Population level genetic memory of prior metabolic adaptation in *E. coli*

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Abstract

Bacteria must often survive following the exhaustion of their external growth resources. Fitting with this need, many bacterial species that cannot sporulate, can enter a state known as long term stationary phase (LTSP) in which they can persist for years within spent media. Several recent studies have revealed the dynamics of genetic adaptation of *Escherichia coli* under LTSP. Yet, the metabolic consequences of such genetic adaptation were not addressed. Here, we characterized the metabolic changes LTSP populations experience and link them to their genetic adaptation. We observed that during growth within fresh resources *E. coli* produces the short chain fatty acid butyrate, which wildtype *E. coli* cannot consume. Once resources are otherwise exhausted, *E. coli* adapts genetically to consume butyrate through the convergent, temporally precise emergence of mutation combinations within genes that regulate fatty acid metabolism. These mutations appear to negatively affect bacterial fitness when butyrate is not available, and hence rapidly decrease in frequency, once all butyrate is consumed. Yet despite this, *E. coli* populations show a remarkable capability of maintaining a population-level genetic 'memory' of prior adaptation to consume butyrate. The maintenance of such a 'memory' allows bacteria to rapidly re-adapt, at an ecological, rather than an evolutionary timeframe, to re-consume previously encountered metabolites.

Introduction

The ability of bacteria to survive within nutrient limited environments has fascinated the scientific community for the past 90 years (Zobell and Grant 1943). This ability results from bacterial evolution in natural environments, where bacteria are likely repeatedly exposed to short periods of feast and long periods of famine. Bacteria pursue several strategies to face long periods of starvation. Some bacterial species can undergo sporulation, allowing them to maintain complete or near complete dormancy for many years. While sporulation has rightfully received much attention, most known bacteria species cannot sporulate, yet many can still survive for years following the exhaustion of their nutrients, by entering a state termed long term stationary phase (LTSP) (Zambrano, et al. 1993; Finkel 2006). In LTSP a sub-population of cells can survive by recycling the remains of their deceased brethren.

We and others have previously reported on evolutionary experiments designed to probe the dynamics of *E. coli* genetic adaptation under LTSP (Avrani, et al. 2017; Chib, et al. 2017; Avrani, et al. 2020; Gross, et al. 2020; Katz, et al. 2021; Ratib, et al. 2021). In one such experiment, initiated in July 2015 and ongoing until this day, we established five independent populations by inoculating *E. coli* cells into rich media and incubating them within a shaking incubator. ~10 clones from each of these five populations were sequenced, at nine time points, spanning the first three years of the experiments. Results of these experiments thus far show that *E. coli* LTSP populations rapidly adapt genetically, in an extremely convergent manner, through mutations to several important regulatory and metabolic genes (Avrani, et al. 2017; Katz, et al. 2021).

The first adaptation we observed to occur in *E. coli* under LTSP, involves mutations within highly specific sites of the RNA polymerase core enzyme (RNAPC) (Avrani, et al. 2017). A second, highly convergent adaptation that emerges with great temporal precision involves mutations to fatty acid metabolism genes. Specifically, at day 22 of our experiments we observed that, across independently evolving populations, ~80% of sequenced clones carry, in addition to the RNAPC adaptation, a mutation in the *fadR* gene which encodes a master regulator of fatty acid metabolism (van Aalten, et al. 2000), combined with a second mutation in either *atoC* or *atoS*, that together encode a two-component system that regulates fatty acid metabolism (Lioliou and Kyriakidis 2004). The adaptations within *fadR* and *atoC*/*atoS* that appear with high

63 convergence at day 22 were not seen at the previous (day 11) or subsequent (day 32) sampled
64 time points (Avrani, et al. 2017).

65 The ability of bacteria to enter LTSP and persist in the absence of external nutrients for many
66 years constitutes a metabolic mystery. Yet the metabolic particulars of LTSP have not been
67 addressed. Here we provide the first characterization of the metabolites present within LTSP
68 exhausted media, over the first 32 days under LTSP. This in turn allowed us to ascertain the
69 function of the *fadR* + *atoC*/*atoS* adaptations, demonstrating that these enable the consumption
70 of the short chain fatty acid butyrate, which we show *E. coli* produces during the first 24 hours of
71 growth in rich media. We could further show that despite these adaptations demonstrating an
72 apparent cost, and despite our populations being well mixed, *E. coli* LTSP populations are able
73 to maintain minorities of cells carrying these adaptations, even when no butyrate is present. Such
74 maintenance allows populations that adapted to consume butyrate, to re-adapt to consuming this
75 metabolite much more rapidly than populations that never encountered butyrate before.

76 Results

77 Characterization of the metabolites present within LTSP media as a function of time spent 78 under LTSP

79 To examine the metabolic consequences of LTSP adaptations, we initiated new LTSP
80 experiments and measured changes of different metabolites in the media over 32 days (Materials
81 and Methods). Sugars serving as main carbon sources such as glucose and its disaccharide
82 trehalose (a major sugar component of LB (Puentes-Tellez, et al. 2013)) were rapidly consumed,
83 following three and 24 hours of growth respectively (**Figure 1A and Supplementary Table S1**).
84 In contrast, uptake of fructose, and of sugar alcohols, considered to be metabolic products
85 (Hanko and Rohrer 2000), was minimal even following 32 days. A significant increase in the
86 concentration of the pentoses, ribose and xylose was observed towards day 32, likely due to the
87 breakdown of dying cells and to the deficient utilization of these sugars, relative to other
88 available carbon sources (Ammar, et al. 2018; Fujiwara, et al. 2020). Amino acids, that are
89 relatively not bioenergetically costly to produce, such as glycine, serine, and threonine, were
90 rapidly consumed. In contrast, very little, if any uptake occurred for phenylalanine, histidine, and
91 the branched-chain amino acids leucine, isoleucine and valine (**Figure 1B and Supplementary**

92 **Table S2**), all of which are much more costly to produce (Kaleta, et al. 2013). This observation
93 fits well with previous data suggesting a compromise between the cost and the potential
94 energetic benefit of degrading amino acids (Zampieri, et al. 2019).

95 ***E. coli* populations produce butyrate during initial growth in fresh rich media and adapt to**
96 **consume it through the acquisition of mutations within *fadR* and *atoC*/*atoS***

97 The concentration of medium- and long-chain free fatty acids did not significantly reduce with
98 time (**Figure 1C and Supplementary Table S3**). However, the short chain fatty acids (SCFA),
99 formate (C1), acetate (C2) and propionate (C3), were consumed by 24 hours (**Figure 1D and**
100 **Supplementary Table S3**). Intriguingly, The SCFA butyrate (C4) was massively produced
101 during the first 24 hours of growth and was then consumed in a temporally precise manner
102 following 18-22 days of culture (**Figure 2A**). Butyrate serves as the main energy source for
103 colonocytes (Bultman 2016). Yet, despite its importance to homeostasis, the human body cannot
104 synthesize butyrate and relies on anaerobic fermentation by commensal gastrointestinal bacteria,
105 of the phyla Bacteroidetes and Firmicutes (Zhang, et al. 2009). Wildtype *E. coli* is not known to
106 produce butyrate ()Jawed, et al. 2016(or to consume it (Overath and Raufuss 1967).
107 Accordingly, the ancestral *E. coli* K12 MG1655 strain, used to initiate our experiments, is not
108 able to consume butyrate (**Figure 2B**). However, our experiments with LTSP evolved clones,
109 carrying the *fadR* + *atoC*/*atoS* mutations demonstrated their acquired ability to consume butyrate
110 (**Figure 2B**). Population samples from day 22, in which these butyrate consuming clones are
111 present at high frequencies can also consume butyrate, while samples extracted from day 11 and
112 day 32 of LTSP cannot (**Figure 2B**). Taken together, these results suggest that LTSP populations
113 rapidly adapt to consume the butyrate they generated during the first day of growth and that they
114 do so through the convergent acquisition of a specific combination of mutations. Once butyrate
115 is fully consumed, the *fadR* + *atoC*/*atoS* mutations are no longer useful and they undergo sharp
116 reductions in frequencies by day 32 of our original experiment (Avrani, et al. 2017). That this
117 reduction in frequency occurred so rapidly, across multiple populations, suggests that in the
118 absence of butyrate, these mutations come at a relative cost.

119 **Butyrate consumption adaptations provide a growth advantage when butyrate is provided,**
120 **but are rapidly outcompeted once butyrate is no longer available**

121 Next, we sought to examine what would happen if butyrate was continuously available. We
122 established six new LTSP populations. For three of them we supplemented the media daily, from
123 day 18 to day 55, with 1 mM butyrate (a concentration similar to that produced during the first
124 24 hours of growth (**Figure 2A**). The remaining three populations served as controls and were
125 allowed to evolve without further intervention. In two of the three butyrate-supplemented
126 populations, the butyrate produced during the first 24 hours of growth was consumed at days 17
127 and 18 of the experiment, and the supplemented butyrate was then continuously consumed daily
128 (**Figure 3A**). Starting at day 18 and up to day 55, bacteria from these two butyrate-supplemented
129 populations were able to grow substantially more than in the absence of butyrate, reflected in
130 much higher colony forming unit (CFU) counts (**Figure 3B**). In the third population, butyrate
131 produced during the first 24 hours of growth was consumed earlier, at day seven, and butyrate
132 supplemented Starting at day 18, initially accumulated, and was only consumed from day 26
133 onwards (**Figure S1A**). In line with this, the increase in CFU was observed for this population
134 only from day 26 (**Figure S1B**). Combined, these results demonstrate that LTSP bacteria that
135 adapt to consume butyrate can grow on butyrate when it continuously made available.

136 We fully sequenced 9-10 clones from each of the six populations at each of four time points:
137 days 26, 55, 62 and 121. At days 26 and 55, clones carrying the *fadR* + *atoC*/*atoS* mutation
138 combination were seen at high frequencies (ranging from 66.7% to 100%) within all three
139 populations supplemented with butyrate (**Figure 3C**, and **Figure S1C Supplementary table**
140 **S4**). Strikingly, in each of the three populations, more than a single genotype carrying these
141 mutation combinations appeared to segregate simultaneously (**Figure S1C**), highlighting the
142 remarkably extensive genetic variation present within LTSP populations. In contrast, no butyrate
143 consumption genotypes were observed at days 26 or 55 within the control populations (**Figure**
144 **3D**, and **Figure S1D Supplementary table S4**). In our original experiments, the *fadR* +
145 *atoC*/*atoS* mutation combination was found within day 22 clones that also carried an RNAPC
146 adaptation (Avrani, et al. 2017). However, all clones sequenced from our butyrate-supplemented
147 populations carried either the *fadR* + *atoC*/*atoS* mutations, or an RNAPC adaptation or, in rarer
148 cases, neither of the two (**Figure 3C** and **Supplementary table S4**). We have previously shown
149 that LTSP RNAPC adaptations severely reduce growth rates in rich media, where nutritional
150 resources are readily available (Avrani, et al. 2017; Avrani, et al. 2020). This may explain why
151 these RNAPC adaptations would be disfavored, in butyrate supplemented LTSP populations, in

152 clones that have adapted to be able to grow on butyrate. Yet, clones that carry an RNAPC
153 adaptation, but do not carry mutations enabling them to consume butyrate still persist within our
154 populations at sufficiently high frequencies to allow their detection through the sequencing of a
155 handful of clones out of ~$400*10^7$ cells present within our populations. This suggests that these
156 clones either occupy a niche that provides them with an advantage despite their inability to
157 consume butyrate, or that they are maintained at high frequencies for more than five weeks,
158 despite carrying substantial costs to fitness. In contrast, once butyrate supplementation stops (at
159 day 55), clones carrying the butyrate consumption adaptations reduce in frequencies rapidly, so
160 by day 62, no such clones were observed in two of the butyrate-supplemented populations and
161 only a single such clone was detected in the third population (**Figure 3C** and **Figure S1C**
162 **Supplementary table S4**). This suggests that butyrate consumption adaptations are associated
163 with costs that rapidly drive their frequencies down once butyrate is no longer available.

164 **Despite apparent costs, LTSP populations are able to maintain a relatively long standing**
165 **'memory' of their prior ability to consume butyrate**

166 Bacteria often exist within fluctuating environments, in which a metabolite that became available
167 once, is more likely to become available again later. Being able to rapidly re-adapt to consume a
168 metabolite that a population already adapted to consume once before, may thus be beneficial.
169 Yet, as seems to be the case for the butyrate-consumption adaptations, adapting to consume a
170 new metabolite may often come at a cost to fitness. We wanted to understand whether despite the
171 apparent costs associated with the butyrate-consumption adaptations, populations are able to
172 maintain a genetic population-level 'memory' of their previous adaptations to consume butyrate.
173 In other words, we aimed to examine whether populations that once grew in the presence of
174 butyrate and adapted to consume it, would re-adapt more rapidly to consume it, once it was again
175 available. To test this, we needed to obtain control populations that were never exposed to
176 butyrate. Towards this end, we established six new LTSP populations, as usual starting by
177 growing cells in LB. After 24 hours of growth, we filtered the bacteria out of their media,
178 washed and re-inoculated them into M9 minimal media (that contains no butyrate and no other
179 carbon sources). To three of the six populations we added butyrate at similar concentrations to
180 those produced by *E. coli* during the first 24 hours of growth in LB (1 mM). Butyrate levels were
181 monitored for all six populations. As expected, no butyrate was detected for the control

182 populations. The M9 populations to which butyrate was added, consumed it earlier than LB-
183 maintained populations, at day six for one of the M9 populations and day 11 for the remaining
184 two (**Figure 4A**). Once butyrate was consumed from these three populations, we waited an
185 additional four weeks and then sampled each of the six populations and added butyrate to each of
186 the samples, either once (**Figure 4**), or daily for seven days (**Figure S2**). As shown in **Figure 4B**
187 **and Figure S2B**, the populations that never saw butyrate, could not consume it for the seven
188 days examined. In contrast, the populations that had seen butyrate and had adapted to consume it,
189 re-adapted to consume it rapidly, doing so within two to five days (**Figure 4A** and **Figure S2A**).

190 Focusing on the populations that initially received butyrate and then received it again only once,
191 we sequenced 9-10 clones from each population at the time point in which they consumed the
192 butyrate for the second time. In two of the three populations 33.3% to 60% of the clones
193 sequenced carried a *fadR* + *atoC*/*atoS* mutation combination (**Supplementary table S5**).
194 Furthermore, in one of these two populations we could also see the exact same mutation
195 combination at the time at which that population initially consumed the butyrate (day 11 of the
196 experiment (**Figure 4C Supplementary table S5**)). Clones carrying the butyrate-consumption
197 genotype were not observed when sequencing 10 clones from the sampling time-point, prior to
198 the addition of butyrate (**Figure 4C Supplementary table S5**). Combined, this demonstrates that
199 the genotypes that arise in frequency to consume the butyrate initially, decrease in frequency, so
200 they can no longer be observed four weeks later, but are still maintained within their populations
201 at lower frequency and can thus again increase in frequency to consume the butyrate the second
202 time. No mutations of note were identified when sequencing ~10 clones from the other
203 populations at the time they initially consumed the butyrate (**Supplementary table S5**). For the
204 third population we did not observe any clones carrying the *fadR* + *atoC*/*atoS* mutation
205 combination, even once they consumed the butyrate for the second time.

206 Our inability to always identify clones carrying the *fadR* + *atoC*/*atoS* mutation combination
207 when butyrate is being consumed, may be explained by the fact that we sequenced only 9-10
208 clones out of a population of ~10^8. This means that any mutation that is segregating at slightly
209 lower frequencies might not be detectable. Nevertheless, combined our results here demonstrate
210 that despite the costs associated with the butyrate-consumption adaptations, they remain within
211 our populations for at least four weeks, following the consumption of butyrate. This in turn

212 allows the populations that previously adapted to consume butyrate to establish a population-
213 level genetic 'memory' of their prior adaptation, and more rapidly re-adapt to consume butyrate,
214 once it again becomes available.

215 Discussion

216 Wildtype *E. coli* cannot consume the short chain fatty acid butyrate. Here, we show that *E. coli*
217 produces butyrate during the first 24 hours of growth in fresh LB. This butyrate remains within
218 the media until, in a remarkably convergent and temporally precise manner, LTSP clones adapt
219 to consume it, through the acquisition of mutation combinations within the *fadR* and *atoC*/*atoS*
220 genes. These adaptations allow *E. coli* to grow on butyrate, when it is daily supplemented into
221 the LTSP media. However, once supplementation stops and butyrate is no longer available, the
222 frequency of the butyrate consumption genotypes convergently reduces within a very short
223 period of time, strongly suggesting that these adaptations carry a cost. Despite this apparent cost,
224 and despite the fact that our populations are well mixed, and that previous studies have
225 demonstrated them to be subject to very strong selection (Avrani, et al. 2017; Katz, et al. 2021),
226 butyrate consumption genotypes remain at lower frequencies within our populations for a
227 substantial period of time. This in turn, allows these populations to re-adapt more rapidly to
228 consume butyrate, once it again becomes available, compared to populations that never
229 encountered butyrate before, and never adapted to consume it.

230 It has long been understood that environmental heterogeneity can support the long-term
231 maintenance of different genotypes, each of which is specialized to a different set of conditions
232 (reviewed in (Hedrick, et al. 1976; Kassen 2104)). At the same time, within well mixed
233 populations, of the type studied here, for which conditions change only temporally, the co-
234 existence of specialist genotypes is thought to be more limited, and possible only under specific
235 sets of circumstances (Hedrick 1976; Reboud and Bell 1997; Kassen 2002, 2104). Our results
236 indicate that even for such well-mixed populations genotypes specialized towards previously
237 encountered conditions may be maintained for substantial periods of time. It is possible that our
238 experimental design that, similar to most natural environments, does not dilute out slow growing
239 or dormant cells, enables this maintenance. While most evolutionary experiments rely on
240 continuous culturing or on serial dilution (reviewed in (Barrick and Lenski 2013; Gresham and
241 Dunham 2014; Kassen 2104)), which do not allow variation in growth rates to freely persist

242 within the studied populations, our experiment does not artificially limit such variation (Katz, et
243 al. 2021). Dormant spores or bacterial cells that enter a dormant or extremely slow growing state
244 are at least partially immune from selection (Kussell, et al. 2005; Jones and Lennon 2010; Gray,
245 et al. 2019), allowing them to survive despite carrying deleterious genotypes. Dormancy or
246 extreme slow growth is thus one manner in which populations may be able to maintain within
247 them non-favored genotypes, specialized towards a previously encountered condition (Miller and
248 Klausmeier 2017; Shoemaker and Lennon 2018). Balancing selection can also enhance the co-
249 existence of specialist genotypes (Good, et al. 2017; Turner, et al. 2020). Enabling populations to
250 maintain higher variation in growth rate may increase the opportunities for such balancing
251 selection (Levy, et al. 2012; Merot, et al. 2020) and may therefore enhance the ability of
252 populations to maintain within them longer-term genetic variation, including genotypes adapted
253 to previous conditions.

254 Importantly, our study revealed that the previous metabolic conditions toward which the bacteria
255 are adapted to under LTSP were, in fact, generated by the bacteria themselves. Butyrate is
256 produced and secreted to the media at times of nutritional abundance, and it forms a metabolic
257 pressure selecting for genetic adaptations that enable later butyrate consumption under LTSP. It
258 is therefore plausible that once bacteria are adapted to thrive on a nutritional resource that was
259 initially produced by the same population, they are likely to maintain genetic memory for such
260 conditions, even if it comes with a cost once the nutrient is fully consumed.

261 That large *E. coli* LTSP populations can maintain a population-level genetic 'memory' of their
262 previously acquired ability to consume a particular metabolite, may very well reflect a broader
263 ability of many bacterial populations to "remember" their previous metabolic adaptations. When
264 such a 'memory' is maintained, genetic adaptation to fluctuating conditions can occur at an
265 ecological rather than an evolutionary time scale. Establishment of a genetic population-level
266 'memory' of adaptations to consume metabolites that are periodically encountered within a
267 certain environment could also enable bacterial populations to obtain a better 'foot-hold' within
268 their environment, by offering a type of protection from invasion by outside populations. After
269 all, populations that already established a 'memory' of the metabolites which are most
270 commonly available within a particular environment, will have an advantage in their ability to

271 re-adapt more rapidly to consume these metabolites, compared to an entirely naïve external
272 population.

273 Materials and Methods

274 Media preparation

275 Luria-Bertani (LB) medium was prepared with 10 g L^{-1} tryptone (BD), 5 g L^{-1} yeast extract (BD),
276 and 5 g L^{-1} NaCl. For (LB) agar plates, 15 g L^{-1} agar (BD) were added to the same formulation.
277 Minimal medium (M9) was prepared according to manufacturer instructions (Difco™ M9
278 Minimal Salts, 5× Cat. No. 248510), and supplemented with 2 mL L^{-1} $MgSO_4$ 1 M, and 0.1 mL
279 L^{-1} $CaCl_2$ 1 M. Butyrate stock solution (Sigma Aldrich CAS no. 107-92-6) was prepared in ultra-
280 pure water at a concentration of 100 mM, and added for a final concentration in the culture medium
281 of 1 mM. All media and solutions were either filter sterilized (0.45 µm pore size) or autoclaved
282 before use.

283 LTSP experiments

284 Single colonies of *E. coli* K12 MG1655 (WT, ancestor) were grown in test tubes containing 4 mL
285 of fresh LB medium in a shaking incubator (225 rpm at 37 °C), until they reached an OD ~ 0.4.
286 Then, 1 mL was taken to a 2 L-polycarbonate breathing flask containing 400 mL of fresh LB, for
287 a final concentration of ~ $2x10^6$ cells/mL. Flasks were placed in an incubator set at 37°C, with
288 shaking at 225 rpm. No new nutrients or resources were added to the cultures with time, except
289 for sterile water that was added to compensate for evaporation every 10–15 days, according to the
290 weight lost by each flask during that time period.

291 Samples from the culture media were taken at regular intervals, centrifuged (5 min at 5000*g*/ 4°C),
292 and the supernatant was stored at -20°C for further metabolomics studies. To estimate bacterial
293 viability, 1 mL of each culture was sampled. Dilutions were plated using a robotic plater to evaluate
294 viability through live counts. Samples were stored in 50% glycerol at -80°C for future analysis.

295 For the daily supplementation of butyrate experiment, single colonies were allowed to recover in
296 4 mL-fresh LB in a shaking incubator (225 rpm at 37 °C), until an OD ~ 0.4 was reached. From
297 each of three ancestral clones, two independent LTSP experiments were started (for a total of six
298 experiments), as described, by inoculating *E. coli* K12 MG1655 into 400 mL-fresh LB medium,

299 to a final concentration of ~ $2x10^6$ cells/mL. Flasks were kept in a shaking incubator at 37°C 225
300 rpm. Starting at day 18 of these experiments, three populations (each one from a different ancestor)
301 were supplemented daily with 1 mL of butyrate (for a final concentration of 1mM), up to day 55.
302 For each butyrate-supplemented population, an additional flask with no supplementation was kept
303 as a control. Population sampling was done regularly.

304 For the M9 experiments, single colonies were allowed to recover in 4 mL-fresh LB in a shaking
305 incubator (225 rpm at 37 °C), until an OD ~ 0.4 was reached. From each of three ancestral clones,
306 2 independent LTSP experiments were started (for a total of six experiments), as described, by
307 inoculating *E. coli* K12 MG1655 into 400 mL-fresh LB medium, to a final concentration of ~
308 $2x10^6$ cells/mL. Flasks were kept in a shaking incubator at 37°C/ 225 rpm during 24 h. Next, the
309 total population in each flask was harvested by centrifugation (20 min at 4000g) and rinsed once
310 in M9. All populations were resuspended in 400 mL of M9, three of them (each one from a
311 different ancestor) received butyrate at a final concentration of 1 mM. At day 40 of the LTSP-M9
312 experiments, 20 mL of each of the six populations were withdrawn and split into two 50 mL-
313 Erlenmeyer flasks (10 mL per flask). Each flask received either a single or a daily dose of butyrate
314 (1 mM final concentration). The Erlenmeyer cultures were kept in a shaking incubator at 37°C/
315 225 rpm, for one week. Culture media samples and CFU measurements were conducted as
316 described above.

317 **Monitoring butyrate consumption**

318 In order to monitor the consumption of butyrate, Frozen cultures from our previous study(Avrani,
319 et al. 2017), of *E. coli* K12 MG1655 (WT, ancestor), LTSP populations 1,2 and 4 at days 11, 22
320 and 32, and isolated colonies from the clones summarized in **Supplementary table S6**, were
321 thawed and allowed to grow overnight in liquid LB. Additionally, the wildtype ancestral strain
322 was also thawed and grown overnight. At the following day, each type of resulting culture was
323 diluted 1:50 into fresh LB media and recovered for an additional two hours, until they reached an
324 OD ~ 0.2. Cells were harvested by centrifugation (5 min at 5000*g*) and rinsed twice in M9 media.
325 The cells were then resuspended in 5 mL of M9 (baseline) or M9 supplemented with butyrate
326 (1mM final concentration). Tubes were placed in a shaking incubator (225 rpm at 37 °C) and
327 sampled at time 0, 3 and 24 hours. Culture media samples were obtained after cell centrifugation
328 (5 min at 5000*g*/ 4°C) and stored at -20°C for further metabolomics analysis.

329 **Profiling short-chain fatty acids (SCFA) by GCMS**

330 Representative metabolite standards were used for quality control. ^{13}C-labeled isotopes of formate,
331 propionate, butyrate, and $^{2}H_3$-acetate, were used as internal standards for quantification.

332 ***Chemical derivatization for SCFA.*** The chemical derivatization procedure of samples using ethyl
333 chloroformate (ECF) was adapted from the protocol described in(Tumanov, et al. 2016). Briefly,
334 200 µL of medium sample was added to a 1.5 mL microfuge tube, followed by addition of 10 µL
335 of internal standard, 10 µL of benzyl alcohol, 10 µL of sodium hydroxide 1 M, and 50 µL of
336 pyridine. The tube was then placed on ice for 5 min. Next, 20 µL of ECF were added immediately
337 followed by vigorous vortexing for 30 s. As gas builds up in the microfuge tube during the
338 derivatization reaction, it is very important that samples are cold enough. Tubes may be carefully
339 opened in the middle of the vortexing period to relieve pressure and put back to vortex to complete
340 the required length of time. After vortexing, 200 µL of MTBE were added, the sample vortexed
341 for another 20 s, and centrifuged at 10,000*g* or max speed for 5 min. 120 µL microliters of the
342 resulting upper layer were transferred to a GC vial with insert for analysis.

343 ***SCFA detection and quantification.*** Derivatized samples were analyzed with an Agilent 7890B
344 GC system coupled to a 7000 Triple Quadrupole GC-MS system. The column was Phenomenex
345 ZB-1701 column (30 m x 0.25 mm x 0.25 µm), with the following oven program: initial
346 temperature 50°C/ hold 2 min; increment at 10°C/min to 140°C/ hold 0 min; increment at
347 20°C/min to 182°C/ hold 1 min; and increment at 50°C/min to 280°C/ hold 0 min. Run time 16.1
348 min. Samples (1 µL) were injected using split mode (20:1, 28 mL/min split flow). The column gas
349 flow was held at 1.4 mL/min of He. The temperature of the inlet was 280°C, the interface
350 temperature 230°C, and the quadrupole temperature 150°C. The column was equilibrated for 2
351 min before each analysis. The mass spectrometer was operated in single-ion monitoring (SIM)
352 mode between 9.9 and 14.0 min. A segment for each compound was defined: benzyl-formate at
353 9.9 min, fragments *m/z* 136 and 137; benzyl-acetate at 11.0 min, fragments *m/z* 150, 151, 152 and
354 153; benzyl-propionate at 12.2, fragments *m/z* 164, 165, 166 and 167; and benzyl-butyrate at 13
355 min, fragments *m/z* 178,179,180, and 182. All segments at 2.5 cycles/s. Agilent MassHunter
356 Workstation Software (version B.07.01 SP1) was employed for automated data processing, using
357 peak area for absolute concentrations calculations.

358 **Metabolites profile by LCMS**

359 ***Metabolite extraction.*** For the extraction of secreted metabolites, 50 µL of medium was diluted in
360 950 µL of ice-cold extraction solvent (50% methanol, 30% acetonitrile, 20% water), vortexed at
361 high speed/ 4°C during 10 min, centrifuged at 16 000*g* for 10 min at 4°C. Supernatant was
362 transferred into LC-MS glass vials and stored at −80°C until measurement. For the extraction of
363 intracellular metabolites cell pellets were vortexed with 200 µL of ice-cold extraction solvent
364 during 10 min, and incubated at -20°C for another 10 min. These cold-vortex / incubating steps
365 were repeated three times. Pellets were subsequently centrifuged at 16000*g* for 10 min at 4°C, and
366 the supernatant were transferred into LC-MS glass vials with inserts and stored at −80°C until
367 measurement.

368 ***LC-MS measurements.*** LC-MS metabolomics analysis was performed as described
369 previously(Mackay, et al. 2015; Meiser, et al. 2016) (Mackay et al., 2015; Meiser et al. 2016).
370 Briefly a Q Exactive Orbitrap mass spectrometer (Thermo Fisher Scientific) was used, together
371 with a Thermo UltiMate 3000 high-performance liquid chromatography (HPLC) system. The
372 HPLC setup consisted of a ZIC-pHILIC column (SeQuant, 150 mm x 2.1 mm; Merck KGaA, 5
373 mm), with a ZIC-pHILIC guard column (SeQuant, 20 mm x 2.1 mm) and an initial mobile phase
374 of 20% of 20 mM ammonium carbonate (pH 9.2) and 80% acetonitrile. Cell and medium extracts
375 (5 µL) were injected, and metabolites were separated over a 15-min mobile phase gradient,
376 decreasing the acetonitrile content to 20%, at a flow rate of 200 µL/min and a column temperature
377 of 45°C. The total analysis time was 27 min. All metabolites were detected across a mass range of
378 75 to 1 000 mass/charge ratio (*m/z*) using the Exactive mass spectrometer at a resolution of 35 000
379 (at 200 *m/z*), with electrospray ionization and polarity switching to enable both positive and
380 negative ions to be determined in the same run. Lock masses were used, and the mass accuracy
381 obtained for all metabolites was below 5 ppm. Data were acquired using Thermo Xcalibur
382 software. The peak areas of different metabolites were determined using Thermo TraceFinder 4.1
383 software, where metabolites were identified by the exact mass of the singly charged ion and by
384 known retention time on the HPLC column, using an in-house MS library built by running
385 commercial standards of all metabolites detected.

386 **DNA extraction, sequencing library preparation and mutation calling**

387 Frozen cultures were thawed and dilutions were plated and grown over night. Colonies to be
388 sequenced were used to inoculate 4 ml of LB medium in a test tube and were grown until they

389 reached an OD of 1. One milliliter of the culture was centrifuged at 5,000 g for 5 min and the pellet
390 was used for DNA extraction. The remainder of each culture was then archived by freezing in 50%
391 of glycerol at -80°C. DNA was extracted using the Macherey-Nagel NucleoSpin 96 Tissue Kit.
392 Library preparation followed the protocol outlined in Baym et al(Baym, et al. 2015). Sequencing
393 was carried out at Admera Health (New Jersey USA) using an Illumina HiSeq machine. Clones
394 were sequenced using paired end 150 bp reads. In order to call mutations, the reads obtained for
395 each clone were aligned to the *E. coli* K12 MG1655 reference genome (accession NC_000913).
396 Mutations were recorded if they appear within a clone's genome, but not within the ancestral
397 genome. Alignment and mutation calling were carried out using the Breseq platform, which allows
398 for the identification of point mutations, short insertions and deletions, larger deletions, and the
399 creation of new junctions(Deatherage and Barrick 2014).

400 **Competing interest declaration**

401 The authors declare no competing financial and/or non-financial interests in relation to the work
402 described in the submitted manuscript.

403 **Data availability statement**

404 Metabolomics data have been deposited to the EMBL-EBI MetaboLights database, under the
405 identifier MTBLS3041.
406 Raw sequencing reads were deposited to the sequence read archive (SRA) BioProject ID:
407 PRJNA746737.

408 **Acknowledgements**

409 This work was supported by ISF grants (No. 1860/21, to R.H and No. 824/19, to E.G) and by the
410 Rappaport Family Institute for Research in the Medical Sciences (to R.H). C.GI was supported
411 by a TICC - Rubenstein Postdoctoral Fellowship.

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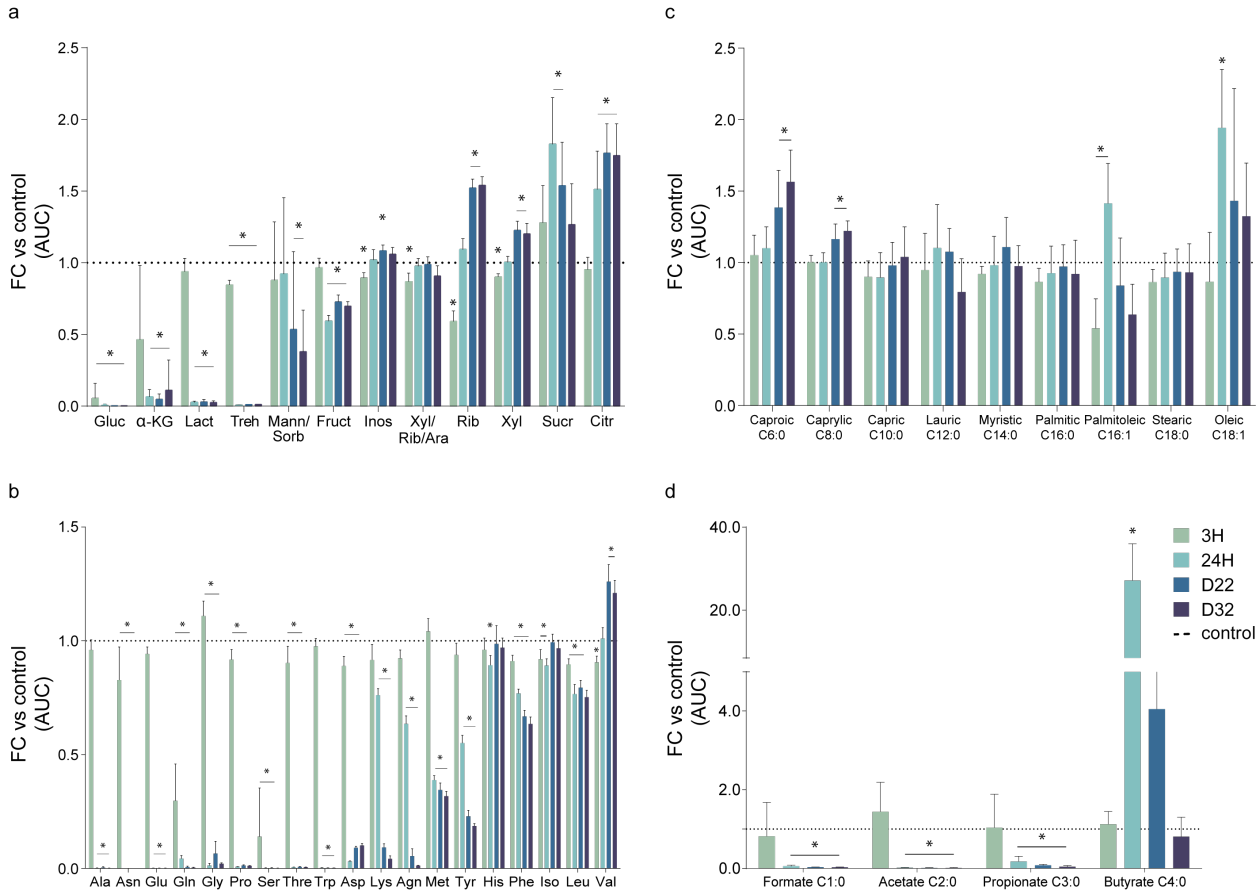

Figure 1. Changes in the presence of metabolites within LTSP media, during the first 32 days of adaptation. Data obtained from five to nine independent LTSP populations, which were established as described in the text. For each population, consumed and excreted metabolites were identified in the media, following 3 h, 24 h, 22 days and 32 days of culture. Six flasks containing only LB without bacteria were used as controls. The fold change (FC) area under the curve (AUC), compared to the controls, was calculated for each identified metabolite at the indicated time point. (A) Glucose and other sugars and derivatives, (B) proteogenic amino acids, and (C) medium- and long-chain free fatty acids were analyzed by targeted LC-MS. (D) Short-chain fatty acids were identified by targeted GC-MS, previous derivatization. Mean values with error bars representing standard deviations around the mean are shown. Statistical significance between two samples was calculated using a Mann-Whitney two-sided test, to compare the

517 absolute values from each timepoint versus control. * = P <0.05. (The detailed data and their
518 statistical analyses can be found in **Supplementary tables S1-S3**).

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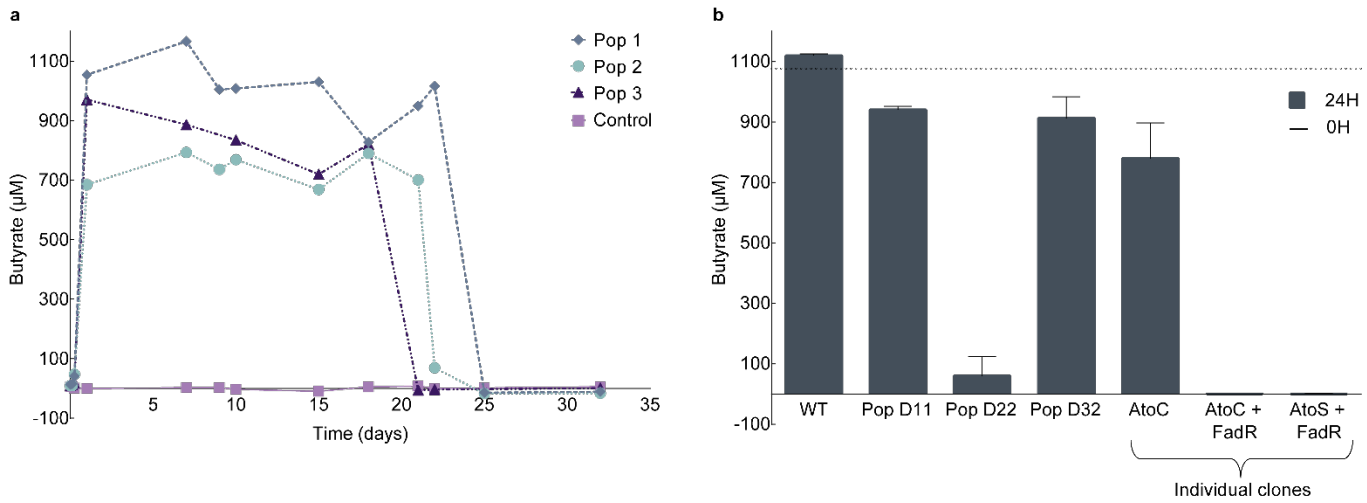

Figure 2. Butyrate kinetics. (A) Butyrate is produced during the first 24 hours of growth in LB and then consumed at days 18-22. Butyrate kinetics were monitored for 32 days by GCMS analyses on three independent LTSP populations, with LB media without inoculated bacteria serving as a control (purple). **(B) day 22 LTSP population samples and clones carrying *fadR* + *atoC* or *fadR* + *atoS* mutation combinations can consume butyrate.** The ability to consume butyrate was examined for each sample or clone, through GCMS measurements of the levels of butyrate remaining after 24 hours. Mean values across three biological replicas, per sample or clone, are presented with error bars representing standard deviations around the mean. The levels of butyrate added at day 0 are marked with a dotted line.

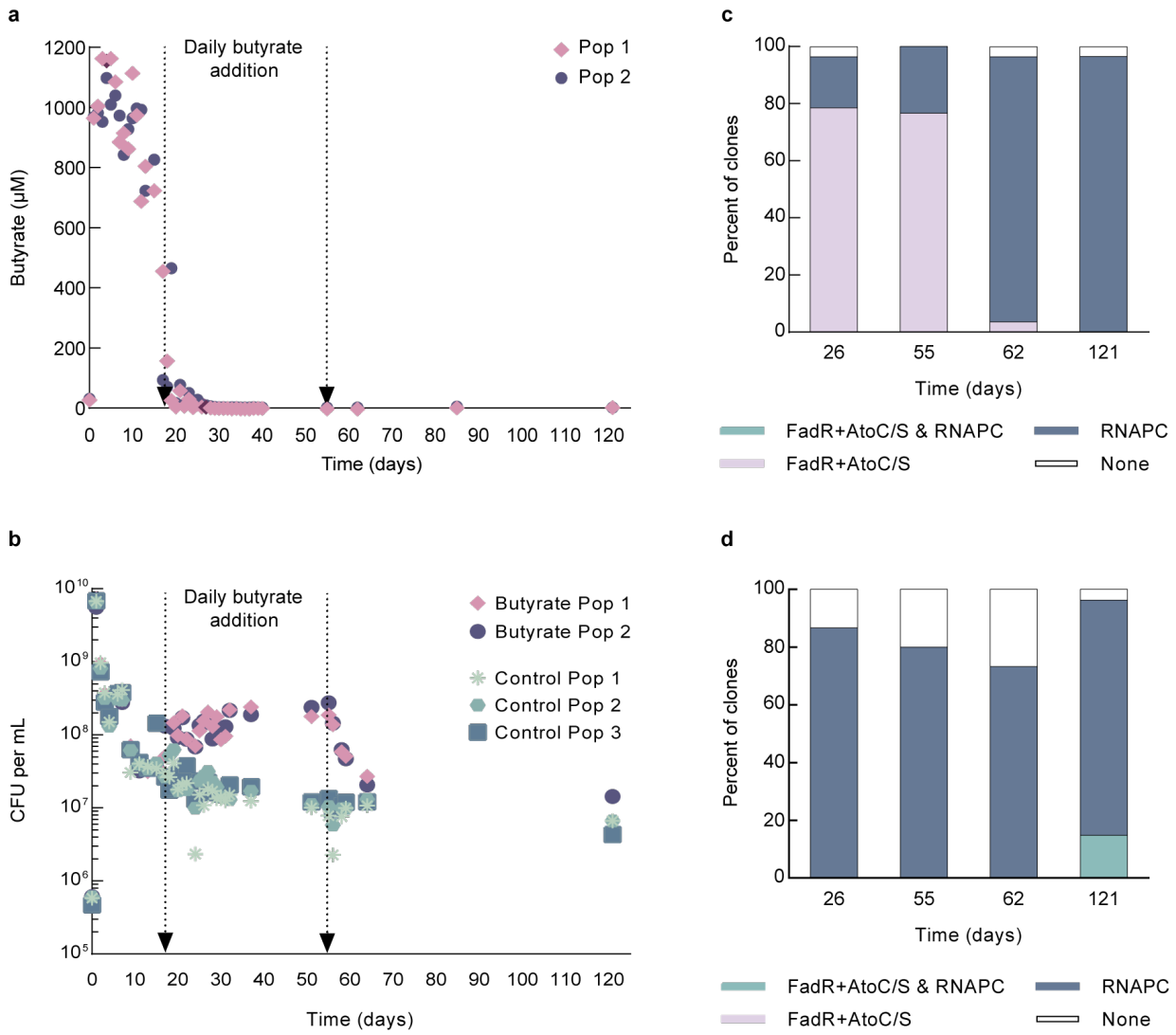

Figure 3. Daily supplementation of butyrate and its effect on LTSP populations. Six LTSP populations were established. To three of the six populations, butyrate was added daily starting at day 18 and ending at day 55. **(A)** Levels of butyrate measured within two populations to which butyrate was added, as a function of time. The time range in which butyrate was added is marked on the graph (A similar plot for the third population is presented in **Supplementary Figure S1A**). **(B)** Mean number of viable cells with time in the two LTSP populations supplemented daily with butyrate (pink/purple) or in control LTSP populations which were allowed to evolve without interference (green). Numbers of viable cells were estimated by counting colony-forming units (CFU). The time range in which butyrate was added is marked on the graph. (A similar plot for the third population is presented in **Supplementary Figure S1B**). **(C)** In the

553 three populations to which butyrate is added between days 18 and 55, a high frequency of clones
554 sequenced at day 26 and 55 carry butyrate consumption adaptations but do not carry any
555 adaptations within the RNAPC. Clones sequenced at day 62 very rarely carry butyrate
556 consumption adaptations and often carry an RNAPC adaptation. **(D)** Control populations to
557 which butyrate was not added almost never contain butyrate consumption adaptations at
558 observable frequencies. In contrast, these populations contain high frequencies of clones carrying
559 RNAPC adaptations at all time points.

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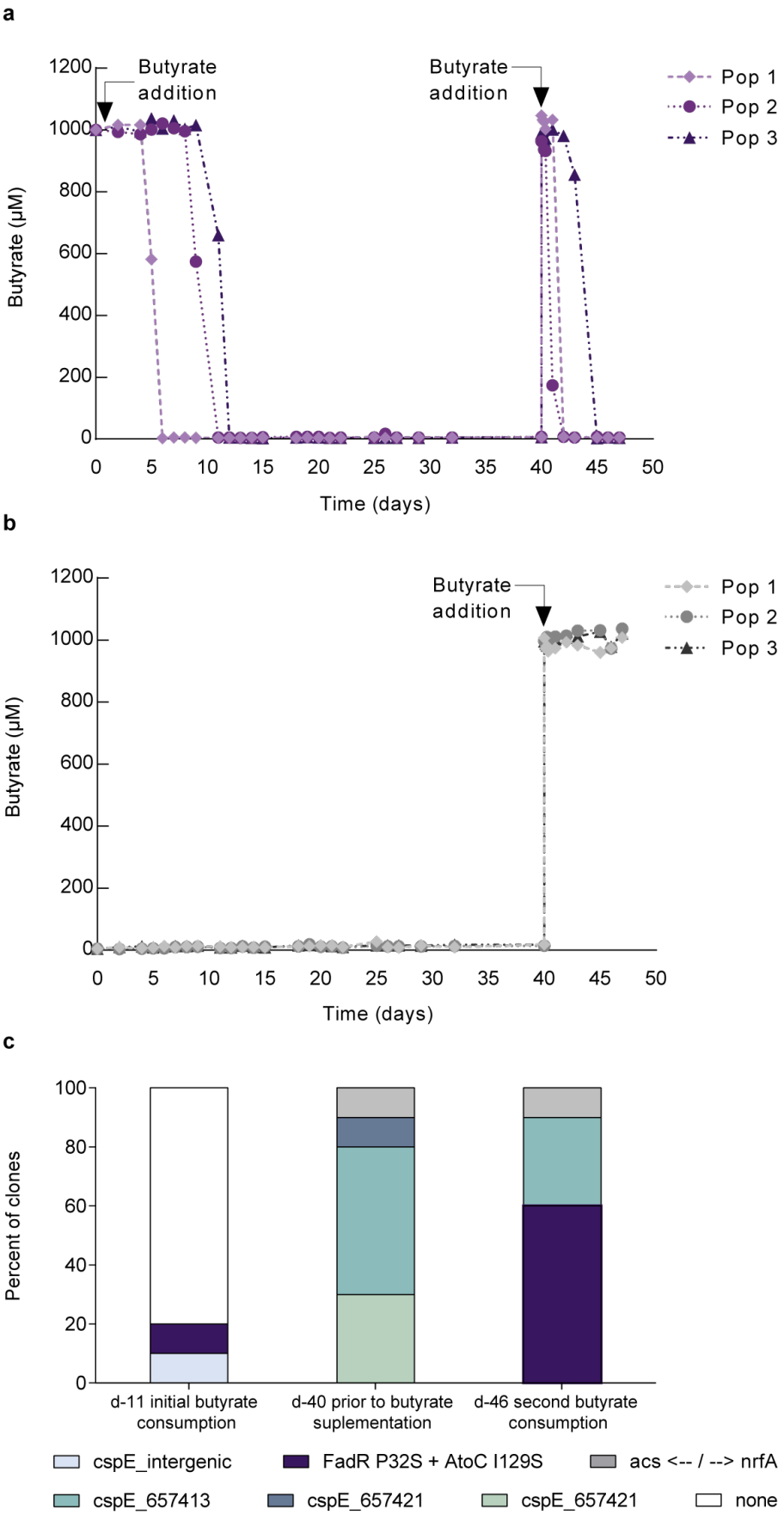
a
Butyrate addition
Butyrate addition
Pop 1
Pop 2
Pop 3
Butyrate (µM)
Time (days)
b
Butyrate addition
Pop 1
Pop 2
Pop 3
Butyrate (µM)
Time (days)
c
Percent of clones
d-11 initial butyrate consumption
d-40 prior to butyrate suplementation
d-46 second butyrate consumption
cspE_intergenic
FadR P32S + AtoC I129S
acs <-- / --> nrfA
cspE_657413
cspE_657421
cspE_657421
none

562

563 **Figure 4. Populations that previously encountered butyrate adapt more rapidly to consume**
564 **it, once it again becomes available**. One day after initiation, cells from three LTSP experiments
565 were filtered out of their LB media and re-inoculated into three flasks of M9 minimal media,
566 either supplemented with 1 mM butyrate **(A)**, or not **(B)**. When butyrate was provided, it was
567 initially consumed by day 12. Following four additional weeks, populations were sampled and
568 assayed for their ability to consume butyrate. As shown, only populations that initially received
569 butyrate could then consume it, during the week following its addition into their media (**A**,
570 arrows represent time points in which butyrate was added). **(C)** Sequencing data from butyrate-
571 supplemented population 3 reveals that the same mutation combination was responsible for
572 initial (day 11) and secondary (day 46) butyrate consumption. Yet, this mutation combination
573 was not present at observable frequencies at day 40, immediately prior to the second
574 supplementation with butyrate. The full list of mutations identified can be found in **Table S5**.

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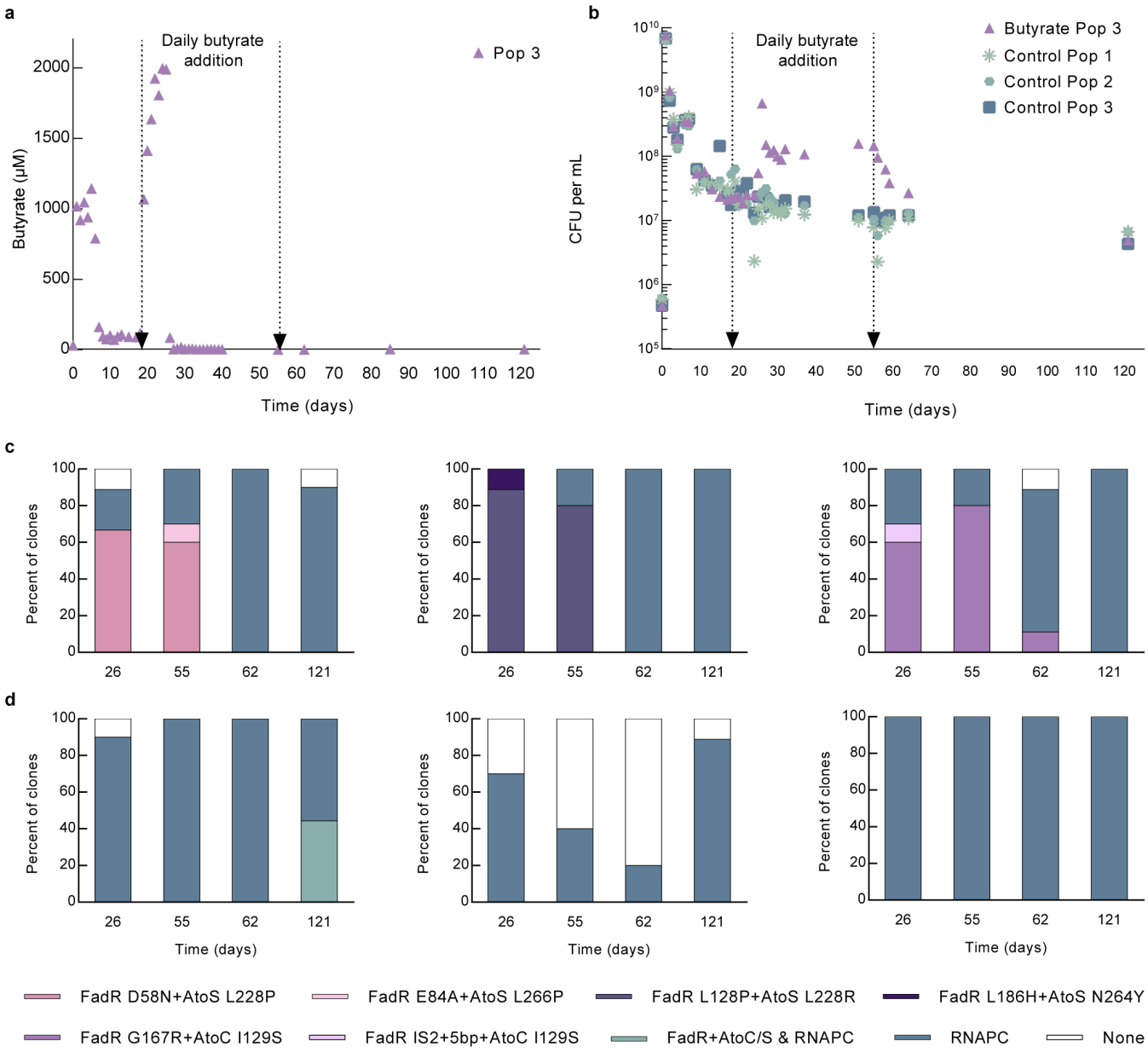
a
Daily butyrate addition
Pop 3
Butyrate (μM)
Time (days)
b
Daily butyrate addition
Butyrate Pop 3
Control Pop 1
Control Pop 2
Control Pop 3
CFU per mL
Time (days)
c
Percent of clones
d
Percent of clones
Time (days)
FadR D58N+AtoS L228P
FadR E84A+AtoS L266P
FadR L128P+AtoS L228R
FadR L186H+AtoS N264Y
FadR G167R+AtoC I129S
FadR IS2+5bp+AtoC I129S
FadR+AtoC/S & RNAPC
RNAPC
None

600 **Figure S1. Daily butyrate supplementation and its effect on LTSP populations.**

601 **(A)** Levels of butyrate and **(B)** Mean number of viable cells, as measured through CFU
602 calculations for the third butyrate-supplemented population. As can be seen, as with the
603 remaining two populations (Figure 3A and 3B), for this population as well, CFU increases, once
604 butyrate begins to be consumed. Dashed lines mark the time frame (days 18-55) during which
605 butyrate was daily added to the media. **(C)** The frequency of different genotypes within the three
606 populations to which butyrate was added between days 18 and 55. **(D)** The frequency of different
607 genotypes within the three control populations to which butyrate was not added.

608

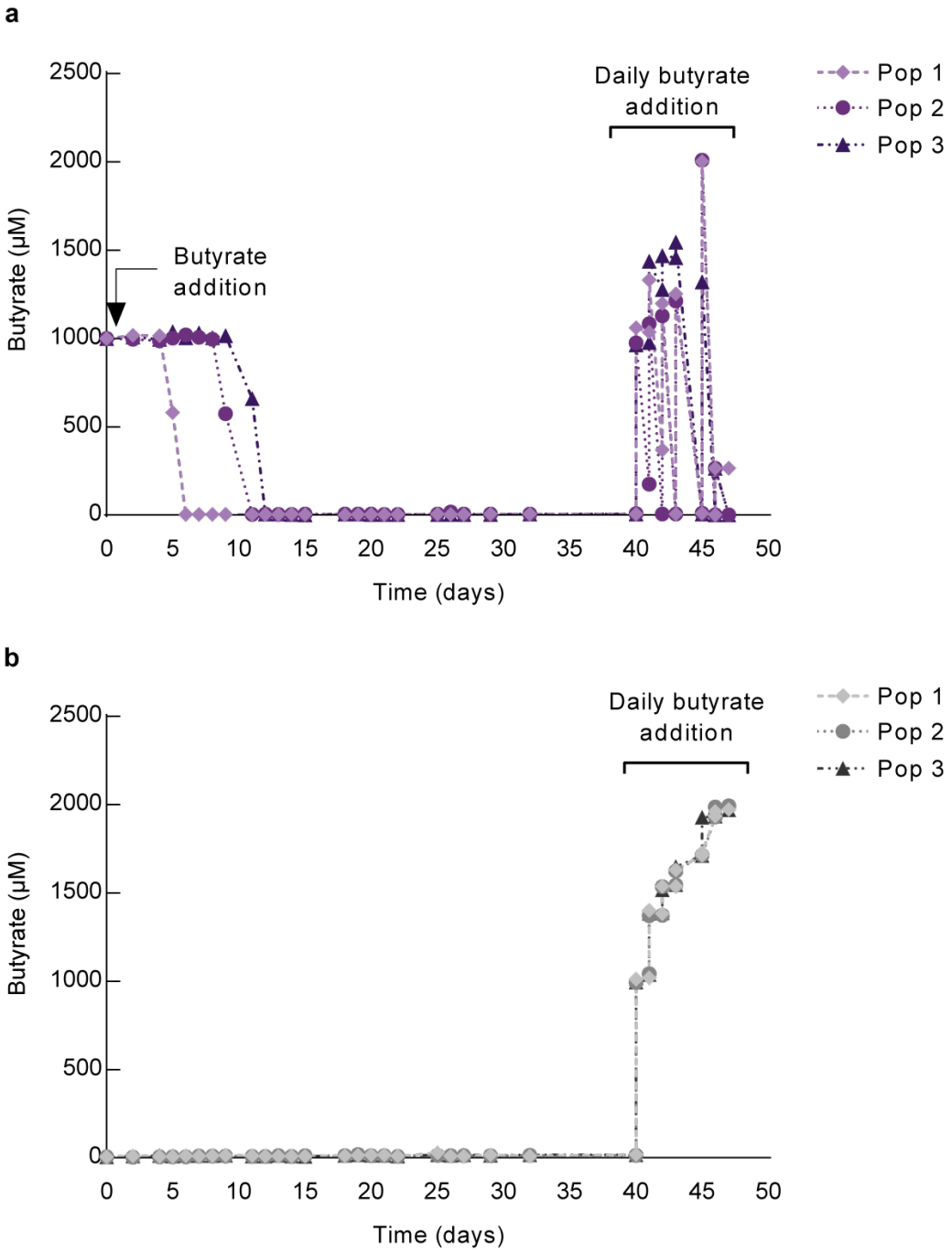

Figure S2. Populations that previously encountered butyrate adapt more rapidly to consume it, once they again encounter butyrate. One day after initiation, cells from three LTSP experiments were filtered out of their LB media and re-inoculated into three flasks of M9 minimal media, either supplemented with 1 mM butyrate **(A)**, or not **(B)**. When butyrate was provided, it was initially consumed by day 12. Following four additional weeks, populations

were sampled and assayed for their ability to consume butyrate, which was supplemented daily into the sampled populations. As shown, only populations that initially received butyrate **(A)** could then consume it, during the week following its addition into their media (arrows represent time points in which butyrate was added).