture (Fig.2A), and OD_{600} and medium pH were monitored. We observed that translation-inhibited cells pre-incubated in acidic medium did not grow but increased medium pH to approximately 4.4 in 14 hours (Fig. 3A-B). This rate of deacidificaiton was similar to that observed during the mid-lag phase of growing cultures (Fig. 2B and Fig. 3C). In contrast, the cultures that were not pre-exposed to acidic pH prior to the inhibition of translation could not efficiently change pH of the medium, suggesting that the growth-dependent pre-induction of the AR system(s) may be essential for subsequent deacidification of the medium by non-growing cells.

Importance of individual AR systems in multiphasic growth under acid stress

To assess which AR system(s) contribute to the acid tolerance of *E. coli* during multiphasic growth under near-lethal acid stress, we first tested *E. coli* strains deficient in the expression of individual AR systems. A strain lacking *cadC*, encoding the transcriptional activator of the AR4 system, showed much reduced growth in LB pH 4.3 even at high inoculum size (Fig. 4A), suggesting

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158   reduced acid tolerance of this mutant. In contrast, no effect was observed for strains deleted for

159   genes encoding the transcriptional activators of AR2 (Δ*gadE*) or AR3 (Δ*adiY*). Consistent with

160   this, supplementation of LB pH 4.2 with lysine, the substrate of AR4, resulted in a strong

161   improvement in growth (Fig. 4B and Fig. S4), with a reduced duration of the mid-lag phase. The

162   duration of the mid-lag phase was also shortened in *E. coli* cells overexpressing *cadC* (Fig. 4C).

163   Although the addition of glutamate (substrate of AR2) even caused a growth reduction (Fig. S4),

164   the overexpression of *gadE* could improve growth at low pH (Fig. S5).

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Other *E. coli* and *Salmonella* strains exhibit cell-density dependent growth and

167   **morphological changes at near-lethal pH**

168   To test whether other *E. coli* isolates also exhibit cell-density dependent multiphasic growth at

169   near-lethal pH, we monitored the growth dynamics of two uropathogenic *E. coli* strains, UTI89

170   (16) and CFT073(17) (Fig. 5 and Fig. S6). Although these strains showed slightly higher acid

171   tolerance than MG1655, the dependence of growth on cell density was observed for both strains at

172   pH of 4.1 (Fig. 5A, B and Fig. S6A, B). However, the initial phase of the multiphasic growth was

173   less pronounced, and in contrast to MG1655 these strains primarily exhibited biomass

174   accumulation in the center of the cells and only minor elongation (Fig. 5C-H). Nevertheless, also

175   for these strains the resumption of the normal growth coincided with the restoration of the normal

176   cell size and with increase in pH (Fig. 5E-H and Fig. S6C, D; high-density culture).

177   Given this difference in the acid-induced phenotype between the K-12 and UTI89 and CFT073

178   strains, we further tested morphological changes for a number of strains from the ECOR collection

179   of natural isolates of *E. coli* (18) grown in LB at pH 4.1. Both cell elongation and widening, or

180   their combination, were observed in the ECOR strains belonging to phylogroups A (which also

181   include *E. coli* K-12), B1 and D (Fig. S7A, B and D). Strains from B2 phylogroup showed primarily

182   cell widening (Fig. S7C), consistent with the phenotype of UTI89 and CFT073 that also belong to

183   this group.

184   Because *Salmonella* and *E. coli* species share acid resistance mechanisms (2, 5, 19), we further

185   tested growth of *S. Typhimurium* at conditions ranging from neutral to near-lethal acidic pH. We

186   observed the growth rate reduction with lower initial medium pH, although at lower pH than for *E.*

187   *coli* strains, as well as the density-dependence of growth (Fig. S8 and Fig. S9A). The initial growth

188   phase was less pronounced in *S. Typhimurium* than in *E. coli* K-12, but there was both a transient

189   change in the cell size (Fig. S9C-D) and an increase in the medium pH (Fig. S9B) before

190   resumption of normal division.

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192   **Cross-protection between acid-tolerant and acid-sensitive strain in a mixed population**

193   Given our observation that *E. coli* growth at near-lethal pH relies on collective deacidification of

194   the medium, we hypothesized that, in a coculture, acid-sensitive cells could be complemented for

195   growth by acid-tolerant cells. To test for such cross-complementation, we cocultured wild-type

196   (WT) and Δ*cadC* *E. coli* MG1655 at equal amounts (WT 50 : KO 50) in LB at pH 4.3, and

197   compared growth of this coculture to the monocultures of either WT or Δ*cadC* (Fig. 6). Consistent

198   with our previous results (Fig. 4A), growth of the Δ*cadC* monoculture at low pH was severely

199   reduced (Fig. 6A) and no deacidification of the medium was observed over 9 h of incubation (Fig.

200   6B). Overall growth and medium deacidification for the WT 50: KO 50 coculture was intermediate

201   between the WT 100 (i.e., twice the WT density) and WT 50 monocultures, suggesting that Δ*cadC*

202   cells contribute to medium deacidification, probably via other AR systems. Most importantly,

203   quantification of the colony-forming units (CFUs) revealed nearly identical growth of the WT and

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204     $\Delta cadC$ strains in the coculture at each time point, further confirming the entirely collective nature

205   of the acid tolerance under these conditions.   Consistently, growth complementation was also

206   observed in the coculture of WT 20: KO 80 (Fig. S10), demonstrating that even a minor fraction

207   of acid-tolerant bacteria can support the growth of the entire population at low pH.

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Discussion

210   Many neutralophilic bacteria, including pathogens, are naturally exposed to acidic environments

211   such as the mammalian colon and acidified foods, soil and water (2–6). To date, *E. coli* growth at

212   near-lethal pH has only been studied for growth or no-growth outcomes (15) or for the overall

213   reduction in growth rate (4, 11, 12, 15). Here, we systematically examined the dependence of *E.*

214   *coli* growth not only on pH but also on cell density.

215   We observed that *E. coli* growth at the near-lethal acid pH is multiphasic, consisting of an initial

216   phase of optical density increase, a mid-lag phase of apparent growth arrest, and a phase of growth

217   resumption. This multiphasic growth was not solely determined by the medium pH, but also by the

218   cell density of the culture. While the first and the third phases of growth showed little density

219   dependence, low cell density cultures displayed higher duration of the mid-lag phase compared to

220   high cell density cultures. Our results suggest that this density dependence could be explained by

221   gradual collective deacidification of the medium during the mid-lag phase, which allows the

222   resumption of growth above pH of 4.4. This deacidification depends on the number of deacidifying

223   cells, but also on the buffering capacity of the medium, and on the presence of the AR systems,

224   primarily AR4 (Cad) system.

225   We could further show that the ability of *E. coli* cells to deacidify the medium depends on their

226   pre-exposure to acidic pH, likely because of the need to induce the expression of acid resistance

227 system(s). Consistent with that, the non-growing, translation-inhibited *E. coli* cells could deacidify

228 the medium similarly to the deacidification observed during the mid-lag phase of growth, but only

229 when pre-exposed to acidic pH before the inhibition of translation. We propose that, during the

230 multiphasic growth of the culture, this induction of the acid resistance systems occurs during the

231 initial, density-independent phase of increase in the optical density. In all tested strains of *E. coli*

232 as well as in *S. Typhimurium*, this first stage corresponded to changes in cell morphology. For *E.*

233 *coli* MG1655 where it was most pronounced, we observed an extensive (up to ~10 fold) cell

234 elongation that could account for a similar-fold increase in optical density. Such elongation at low

235 pH is consistent with a recent report that described it for several *E. coli* strains and for *Citrobacter*

236 *rodentium* but not for *Salmonella*, and explained it by the inhibition of the essential divisome

237 protein FtsI (penicillin-binding protein 3, PBP3) that is involved in septal peptidoglycan

238 biosynthesis (20). Our observation that some *E. coli* strains, particularly those from B2 phylogroup

239 that contains many uropathogens, increase in the cell width suggest that in those instances lateral

240 rather than septal biosynthesis of peptidoglycan is inhibited at low pH. Nevertheless, in all cases,

241 also including *Salmonella*, an increase in the cell size correlating with the initial phase of optical

242 density increase is observed. Our results suggest that this increase of the biomass may be required

243 to allocate additional protein resources in acid resistance. Notably*, E. coli* and *Salmonella* are

244 known to transiently increase cell size in response to other stress conditions (21–23), indicating

245 that the morphological response described here may be a general mechanism to enable bacteria to

246 allocate resources in stress response.

247 The observed collective acid tolerance of *E. coli* growth at near-lethal pH may be related to the

248 recently shown heterogeneous expression of the Cad system due to the low copy number of its

249 regulator CadC (13, 24). It was suggested that stochastic differentiation of *E. coli* population into

250 distinct *cad-on* and *cad-off* subpopulations (25) might represent division of labor in a bacterial

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251 population (13). Our results indeed support this hypothesis, demonstrating that even a minor

252 fraction of Cad-positive cells is sufficient to enable growth of the entire population.

253 Taken together, here we could identify collective dynamics and morphological changes that are

254 important for *E. coli* growth under acidic conditions, contributing to understanding of the

255 mechanisms that enable *E. coli* strains to invade acidic intestinal microenvironments (26–28). The

256 observed response could also affect bacterial interactions with immune cells, by counteracting

257 phagocytosis (29) and/or by enabling survival within the acidic phagosome (26, 30).

258

Materials and methods

260 ***Bacterial strains and plasmids:*** All strains and plasmids used in this study are listed in Table S2.

261 Genetically modified strains were derived from *E. coli* MG1655 using P1 transduction (31).

262 pTrc99a-gadE was constructed by PCR amplification of *gadE* from MG1655 genome followed by

263 Gibson assembly to clone it into pTrc99a expression vector inducible by isopropyl β-d-1-

264 thiogalactopyranoside (IPTG).

265

266 ***Culture conditions:*** Bacteria were cultured in lysogeny broth (LB) medium (10 g/L tryptone, 5 g/L

267 yeast extract, and 5 g/L NaCl) at pH 7.0 (unless otherwise stated). HCl was used to adjust pH.

268 Where indicated, lysine or other amino acids were added to a final concentration of 20 mM before

269 adjusting the pH to 4.3. Pre-cultures were prepared by inoculating 2% overnight culture into LB

270 and incubated for ~1.5 hours until OD_{600} 0.50 - 0.70 at 200rpm and 37 °C. Cultures were washed

271 three times (1500 x g, 3 min, 20 °C) using medium with the target pH and OD_{600} was adjusted to

272 0.50 using a 10 mm cuvette (REF 67742, Sarstedt). These OD-adjusted cultures were inoculated

273 into LB with the target pH at 10% (OD_{600}=0.05), 5% (OD_{600}=0.025), 2.5% (OD_{600}=0.0125), or

274   1.25% (OD_{600}=0.00625) to generate inoculum sizes of ~1.00×10^7 cells/mL, 5.00×10^6 cells/mL,

275   2.50×10^6 cells/mL and 1.25×10^6 cells/mL, respectively. HCl was utilized to acidify the medium.

276   Growth was monitored in 48-well plates (500 µL/well, REF 677102, Greiner bio-one) in a plate

277   reader (Infinite, Tecan GmbH) using alternating cycles of 150 s linear and orbital shaking at 37°C.

278   When indicated, homopiperazine-1,4-bis(2-ethanesulfonic acid) (AB117062, Homo-PIPES, abcr

279   GmbH) was added as buffer.

280   For pH measurements, the lids of 48-well plates were pierced with a sterile twisted drill bit to make

281   sampling holes in each of the wells. The border of the plate and the holes in the wells were then

282   sealed with parafilm to prevent evaporation. Samples were taken at specified intervals and pH was

283   measured using a standard pH meter (ThermoScientific) or an Ultra-Micro-ISM pH meter (Mettler-

284   Toledo) for small volumes. Quantification of CFUs for coculture experiments was done by plating

285   serial dilutions on LB agar plates containing the appropriate antibiotics and manual counting of

286   colonies.

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288   ***Test of pH changes by translation-inhibited cells:*** Pre-cultures grown as above were first

289   transferred to LB medium with either pH 4.2 or pH 7.0 and incubated at 200 rpm for 1 hour at 37

290   °C. Cells were then transferred to LB containing 200 mg/mL chloramphenicol (Cm) at pH 4.2 and

291   incubated at 200 rpm, 37 °C for 3 hours. The OD_{600} was then adjusted to 0.50, the cultures were

292   inoculated at the specified cell density and OD_{600} and pH were monitored during incubation in the

293   plate reader as described above.

294

295   ***Bright field microscopy:*** Cells were collected from 50 µL of culture by centrifugation (4500 *g*, 1

296   min, 20 °C) and the supernatant was used for pH measurement. The pellet was immediately

297   resuspended in 10 µL PBS pH 5.0. A 5µL aliquot was placed on an agarose pad for microscopy.

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298   Images were captured with a 20X objective using Zeiss observer Z1 or a Zeiss Elyra 7. Length and

299   width of 100 to 150 cells were measured using Fiji 1.53 segmented lines tool.

300

301   *Growth complementation assay:* Cultures adjusted to $OD_{600} = 0.50$ were used to generate 50:50

302   and 20:80 cocultures of acid-tolerant and resistant strains at a total density of $OD_{600} = 0.05$ in LB

303   pH 4.3. At each time point, serial dilutions were made and colonies were counted on LB agar plates

304   containing the appropriate antibiotics.

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311   **Author contributions**

312   Rafael R. Segura Munoz, Conceptualization, Formal analysis, Funding acquisition, Investigation,

313   Methodology, Visualization, Writing – original draft, Writing – review and editing | Victor Sourjik,

314   Conceptualization, Funding acquisition, Project administration, Supervision, Writing – original

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Data availability:

322   All of the data are included in this article.

323

324   **Abbreviations**: GDAR, glutamate-dependent Gad system; ADAR, arginine-dependent Adi

325   system; LDAR, lysine-dependent Cad system; Homo-PIPES, homopiperazine-1,4-bis(2-

326   ethanesulfonic acid); Cm, Chloramphenicol.

327

328   **Conflict of interest**: The authors do not declare any conflict of interest.

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References

331   1.   Neidhardt FC, Curtiss R. 1996. *Escherichia coli* and *Salmonella*: cellular and molecular

332   biology, 2nd ed. ASM Press, Washington, D.C.

333   2.   Schwarz J, Schumacher K, Brameyer S, Jung K. 2022. Bacterial battle against acidity.

334   FEMS Microbiol Rev 46:fuac037. https://doi.org/10.1093/femsre/fuac037.

335   3.   Guan N, Liu L. 2020. Microbial response to acid stress: mechanisms and applications.

336   Appl Microbiol Biotechnol 104:51–65. https://doi.org/10.1007/s00253-019-10226-1.

337   4.   Kanjee U, Houry WA. 2013. Mechanisms of Acid Resistance in *Escherichia coli*. Annu

338   Rev Microbiol 67:65–81. https://doi.org/10.1146/annurev-micro-092412-155708.

15

339   5.   Lund P, Tramonti A, De Biase D. 2014. Coping with low pH: molecular strategies in

340   neutralophilic bacteria. FEMS Microbiol Rev 38:1091–1125. https://doi.org/10.1111/1574-

341   6976.12076.

342   6.   Lin J, Smith MP, Chapin KC, Baik HS, Bennett GN, Foster JW. 1996. Mechanisms of acid

343   resistance in enterohemorrhagic *Escherichia coli*. Appl Environ Microbiol 62:3094–3100.

344   https://doi.org/10.1128/aem.62.9.3094-3100.1996.

345   7.   Shalon D, Culver RN, Grembi JA, Folz J, Treit P V, Shi H, Rosenberger FA, Dethlefsen L,

346   Meng X, Yaffe E, Aranda-Díaz A, Geyer PE, Mueller-Reif JB, Spencer S, Patterson AD,

347   Triadafilopoulos G, Holmes SP, Mann M, Fiehn O, Relman DA, Huang KC. 2023.

348   Profiling the human intestinal environment under physiological conditions. Nature

349   617:581–591. https://doi.org/10.1038/s41586-023-05989-7.

350   8.   Bown R, Gibson J, Sladen G, Hicks B, Dawson A. 1974. Effects of lactulose and other

351   laxatives on ileal and colonic pH as measured by a radiotelemetry device. Gut 15:999–

352   1004.

353   9.   Lin J, Lee IS, Frey J, Slonczewski JL, Foster JW. 1995. Comparative analysis of extreme

354   acid survival in *Salmonella typhimurium*, *Shigella flexneri*, and *Escherichia coli*. J

355   Bacteriol 177:4097–4104. https://doi.org/10.1128/jb.177.14.4097-4104.1995.

356   10.   Mates AK, Sayed AK, Foster JW. 2007. Products of the *Escherichia coli* acid fitness

357   island attenuate metabolite stress at extremely low pH and mediate a cell density-

358   dependent acid resistance. J Bacteriol 189:2759–2768. https://doi.org/10.1128/JB.01490-

359   06.

360   11.   Xu Y, Zhao Z, Tong W, Ding Y, Liu B, Shi Y, Wang J, Sun S, Liu M, Wang Y, Qi Q,

16

361   Xian M, Zhao G. 2020. An acid-tolerance response system protecting exponentially

362   growing *Escherichia coli*. Nat Commun 11:1496. https://doi.org/10.1038/s41467-020-

363   15350-5.

364   12.   Castanie-Cornet M-P, Penfound TA, Smith D, Elliott JF, Foster JW. 1999. Control of Acid

365   Resistance in *Escherichia coli*. J Bacteriol 181:3525–3535.

366   https://doi.org/10.1128/JB.181.11.3525-3535.1999.

367   13.   Brameyer S, Schumacher K, Kuppermann S, Jung K. 2022. Division of labor and

368   collective functionality in *Escherichia coli* under acid stress. Commun Biol 5:327.

369   https://doi.org/10.1038/s42003-022-03281-4.

370   14.   Schumacher K, Brameyer S, Jung K. 2023. Bacterial acid stress response: from cellular

371   changes to antibiotic tolerance and phenotypic heterogeneity. Curr Opin Microbiol

372   75:102367. https://doi.org/https://doi.org/10.1016/j.mib.2023.102367.

373   15.   Skandamis PN, Stopforth JD, Kendall PA, Belk KE, Scanga JA, Smith GC, Sofos JN.

374   2007. Modeling the effect of inoculum size and acid adaptation on growth/no growth

375   interface of *Escherichia coli* O157:H7. Int J Food Microbiol 120:237–249.

376   https://doi.org/10.1016/j.ijfoodmicro.2007.08.028.

377   16.   Chen SL, Hung C-S, Xu J, Reigstad CS, Magrini V, Sabo A, Blasiar D, Bieri T, Meyer

378   RR, Ozersky P, Armstrong JR, Fulton RS, Latreille JP, Spieth J, Hooton TM, Mardis ER,

379   Hultgren SJ, Gordon JI. 2006. Identification of genes subject to positive selection in

380   uropathogenic strains of *Escherichia coli*: A comparative genomics approach. Proc Natl

381   Acad Sci 103:5977–5982. https://doi.org/10.1073/pnas.0600938103.

382   17.   Welch RA, Burland V, Plunkett G, Redford P, Roesch P, Rasko D, Buckles EL, Liou S-R,

383   Boutin A, Hackett J, Stroud D, Mayhew GF, Rose DJ, Zhou S, Schwartz DC, Perna NT,

384   Mobley HLT, Donnenberg MS, Blattner FR. 2002. Extensive mosaic structure revealed by

385   the complete genome sequence of uropathogenic *Escherichia coli*. Proc Natl Acad Sci

386   99:17020–17024. https://doi.org/10.1073/pnas.252529799.

387   18.   Ochman H, Selander RK. 1984. Standard reference strains of *Escherichia coli* from natural

388   populations. J Bacteriol 157:690–693. https://doi.org/10.1128/jb.157.2.690-693.1984.

389   19.   Small P, Blankenhorn D, Welty D, Zinser E, Slonczewski JL. 1994. Acid and base

390   resistance in *Escherichia coli* and *Shigella flexneri*: role of rpoS and growth pH. J

391   Bacteriol 176:1729–1737. https://doi.org/10.1128/jb.176.6.1729-1737.1994.

392   20.   López-Escarpa D, Castanheira S, García-del Portillo F. 2022. OmpR and Prc contribute to

393   switch the *Salmonella* morphogenetic program in response to phagosome cues. Mol

394   Microbiol 118:477–493. https://doi.org/10.1111/mmi.14982.

395   21.   Zhang D, Yin F, Qin Q, Qiao L. 2017. Molecular responses during bacterial filamentation

396   reveal inhibition methods of drug-resistant bacteria. Proc Natl Acad Sci 120:e2301170120.

397   https://doi.org/10.1073/pnas.

398   22.   Dev Kumar G, Macarisin D, Micallef SA. 2019. *Salmonella enterica* Filamentation

399   Induced by Pelargonic Acid Is a Transient Morphotype. Appl Environ Microbiol

400   85:e02191-18. https://doi.org/10.1128/AEM.02191-18.

401   23.   Mattick KL, Rowbury RJ, Humphrey TJ. 2003. Morphological Changes to *Escherichia*

402   *coli* O157:H7, Commensal *E. coli* and *Salmonella* spp in Response to Marginal Growth

403   Conditions, with Special Reference to Mildly Stressing Temperatures. Sci Prog 86:103–

404   113. https://doi.org/10.3184/003685003783238725.

405   24.   Ude S, Lassak J, Starosta AL, Kraxenberger T, Wilson DN, Jung K. 2013. Translation

406   Elongation Factor EF-P Alleviates Ribosome Stalling at Polyproline Stretches. Science

407   339:82–85. https://doi.org/10.1093/femsre/fuaa003.

408   25.   Brameyer S, Hoyer E, Bibinger S, Burdack K, Lassak J, Jung K. 2020. Molecular design

409   of a signaling system influences noise in protein abundance under acid stress in different

410   γ-Proteobacteria. J Bacteriol 202. https://doi.org/10.1128/JB.00121-20.

411   26.   Kittana H, Gomes-Neto CJ, Kari H, Juritsch AF, Sughroue J, Xian Y, Mantz S, Segura

412   Muñoz RR, Cody LA, Schmaltz RJ, Anderson CL, Moxley RA, Hostetter JM, Samodha

413   FC, Clarke J, Kachman SD, Cressler CE, Benson AK, Walter J, Ramer-Tait AE. 2023.

414   Evidence for a Causal Role for *Escherichia coli* Strains Identified as Adherent-Invasive

415   (AIEC) in Intestinal Inflammation. mSphere 8:e00478-22.

416   https://doi.org/10.1128/msphere.00478-22.

417   27.   Kotlowski R, Bernstein CN, Sepehri S, Krause DO. 2007. High prevalence of *Escherichia*

418   *coli* belonging to the B2+D phylogenetic group in inflammatory bowel disease. Gut

419   56:669 LP – 675. https://doi.org/10.1136/gut.2006.099796.

420   28.   O'Brien CL, Bringer M-A, Holt KE, Gordon DM, Dubois AL, Barnich N, Darfeuille-

421   Michaud A, Pavli P. 2017. Comparative genomics of Crohn's disease-associated adherent-

422   invasive *Escherichia coli*. Gut 66:1382 LP – 1389. https://doi.org/10.1136/gutjnl-2015-

423   311059.

424   29.   Möller J, Luehmann T, Hall H, Vogel V. 2012. The Race to the Pole: How High-Aspect

425   Ratio Shape and Heterogeneous Environments Limit Phagocytosis of Filamentous

426   *Escherichia coli* Bacteria by Macrophages. Nano Lett 12:2901–2905.

427   https://doi.org/10.1021/nl3004896.

428   30.   Yu Y, Zhang Z, Walpole GFW, Yu Y. 2022. Kinetics of phagosome maturation is coupled

429   to their intracellular motility. Commun Biol 5:1014. https://doi.org/10.1038/s42003-022-

430   03988-4.

431   31.   Thomason LC, Costantino N, Court DL. 2007. *E. coli* Genome Manipulation by P1

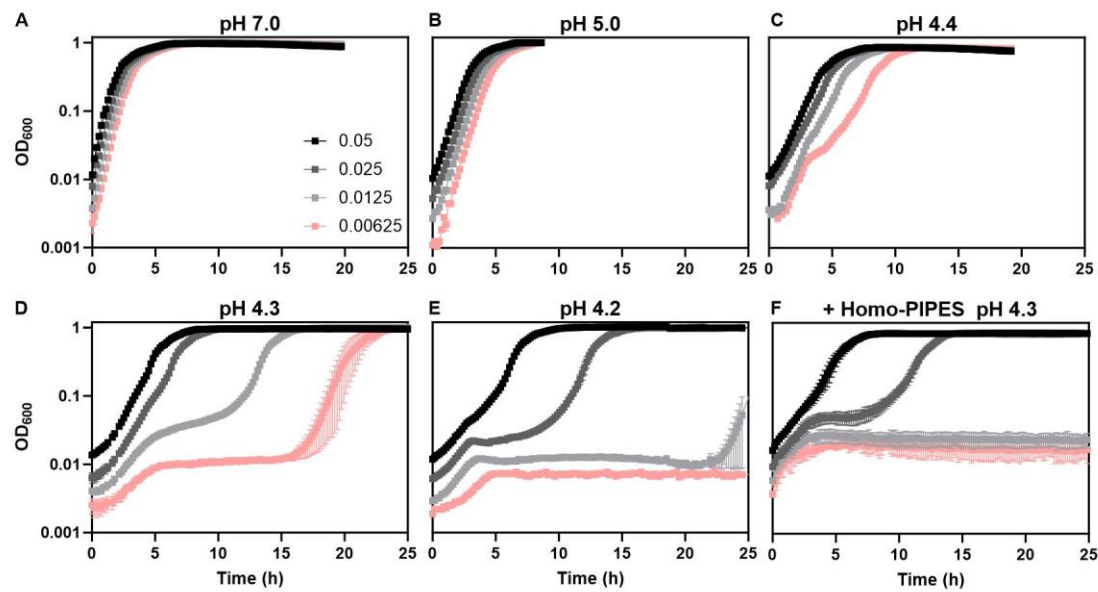432   Transduction. Curr Protoc Mol Biol 79:1–8.

433   https://doi.org/10.1002/0471142727.mb0117s79.

434

Fig. 1 *E. coli* **exhibits density-dependent growth at low pH.** Growth of *E. coli* MG1655 measured using plate reader at indicated inoculum sizes in unbuffered LB adjusted to initial pH of (A) 7.0, (B) 5.0, (C) 4.4, (D) 4.3, (E) 4.2, or in (F) LB buffered with 33 mM Homo-PIPES at pH 4.3. Inoculum size values here and throughout correspond to OD_{600} measurements in a 10 mm cuvette using spectrophotometer, thus deviating from OD_{600} values measured in a plate reader. Mean and SEM of 3 replicates are shown.
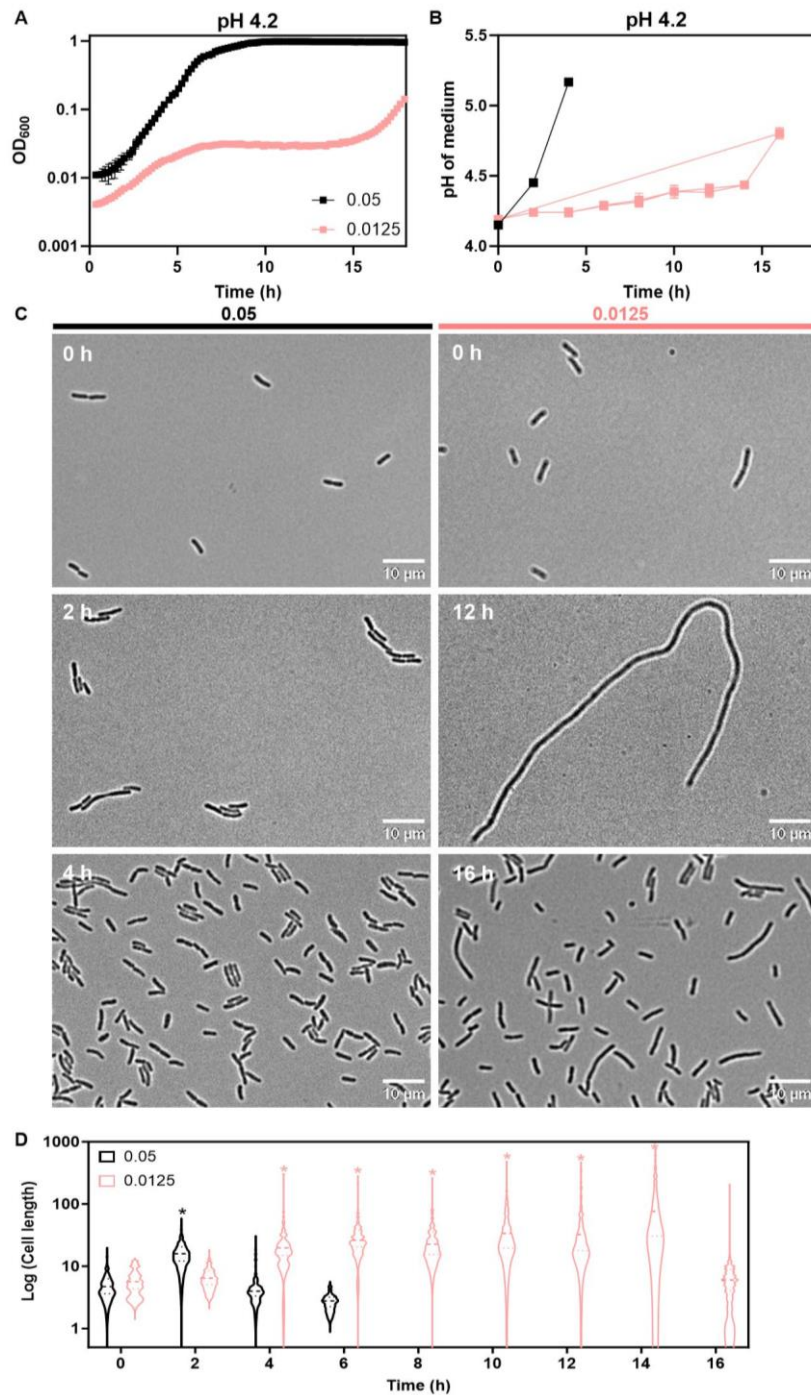
Fig. 2 Multiphasic *E. coli* growth reflects changes of cell size and medium deacidification. *E. coli* MG1655 cultures with indicated inoculum size were grown in LB at pH 4.2. (A) Growth of the culture. (B) Medium pH, measured as described in Materials and Methods. Mean and SEM of 3 replicates are shown. (C) Morphology of *E. coli* cells at indicated time points. Scale bar is 10

µm. (D) Quantification of cell length for 100-150 cells at indicated time points, with the distribution of responses, the average (dashed line) and the upper and lower quartiles (dotted lines) being indicated. Significance of difference compared to $t = 0$, assessed using a paired t-test, is indicated by asterisks (*$p \leq 0.05$).
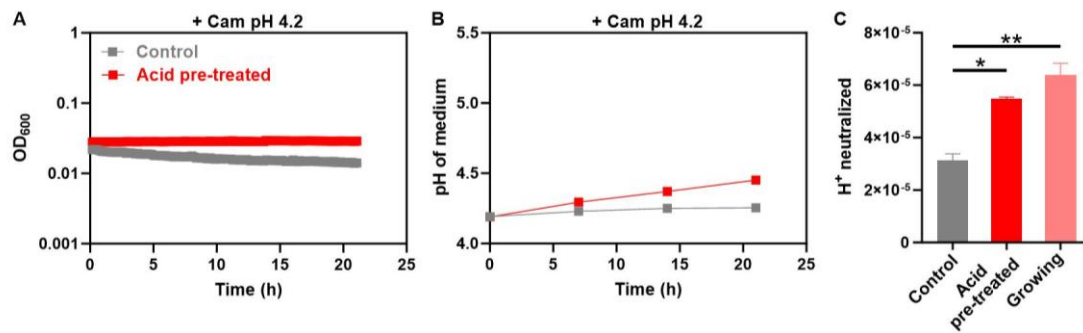
Fig. 3 Non-growing cells can efficiently deacidify the medium. *E. coli* cells treated with chloramphenicol to inhibit translation were incubated in LB at pH 4.2. (A) Growth of the culture. (B) Medium pH. Inoculum size was 0.1. Prior to the chloramphenicol (Cam) treatment, cells were exposed to either pH 4.2 (acid pre-treated) or pH 7.0 (control) for 1 h in LB. (C) Rate of neutralization, calculated as the molar mount of protons neutralized per OD_{600} per hour in translation-inhibited cells, or in untreated cells during the mid-lag phase (data from Fig. 2B). Mean and SEM of 3 replicates are shown. Significance of differences, assessed using an unpaired *t*-test, is indicated by asterisks (*$p \leq 0.05$, **$p \leq 0.01$).
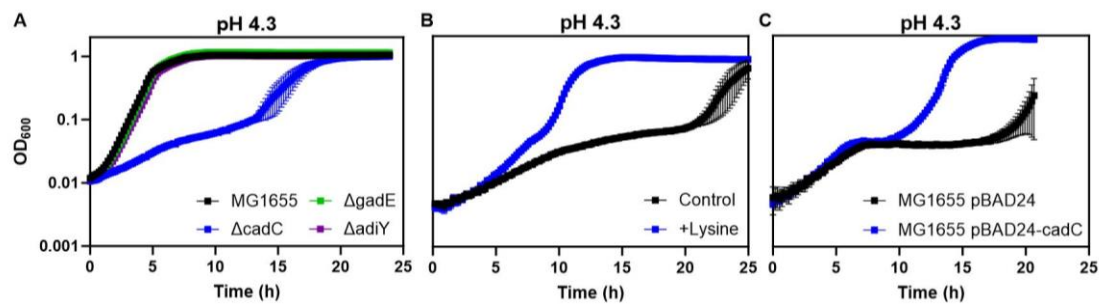
Fig. 4 Cad system is involved in cell density-dependent acid tolerance. (A) Growth of *E. coli* MG1655 wild type or *ΔcadC*, *ΔgadE*, or *ΔadiY* mutants, as indicated, in LB at pH 4.3. Inoculum size was 0.05 for all strains. (B) Growth of *E. coli* MG1655 in LB with or without 20mM lysine supplementation, at inoculum size of 0.0125. (C) Growth of *E. coli* MG1655 carrying either pBAD24 or pBAD24-*cadC*, at inoculum size of 0.0125. Mean and SEM of 3 replicates are shown.
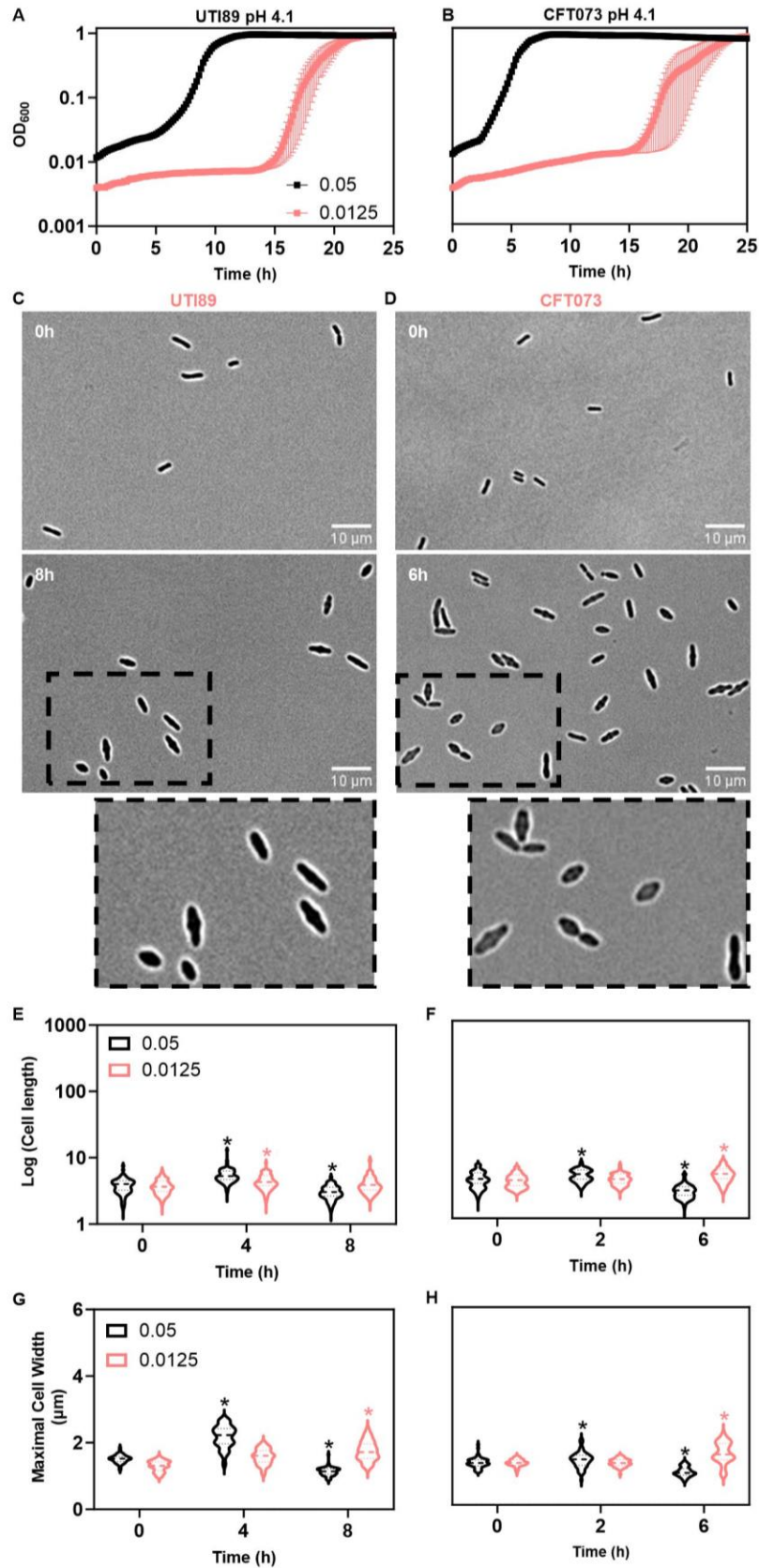
Fig. 5 Uropathogenic *E. coli* strains exhibit cell-density dependent growth and morphology changes at near-lethal pH. Growth of *E. coli* strains UTI89 (A) and CFT073 (B) at indicated inoculum sizes in LB at pH 4.1. Mean and SEM of 3 replicates are shown. Morphology of UTI89 (C) and CFT073 (D) at indicated time points. Scale bar is 10 µm. Dotted lines specified the magnified area. Quantification of cell length for 100-150 cells of UTI89 (E) and CFT073 (F), and of maximal cell width for UTI89 (G) and CFT073 (H), done and represented as in Figure 2.
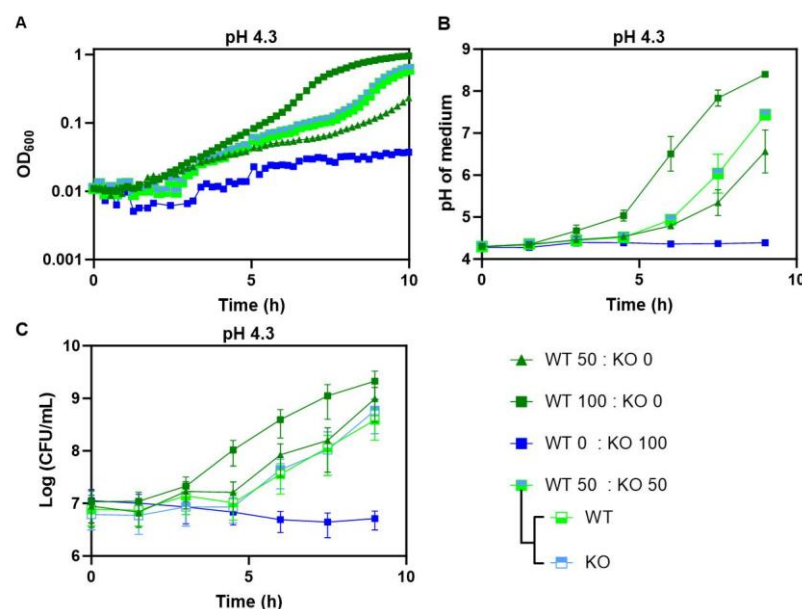
Fig. 6 Acid-tolerant strain complements growth of an acid-sensitive strain in a coculture. Acid-tolerant (WT; MG1655 carrying pBAD24) and sensitive (KO; MG1655 *ΔcadC*) were cocultured at total inoculum size of 0.05 in LB at pH 4.3. Two strains were mixed at equal proportions (50:50); monocultures were inoculated either at half the density or at the full density. (A) Growth of the cocultures. (B) Medium pH. (C) Colony-forming units (CFU) per mL or indicated cultures. The CFUs for WT and KO were distinguished by plating cocultures on LB agar with ampicillin and kanamycin, respectively. Mean and SEM of 3 replicates are shown.