Acetate metabolism and the inhibition of bacterial growth by acetate

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Running head: Acetate metabolism and growth inhibition by acetate
Abstract

During aerobic growth on glucose, *Escherichia coli* excretes acetate, a mechanism called “overflow metabolism”. At high concentrations, the secreted acetate inhibits growth. Several mechanisms have been proposed for explaining this phenomenon, but a thorough analysis is hampered by the diversity of experimental conditions and strains used in these studies. Here, we describe the construction of a set of isogenic strains that remove different parts of the metabolic network involved in acetate metabolism. Analysis of these strains reveals that (i) high concentrations of acetate in the medium inhibit growth without significantly perturbing central metabolism, (ii) growth inhibition persists even when acetate assimilation is completely blocked, and (iii) regulatory interactions mediated by acetyl-phosphate play a small but significant role in growth inhibition by acetate. The major contribution to growth inhibition by acetate may originate in systemic effects like the uncoupling effect of organic acids or the perturbation of the anion composition of the cell, as previously proposed. Our data suggest, however, that in the conditions considered here, the uncoupling effect plays only a limited role.
Importance

High concentrations of organic acids such as acetate inhibit growth of *Escherichia coli* and other bacteria. This phenomenon is of interest for understanding bacterial physiology, but is also of practical relevance. Growth inhibition by organic acids underlies food preservation and causes problems during high-density fermentation in biotechnology. What causes this phenomenon? Classical explanations invoke the uncoupling effect of acetate and the establishment of an anion imbalance. Here we propose and investigate an alternative hypothesis: the perturbation of acetate metabolism due to the inflow of excess acetate. We find that this perturbation accounts for 20% of the growth-inhibitory effect, through a modification of the acetyl phosphate concentration. Moreover, we argue that our observations are not expected based on uncoupling alone.
Introduction

Growth rate is probably the most important physiological parameter characterizing bacteria. The growth rate of a bacterial culture depends on the composition of the growth medium and the genotype of the particular strain. In the most commonly used controlled growth condition, minimal medium supplemented with glucose as the sole carbon source, the model bacterium *Escherichia coli* secretes acetate, a by-product of glycolysis during fast aerobic growth. This “overflow metabolism” is a function of growth rate. Experiments that vary the rate of glucose utilization by *E. coli* cells growing aerobically show a linear increase of growth rate with the rate of glucose utilization up to around 0.6 h\(^{-1}\) [56]. Beyond this growth rate, respiration becomes limiting at 15 mmol of O\(_2\) per gram dry weight (gDW) and per h. Since glucose can no longer be fully oxidized to CO\(_2\), the extra redox potential is eliminated by secreting metabolites such as acetate [18]. These observations have been explained in terms of constraints on proteome allocation. Above a certain glucose uptake rate, the cell favors the use of fermentation pathways that are less efficient than respiration in producing ATP, but also less costly to synthesize [5].

The secretion of acetate and other fermentation acids during growth is common in microorganisms and it has been known for a long time that acid accumulation in the medium inhibits growth. For example, the growth rate of *E. coli* in minimal medium with glucose is reduced with increasing concentrations of acetate, diminishing to half of its reference growth rate in glucose alone when about 100 mM acetate is added to the medium [36]. This inhibitory effect of acetate and other organic acids on microbial growth is of considerable practical interest. The addition of organic acids is widely used in the food industry to inhibit the growth of microbial pathogens [6]. Moreover, growth inhibition by acetate and other organic acids is an important problem in biotechnological fermentation processes, limiting their utilization as a substrate for biorefining applications [57] and reducing the production of recombinant proteins in aerobic high cell density cultures [17, 37]. This has motivated many studies in *E. coli*, searching for genetic modifications capable of reducing the flux to unwanted anaerobic by-products or increasing the acid tolerance of the cell.
Several hypotheses have been advanced in the literature for explaining the inhibition of microbial growth by acetate and other organic acids. The classical explanation invokes the uncoupling effect of organic acids. Acetic acid (HAc), the protonated form of acetate, can diffuse freely across the cell membrane [26]. Inside the cell, HAc dissociates into an acetate anion Ac$^-$ and a proton H$^+$ because the p$K_a$ of HAc (4.76) is much lower than the intracellular pH (around 7.6, [59]). In order to maintain the membrane potential, the excess protons have to be expelled from the cell, which causes an energy expenditure detrimental to growth [2, 29, 51]. The presence of acetate anions inside the cell also increases the internal osmotic pressure, which forms the basis for a second explanation [44, 47]. Roe et al. [44] have observed that, in order to maintain osmotic pressure, the intracellular pools of other anions, most prominently glutamate, are reduced. The resulting perturbation of anion pools may affect the functioning of metabolism and therefore growth. A follow-up study showed that high concentrations of acetate in the cell specifically inhibit a step in the biosynthesis of methionine, leading to the accumulation of the toxic intermediate homocysteine [45]. The authors observed that growth inhibition could be substantially relieved by supplying the medium with methionine.

A recent study showed that, surprisingly, acetate is also taken up and consumed by *E. coli* cells growing on excess glucose [33]. Further work confirmed that the PtA-AckA pathway did not only produce but also consume acetate [20]. The net flux through the pathway was found to be controlled thermodynamically, in the sense that at high concentrations of external acetate, the flux direction is reversed and *E. coli* cells consume acetate while growing on glucose. This suggests a third hypothesis for growth inhibition by acetate, namely the perturbation of acetate metabolism. The influx of excess acetate into the cell may be detrimental to maximum growth on glucose by perturbing fluxes in central metabolism. Moreover, it may change the concentration of acetyl-phosphate (Ac$\sim$P), a signaling metabolite that can transfer phosphate groups to regulatory proteins and thus modulate the expression of many genes or affect other processes, such as motility (as reviewed in [60, 61]). *In-vitro* studies have suggested that Ac$\sim$P may even function as...
an alternative phosphate donor in the uptake of sugars transported by a phosphotrans-
ferase system (PTS) [24]. Moreover, Ac~P is involved in the acetylation of enzymes and
regulatory proteins with broad physiological consequences [7, 34, 58].

The major aim of this manuscript is to investigate this third hypothesis, namely that
growth inhibition by acetate is connected with the influx of excess acetate into central
carbon metabolism and/or with the regulation of cellular functions by Ac~P. To this end,
we constructed a collection of mutant strains with deletions of genes encoding enzymes
involved in acetate metabolism. The metabolic network of acetate excretion and assim-
ilation is represented in Figure 1. We constructed mutants in all relevant genes coding
for enzymes that connect acetate to central carbon metabolism and/or produce Ac~P,
i.e., the genes acs, pta, ackA, and poxB. We reasoned that, if growth inhibition occurs
through the uptake and assimilation of acetate by central carbon metabolism, with the
consequent perturbation of fluxes and Ac~P levels, this effect should be strongly miti-
gated in the mutant strains. Moreover, one would expect the distribution of fluxes within
central carbon metabolism to be strongly perturbed by the addition of acetate to the
growth medium. We tested these predictions by studying the effect of acetate on the
growth of E. coli strain BW25113, in well-controlled growth conditions (minimal medium
with glucose, at pH 7.4 or pH 6.4) using the above-mentioned defined mutants of other-
wise isogenic strains. Moreover, we quantified the extracellular concentrations of the sole
carbon source in our conditions, glucose, and the major fermentation products: acetate,
formate, pyruvate, lactate, and ethanol.

Firstly, we found that in mutant strains devoid of Ac~P (Δpta ackA) growth inhibition is
reduced by 20%. This indicates that Ac~P has a small but significant effect in mediating
the inhibitory effect of acetate. The same effect was found in the single ΔackA mutant,
which suggests that blocking the synthesis of Ac~P from external acetate via AckA
is enough to reduce the Ac~P concentration to a level below which it does no longer
contribute to growth inhibition. Secondly, we computed uptake and secretion rates from
the measurements of extracellular metabolite concentrations, both in the wild-type strain
and in the Δacs pta and Δacs pta ackA strains. When combining the measured uptake
and secretion rates with a genome-scale flux balance model [23], the predicted internal
metabolic fluxes during growth with or without acetate are found to be strongly correlated.
This suggests that, apart from a proportional rescaling of fluxes due to the reduced growth
rate, central carbon metabolism functions in much the same way whether acetate is added
to the medium or not. We conclude that the growth inhibitory effect of acetate is not due
to the influx of excess acetate into central carbon metabolism.

Our results indicate that changes in the concentration of Ac~P account for about 20% of
the reduction in growth rate in the presence of high acetate concentrations in the medium.
Although our data do not allow to unambiguously attribute the remaining 80% of the effect
to either or both of the two classical hypotheses, uncoupling and anion imbalance, they
do provide circumstantial evidence that the uncoupling hypothesis may be less important
than is sometimes assumed, consistent with previous reports [47, 48]. In particular, we
find that the biomass yield, defined as the ratio of the growth rate and the glucose
uptake rate, does not significantly change when adding acetate to the medium, contrary
to what is expected from the uncoupling hypothesis. Moreover, deletion of known acetate
transporters does not noticeably change the growth inhibitory effect of acetate, whereas it
would be expected to affect the futile cycle of acetate uptake and secretion necessary for
uncoupling. These observations, while not conclusive in themselves, provide an interesting
basis for further research, in particular the measurements of the changes in bioenergetic
parameters upon acetate addition and the precise characterization of physiological changes
accompanying the perturbation of anion pools and their regulatory effects at the molecular
level.

Materials and Methods

Bacterial strains and growth media

The bacteria used in this study were E. coli K-12, strain BW25113 [3], that we will
call “wild-type” (rrnB\text{T14} ΔlacZ\text{WJ16} hsdR514 ΔaraBAD\text{AH33} ΔrhaBAD\text{LD78}). The fol-
lowing deletion mutants were constructed by removing the entire open reading frames of the corresponding genes: Δacs, ΔackA, Δpta, Δpta ackA, Δacs pta, Δacs pta ackA, Δacs pta ackA poxB. Δpta ackA is the shorthand notation for the double mutant Δpta ΔackA (an analogous abbreviation is used for the other strains). We also constructed a reversion mutant, Δacs pta ackA::ackA_wt. In this last mutant, a wild-type copy of the ackA gene was re-introduced into the mutant strain in order to verify the strain constructions. The phenotype of the resulting complemented strain should be identical to Δacs pta.

The standard minimal medium contained: 11.1 mg/L CaCl₂, 240.73 mg/L MgSO₄, 5 mg/l thiamine, 1 g/L NH₄Cl, 0.5 g/L NaCl, 3 g/L KH₂PO₄, 8.5 g/L Na₂HPO₄·2 H₂O, 3 mg/L FeSO₄·7 H₂O, 15 mg/L Na₂EDTA·2 H₂O, 4.5 mg/L ZnSO₄·7 H₂O, 0.3 mg/L CoCl₂·6 H₂O, 1 mg/L MnCl₂·4 H₂O, 1 mg/L H₃BO₃, 0.4 mg/L Na₂MoO₄·2 H₂O, 0.3 mg/L CuSO₄·5 H₂O. In order to obtain a growth medium at pH 7.4 or 6.4, the relative concentrations of KH₂PO₄ and Na₂HPO₄·2 H₂O were adjusted appropriately, without changing the total (molar) phosphate concentration. As carbon source, 3 g/L glucose was used. The growth medium was supplemented with methionine to a final concentration of 3.3 mM when appropriate. Acetate was added to the growth medium as a concentrated solution of sodium acetate equilibrated to pH 7.4 or pH 6.4 in order to obtain the desired final concentration of acetate (128 mM in most experiments).

Construction of E. coli mutants

All of our mutants were derived from strains in the Keio collection [3]. The kanamycin resistance cassette replacing the coding sequence of the genes was removed such that none of our mutants carry an antibiotic resistance cassette. The kanamycin resistance cassette is flanked by recognition sites of the Flp recombinase and the cassette can therefore be excised using a plasmid expressing the Flp recombinase (plasmid 705-FLP, [63]). This excision creates an in-frame scar sequence (102 bp), reducing polar effects on downstream gene expression. The first mutants to be constructed were the ΔackA and Δpta mutants, by simply removing the kanamycin resistance cassette from the corresponding Keio clone.
We constructed the Δacs and the Δptaka mutants by replacing the gene acs and the operon pta-ackA with a FRT-flanked kanamycin resistance gene generated by PCR [12]. Primers have 20-nt 3’ ends homologous to the kanamycin resistance cassette used in the Keio collection and 50-nt 5’ ends of homology targeting the chromosomal region of interest. PCR products were transformed into a BW25113 strain expressing the λ Red recombinase (plasmid pSIM5, [50]). Antibiotic-resistant recombinants were then selected and the kanamycin resistance cassette removed.

We constructed the Δacspta, ΔacsptaackA, and ΔacsptaackApoxB mutants by P1 transduction [53]. The P1 lysate was grown on our ΔptaackA::kan mutant and the Δpta::kan mutant from the Keio collection. These lysates were then used to infect the Δacs strain in order to obtain ΔacsptaackA::kan and Δacspta::kan transductants. The kanamycin resistance cassette was removed as described above. The same procedure was used for moving the ΔpoxB::kan mutation from the ΔpoxB Keio mutant to our ΔacsptaackA strain.

We re-introduced the gene ackA into the ΔacsptaackA mutant precisely into the original locus, following a previously described approach [31], thereby effectively restoring a Δacspta mutant. Primers pta-CCDB1 and ackA-KN1 were used to amplify a kan:pBAD::ccdB cassette. The PCR product was transformed into a ΔacsptaackA mutant expressing the λ Red recombinase (plasmid pSIM5). Antibiotic-resistant recombinants were selected. Primers Y2-ACKA and ackA_ppta_left_PCR_verif were used to amplify the sequence between the initiation codon of ackA and the initiation codon of pta of the Δacspta mutant. The PCR product was recombined into the chromosome in place of the cassette. Recombinants were selected on a medium containing arabinose for activation of the suicide-gene ccdB that kills cells that have not recombined the ackA gene.

All mutants were verified by PCR and DNA sequencing. The list of primers used in this study can be found in Table 1.
Growth in shake flasks

For each strain, a seed flask (50 mL capacity), containing 10 mL of filtered minimal medium with glucose, was inoculated from a glycerol stock. The culture in the seed flask was grown overnight at 37°C with orbital agitation of 200 rpm. At the same time, 50 mL of filtered minimal medium with glucose (and methionine when appropriate) were pipetted into different 250-ml flasks (as many as there are experimental conditions and replicates) and stored overnight at 37°C without shaking. The following day, each 250-mL flask was inoculated to an optical density of 0.02 OD₆₀₀ from the seed flask. For each strain, two 250-ml flasks were used: one for the addition of 2 mL of filtered minimal medium with acetate and the other for the addition of 2 mL of filtered minimal medium without any carbon source (control). Cultures were grown at 37°C with orbital shaking at 200 rpm.

Growth of the strains was monitored every 30 minutes by removing a sample of 1 mL. Samples were used to measure the optical density. The remaining volume was centrifuged at 14000 g for 3 min at 4°C. The supernatant was frozen at -20°C for the quantification of metabolites. Acetate stock solution was prepared in concentrated form such that 2 mL of the stock solution, added to the culture, would give a final concentration of 128 mM acetate. Minimal medium with acetate and minimal medium without any carbon source were stored at 37°C before addition to the growing culture. Acetate was added when the optical density reached about 0.2 OD₆₀₀.

pO₂, pH and OD measurements

Cell growth was monitored by measuring the optical density with a spectrophotometer (Eppendorf BioPhotometer) at 600 nm. Dilutions were done when appropriate in order to stay in the range of linearity of the instrument. The partial oxygen pressure, pO₂, and the pH were measured with a Clark electrode (LAMBDA fermentor) and a pH (micro)probe (Mettler Toledo or Thermo Scientific Orion), respectively.
Quantification of metabolite concentrations in the medium

D-glucose, acetic acid, formic acid, pyruvic acid, D-lactate, and ethanol were assayed by enzymatic assay kits according to the manufacturer’s recommendations: Boehringer Mannheim, R-Biophar No 10 716 251 035, Megazyme K-ACETRM, Megazyme K-FORM, Megazyme K-LATE, Megazyme K-PYRUV, and Megazyme K-ETOH, respectively. All of the above measurement procedures are based on coupled enzyme assays.

Quantifications were done in 96-well microplates (clear flat-bottomed, plastic). Depending of the metabolite we wanted to quantify, different enzymatic reactions led to the consumption or the production of NADH. The concentration change of NADH was quantified by measuring the difference in absorbance at 340 nm ($\Delta A_{\text{metabolite}}$) with a microplate reader (Perkin Elmer Fusion Alpha). The concentration of the sample $C_{\text{metabolite}}$ (diluted in order to remain within the linearity region of the assay) is then calculated as:

$$C_{\text{metabolite}} = \frac{\Delta A_{\text{metabolite}}}{\Delta A_{\text{standard}}} \cdot C_{\text{standard}}$$

where $\Delta A_{\text{standard}}$ and $C_{\text{standard}}$ are the measured absorbance difference and the concentration of the metabolite standard. The metabolite standard solution was provided with each kit. In order to compute $\Delta A_{\text{metabolite}}$ and $\Delta A_{\text{standard}}$, the absorbance before starting the reactions ($A_1$) and the absorbance at the end of the reactions ($A_2$) were read ten times at regular time intervals in order to ensure that the reaction had reached equilibrium. In order to compensate for drift in the measurements, we fitted a straight line to the repeated measurements of $A_1$ and $A_2$. Using this straight-line extrapolation, the absorbance difference, $\Delta A = A_2 - A_1$, was calculated at the time of addition of the last enzyme that starts the reactions. Metabolite concentrations were corrected to take into account the dilution due to the addition of 2 mL of medium with or without acetate. Concentrations are given as the mean of at least three independent experiments. Error bars are set equal to twice the standard error of the mean.
Estimation of growth rates and uptake and secretion rates

In order to compute growth rates for the different strains, cultured in the presence or absence of acetate, we used the exponential growth model

$$\frac{d}{dt} B(t) = \mu \cdot B(t),$$

with $B(t)$, $B_0$ the time-varying and initial biomass in units OD$_{600}$, respectively, and $\mu$ the growth rate [h$^{-1}$]. This model has the explicit solution

$$B(t) = B_0 \cdot e^{\mu t}. \quad (1)$$

The above equation was fitted to each individual time-series of optical density measurements. We checked that within the chosen time interval, the underlying assumption of exponential growth at a constant rate is satisfied. The reported growth-rate values are the mean of at least three independent experiments. Error estimates are reported as twice the standard error of the mean.

In order to compute the glucose uptake rate, we combined the growth model with the glucose consumption model

$$\frac{d}{dt} G(t) = -r_{\text{glc}} \cdot B(t),$$

which has the explicit solution

$$G(t) = G_0 - (B_0/Y) \cdot (e^{\mu t} - 1), \quad (2)$$

where $G(t), G_0$ are the time-varying and initial glucose concentrations [mM], respectively, and $Y$ [OD$_{600}$ mM$^{-1}$] the biomass yield, defined as the ratio of the growth rate and the glucose uptake rate $r_{\text{glc}}$ [mM OD$_{600}^{-1}$ h$^{-1}$]. We simultaneously fitted Eqs 1-2 to each individual time-series data set of glucose concentrations and optical densities, obtained in
a single growth experiment. For each of the six conditions considered (the three strains, wild-type, Δacspta, and ΔacsptaackA, in the two growth condition, 0 or 128 mM acetate added to the glucose minimal medium), estimates of μ and Y were obtained. The glucose uptake rate can be directly obtained from these estimates bearing in mind that \( Y = \frac{\mu}{r_{\text{glc}}} \). The reported values are the mean of four independent replicate experiments. Error estimates are reported as twice the standard error of the mean.

In order to obtain the secretion rate of the fermentation by-products, used in the flux balance model, we again fitted Eq. 2 to the data, but replacing the glucose uptake rate by the appropriate secretion rate and using the values of \( \mu \) and \( B_0 \) obtained as described above. All uptake and secretion rates are computed from four independent replicate experiments with error estimates given by twice the standard error of the mean.

### Metabolic flux analysis

All computational analyses were performed with a slightly modified version of the genome-scale reconstruction iAF1260-flux1 of *Escherichia coli* metabolism by Feist *et al.* [23], to which we added a reaction accounting for transport of acetate anions by ActP [49]. The lower bound of exchange fluxes was set to zero, except for components of the *in silico* growth medium (water, vitamin, salts, traces, and glucose), which were left unconstrained, and for the oxygen uptake rate, which was limited to 20 mmol gDW\(^{-1}\) h\(^{-1}\). The upper bound of the exchange fluxes was set to zero for secreted products, except for those detected in the external medium in our experiments, namely acetate, formate, lactate, pyruvate, and ethanol. These fluxes were set to their measured values ± two standard errors of the mean, except for acetate when excess acetate is supplied to the medium. A theoretical exchange flux of carbon dioxide was determined based on the carbon mass balance. Eighteen additional reactions were constrained by literature data to allow normal functioning of the glycolysis and the pentose phosphate pathway (Supplementary File 1). Reactions allowing glycogen consumption and transport of D-glucose through alternative pathways were blocked, as well as fluxes through reactions catalyzed
by putative sugar phosphatase and aldehyde dehydrogenase. The maintenance fluxes were set to their default values (59.81 mmol gDW\(^{-1}\) h\(^{-1}\) and 8.39 mmol gDW\(^{-1}\) h\(^{-1}\) for the growth- and non-growth-associated maintenance fluxes, respectively). We checked that this allows flux balance analysis to reproduce the measured growth rate of the wild-type strain cultured in minimal medium with glucose in the absence of acetate, when the objective function is the maximization of biomass. The biomass function used is \(\text{Ec}_{\text{biomass \_iAF1260\_core\_59p81M}}\) [23].

In order to test the consistency of the metabolite measurements with the network stoichiometry, we performed a metabolic flux analysis [1], where the objective is to minimize the differences between the measured and predicted exchange fluxes and growth rate. Let \(v\) denote the vector of fluxes at steady state, \(N\) the stoichiometry matrix, \(v^l, v^u\) the vectors of upper and lower bounds on fluxes, respectively, and \(\hat{\nu}\) the vector of \(p\) measurements of exchange fluxes. We assume that the first \(p\) elements of \(v\) correspond to the measured exchange fluxes. Moreover, define \(u^+, u^-\) as non-negative dummy variables. Then, following the formulation of Lee et al. [35] (see also [39, 54]), metabolic flux analysis can be formulated as the following linear programming problem:

\[
\min \sum_{j=1}^{p} (u^+_j + u^-_j) \quad \text{subject to:}
\]

\[
N v = 0,
\]

\[
v^l \leq v \leq v^u,
\]

\[
v_j - u^+_j + u^-_j = \hat{\nu}_j, \quad \text{for all } j = 1, \ldots, p,
\]

\[
u^+, u^- \geq 0.
\]

This minimization problem was solved for each of the strains considered, both in the absence and presence of acetate, using the COBRA v3.0 Toolbox [28] with Gurobi 7.5.2 as the linear programming solver (Gurobi Optimization, Inc., Houston, TX, USA).

In order to further characterize the solution space of the above metabolic flux analysis
problem, we used a Monte Carlo sampling approach to estimate for each reaction in the network a distribution of possible flux values [43]. In particular, we performed uniform random sampling by means of the Coordinate Hit-and-Run with Rounding (CHRR) algorithm implemented in the COBRA Toolbox [27]. We computed the distribution of reaction fluxes consistent with the measured growth rate and exchange fluxes for each condition, focusing on reactions in central carbon metabolism. These reactions were determined by their annotation in the iAF1260-flux1 model: Pentose Phosphate Pathway, Anaplerotic Reactions, Glycolysis Gluconeogenesis, Pyruvate Metabolism, Citric Acid Cycle. The maximum of the resulting distributions, one for each reaction, was used for comparing reaction fluxes in the presence or absence of acetate in the growth medium. To this end, the reaction fluxes were rescaled by dividing them by the measured growth rate.

Quantification of Acs expression

In order to quantify Acs expression, we used a fluorescent reporter gene system based on a transcriptional fusion of the acs promoter with a stable green fluorescent protein, GFP-mut2, carried on the low-copy plasmid pUA66 [4]. The wild-type strain was transformed with this plasmid and experiments were carried out in the reference conditions described above. In addition to being used for measuring the optical density, samples taken were transferred to a 96-well plate to quantify the fluorescence emitted by the cultures in a microplate reader (Tecan Infinite 200 PRO). The excitation wavelength was set to 480 nm and the emitted fluorescence measured at 520 nm.

The data were corrected for background fluorescence levels using the wild-type strain as described in [13]. An estimate of the reporter protein concentration, in arbitrary units, was obtained by dividing, at each time-point, the background-corrected fluorescence level by the optical density. Assuming that Acs is a stable protein, like the GFP variant used in this study, the reporter concentration can be assumed proportional to the Acs concentration [52].
**Statistical tests**

In order to test the hypothesis that the growth rates or biomass yields of two strains in a given condition are equal, we used Welch’s t-test, a t-test for normally distributed variables with possibly unequal variances and an unequal number of independent samples (experiments) [46]. A pair of strains failing the test, for significance thresholds of 0.06 or 0.01, are concluded to have significantly different growth rates.

No off-the-shelf statistical method is available for testing the hypothesis that two inhibition indices are equal, as inhibition indices, which quantify the effect of acetate on the growth rate, are defined as ratios of normally distributed variables with non-zero means (Eq. 3). We therefore followed a bootstrap procedure by randomly resampling with replacement the experimentally-determined distributions of the growth rates and computing the corresponding inhibition indices, thus obtaining estimates of the mean and confidence interval of the inhibition indices [25]. These bootstrap distributions were used to test the null hypothesis that the inhibition indices of two strains in a given condition are equal, by computing the p-value corresponding to the probability that the difference between the inhibition indices estimated from the data would occur if the two indices were equal. The inhibition indices were concluded to be different for p-values below 0.03 or 0.005, corresponding to significance thresholds of 0.06 or 0.01, respectively, because of the two-sidedness of the distribution.

**Results**

**Growth inhibition by acetate**

In order to dissect the mechanism of growth inhibition by acetate, we adapted a standardized, well-controlled experimental setup from Luli et al. [36]. We use the *Escherichia coli* BW25113 strain. *E. coli* bacteria from an overnight pre-culture in minimal medium supplemented with glucose were diluted into the same medium and grown in a shake flask
batch culture. The growth characteristics of our strain are identical to those measured for similar strains of *E. coli* [19]. As shown in Supplementary Figure S1, after about 7 hours of growth, the bacteria reach a final optical density of 3.5 OD₆₀₀. pH and oxygen pressure decrease continuously during the exponential growth phase due to the increasing number of bacteria consuming oxygen and secreting acidic by-products. During the entire growth of the culture, the partial oxygen pressure, pO₂, never falls below 40%, meaning that the bacteria grow aerobically [19]. The pH remains close to neutral (pH 7) at the beginning of the experiment, but drops to a value of 6.4 at the end of exponential phase (Supplementary Figure S1).

In order to quantify the effect of acetate on the growth of the culture, identical starting cultures were split at the beginning of the experiment and grown in separate shake flasks. After about 3 hours of growth, a solution with a defined concentration of acetate was added to the medium in one flask and a solution without acetate to the medium in another flask (Figure 2). Growth of both the acetate-treated and the control culture were followed until the end of exponential phase, and the optical density (OD₆₀₀) and extracellular metabolites were measured at regular time intervals (see below). From these measurements we computed the growth rate, as described in the *Materials and Methods*. The growth rate was determined from optical densities below 1, since above this value the oxygen pressure decreases to a point that it no longer allows growth at the maximum rate supported by the medium. Moreover, in the fast-growing control culture, acidification of the medium due to overflow metabolism sets in at higher optical densities (Supplementary Figure S1), thus confounding the effect from external acetate addition.

Growth inhibition by acetate is quantified using a so-called inhibition index [36], defined as

\[ i = \frac{\mu_{\text{control}} - \mu_{\text{acetate}}}{\mu_{\text{control}}}, \]  

where \( \mu_{\text{control}} \) denotes the growth rate in the culture without added acetate and \( \mu_{\text{acetate}} \) the growth rate in the culture with added acetate. Note that the value of the inhibition
index varies between 0 and 1. An inhibition index of 0 means no growth inhibition by acetate, whereas an inhibition index of 1 corresponds to full growth inhibition.

Figure 3 shows the measured growth rate of the wild-type strain after the addition of different concentrations of acetate to a culture growing in the reference conditions at pH 7.4, close to the intracellular pH value enabling maximal growth [59]. As can be seen, the growth rate drops from 0.75 h\(^{-1}\) to 0.4 h\(^{-1}\) when the acetate concentration increases from 0 to 128 mM. This change in growth rate corresponds to an inhibition index of 0.47.

Previous reports [36] had suggested an exponential decrease of growth rate as a function of the concentration of added acetate. When allowing for an offset of the exponential function, we obtain the best exponential fit with a baseline at 0.3 h\(^{-1}\). This baseline value corresponds to the growth rate of \textit{E. coli} in the same medium with 128 mM of acetate as the sole carbon source (0.27 h\(^{-1}\), Figure 3).

Given that growth inhibition is a monotonic function of the acetate concentration and that industrially relevant concentrations of acetate are on the order of 100 mM [11], we have decided to carry out all subsequent experiments at 128 mM acetate. The qualitative effect of acetate is probably the same at all concentrations, but quantitative estimates are much more easily obtained for larger effects. While most measurements were carried out at pH 7.4, we also quantified growth inhibition by acetate at pH 6.4 and found an even stronger effect of acetate: the growth rate drops from 0.74 h\(^{-1}\) to 0.084 h\(^{-1}\) when adding 128 mM of acetate, corresponding to an inhibition index of 0.63 (Figure 3).

Previous results have shown that the addition of methionine to the medium can alleviate the inhibitory effect of acetate [44]. Further work indicated that acetate inhibits methionine biosynthesis, and more particularly the activity of the MetE enzyme [38, 45]. As a further control, we therefore tested whether supplementing the growth medium with methionine could offset the inhibition effect, by adding 128 mM of acetate during growth in minimal medium with glucose at pH 7.4, in the absence of methionine and in the presence of 3.3 mM of methionine (comparable to the concentration of 2 mM used in [44]). We found only a small, non-significant effect of methionine in our conditions, consisting in
an increase of the growth rate from 0.38 h\(^{-1}\) to 0.43 h\(^{-1}\), and a corresponding decrease of the inhibition index from 0.52 to 0.45 (Supplementary Figure S2). Roe et al. [44] observed a much stronger effect, where supplementing methionine could restore 75-80% of the growth-rate diminution due to acetate. More recent data also demonstrate a relief of growth inhibition by methionine, but the magnitude is closer to what we observe (reduction of inhibition index from 0.71 to 0.53) [38]. Note that these experiments were carried out at pH 6, which may account for the difference in strength of the relief of growth inhibition by methionine observed in our conditions. For our purpose, however, the most important conclusion is that methionine is not a major contributor to growth inhibition at physiological pH levels. In the remainder of the paper, we will therefore investigate another hypothesis.

**Mutants of acetate metabolism**

While growing at a high rate in aerobic conditions, *E. coli* redirects some of the glycolytic flux towards the production of acetate, since limited respiration capabilities do not allow all carbon intermediates to enter the tricarboxylic acid (TCA) cycle [18]. This physiological response optimizes the balance between demands for energy production and biomass synthesis [5]. Conversion of acetyl-CoA into acetate involves the phosphotransacetylase, Pta, and the acetate kinase, AckA. Acetate can also be produced from pyruvate, the metabolite just before acetyl-CoA in glycolysis, by pyruvate oxidase, PoxB (Figure 1).

When *E. coli* cells are growing on acetate, the Pta-AckA pathway operates in the reverse direction, converting acetate into acetyl-coenzyme A (AcCoA). For low concentrations of acetate in the medium, on the order of a few mM, the main route for acetate assimilation involves the acetyl-CoA synthetase Acs [61].

Recent work has shown that, during growth of *E. coli* on glucose, acetate metabolism not only produces but also consumes acetate, and acetate uptake or secretion corresponds to the net flux through the Pta-AckA pathway [20] (see also [33]). At high external concentrations, the net flux of acetate was observed to change direction and flow into the
cells. This suggests that excess acetate may flow into central metabolism and possibly perturb fluxes necessary for sustaining maximal growth. Moreover, excess acetate may change the concentration of acetyl-phosphate (Ac\textasciitilde P), the intermediate of the Pta-AckA pathway. Ac\textasciitilde P plays an important role in the regulation of various cellular processes, as mentioned in the Introduction (see [60, 61] for reviews).

Valgapea et al. found that in continuous culture experiments at low dilution rates, even in the absence of acetate secretion, the Pta-AckA and Acs pathways are actively recycling acetate [55], possibly to maintain an appropriate level of Ac\textasciitilde P. In our conditions Acs is not significantly expressed at the reduced growth rate following acetate addition (Supplementary Figure S3). However, the more general point that perturbing the concentration of Ac\textasciitilde P may have consequences on growth remains valid.

The above considerations motivate the main question of the present work: do the perturbations of acetate metabolism, due to the inflow of excess acetate from the medium, play a role in growth inhibition by acetate, either by perturbing the distribution of fluxes in central carbon metabolism or by affecting the regulatory role of Ac\textasciitilde P? In order to answer this question, we constructed mutants that cut different parts of the acetate utilization and synthesis pathways and modify the concentration of Ac\textasciitilde P. All these mutants are derived from the wild-type strain, BW25113, and their genotype is listed in Table 2. We measured the growth rate of all strains in our reference conditions (growth on minimal medium with glucose at pH 7.4). As can be seen in Figure 4(a), none of the mutants significantly reduces the growth rate on glucose, in agreement with previous observations for the BW25113 strain in comparable growth conditions [9].

\Delta ackA mutant partly relieves growth inhibition by acetate

Next, we measured the effect of acetate on the growth rate of the mutant strains by experiments analogous to the one shown in Figure 2. The results are shown in Figure 4(b). The wild-type and all mutant strains are strongly inhibited by the addition of 128 mM...
acetate to the growth medium. Even though all strains are strongly affected, we observe
two distinct groups: strains that carry the ackA deletion grow faster than strains that
carry a wild-type copy of the gene. The inhibition indices of ∆ackA strains are around 0.4,
as compared to the values of around 0.5 for all other strains (Figure 4(c)). The observed
differences in growth rates and inhibition indices, between the wild-type strain on the
one hand and the ackA mutants on the other, are statistically significant (Materials and
methods).

To preclude the possibility of unmapped, secondary mutations being responsible for the
differential effect in ∆ackA strains, we constructed a revertant of the phenotype of the
ackA deletion. In strain ∆acs pta ackA::ackA_{wt}, the wild-type allele of ackA was restored.
The slight growth advantage of the ∆ackA strains in acetate, reflected by the smaller
inhibition indices (0.4 vs 0.5), disappears in the complemented strain. The inhibition
index increases to about 0.47 and reaches the same level as for the equivalent ∆acs pta
strain (Figure 4(c)). We conclude that, even though the construction of the triple mutant
has involved many growth and selection steps, the observed phenotypes of the mutants
are not due to secondary mutations elsewhere on the chromosome.

The differential effect of the ackA deletion is not caused by a decreased flux of acetate
into central carbon metabolism (and a corresponding decrease in ATP consumption) since
the pta deletion, which interrupts the same pathway (Figure 1), does not reduce growth
inhibition by acetate. However, the ackA deletion is expected to modify the intracellular
concentration of Ac∼P. During growth on glucose without excess acetate, Ac∼P is
produced by Pta from acetyl-CoA [32, 61], but in the presence of high concentrations of
acetate in the medium, we expect Ac∼P to be mostly generated by phosphorylation of
acetate. The latter reaction no longer takes place in a ∆ackA strain, so the concentration
of Ac∼P will be lower than in a strain with a functional AckA. In the extreme, deletion
of both ackA and pta, like the ∆pta ackA, ∆acs pta ackA, and ∆acs pta ackA polB strains
in Figure 4, eliminating all reactions that can produce Ac∼P, results in a strain that is
completely devoid of Ac∼P [32].
The 20% decrease of the inhibition index in the ΔackA strains can thus be explained by a (partial) compensation for the increase in Ac∼P expected in the presence of excess acetate in the medium. The growth-inhibitory effect of an increase of the concentration of Ac∼P is consistent with the observation that deleting one of the known deacetylases in E. coli, CobB, reduces the growth rate of E. coli on acetate by almost twofold [8]. Moreover, in the presence of 160 mM acetate, wild-type cells that are in a growth-arrested state (because of a lack of a nitrogen source in the medium) have a much higher degree of lysine acetylation than the ΔackA derivative [58], suggesting an increased Ac∼P concentration.

So far, we have focused on the reactions producing and consuming acetate inside the cell, ignoring questions about acetate uptake. This is motivated by the fact that, as mentioned in the Introduction, the protonated form of acetate acid, HAc, can freely diffuse into the cell. Nevertheless, two specific transporters of acetate, SatP and ActP, have been identified previously [49]. We therefore wanted to ascertain that active acetate uptake or secretion is not necessary for the growth inhibitory effect of acetate in the medium.

Using satP and actP mutants of the wild-type strain [3], we tested in the same conditions as above the effect of adding acetate on the growth rate of the mutant strains as compared to the wild-type strain. We found no significant difference in the growth inhibition index between the wild type and the mutants (Supplementary Figure S4), showing that in our conditions active acetate uptake is not required for growth inhibition.

Growth inhibition by acetate does not involve reorganization of fluxes in central carbon metabolism

As explained above, the results reported in Figure 4 do not support the hypothesis that the influx of excess acetate into central carbon metabolism plays a significant role for growth inhibition. While no such influx can occur in the Δacs pta mutant, in which both the Acs and the Pta-AckA pathways have been eliminated, the growth rate in the presence of acetate and the inhibition index are the same as for the wild-type strain. In order to further characterize central carbon metabolism on a coarse-grained level [30], we
measured the extracellular concentration of glucose and the major fermentation products known to accumulate during aerobic growth of *E. coli* on glucose, in the wild-type strain, in the double mutant ∆*acs pta*, and in the triple mutant ∆*acs pta ackA*. In particular, we quantified acetate, ethanol, formate, lactate and pyruvate during growth experiments as in Figure 2 [9, 16, 42, 62].

The results in Figure 5 (left column) confirm the expected overflow metabolism during growth of the wild-type strain on glucose, namely the secretion of acetate [19]. This overflow metabolism is almost completely abolished in the mutants, confirming that the major pathway of acetate production is interrupted. In order to dissipate the extra reducing equivalents, other metabolites, in particular lactate and pyruvate, are secreted during growth of the mutants on glucose. When 128 mM acetate is added to the growth medium (right column of Figure 5), the growth rate slows and the glucose uptake rate diminishes accordingly. As a consequence of the lower growth rate, there is no longer any detectable overflow metabolism, except for a small amount of pyruvate in the wild type and triple mutant. Notice that changes in acetate concentration are undetectable since there is a large excess of acetate in the growth medium in this condition. Diminished pyruvate excretion in the ∆*acs pta* mutant may be an unknown regulatory effect mediated by Ac∼P and affected by the additional deletion of *ackA* in the triple mutant. The slight relief of growth inhibition by acetate in the triple mutant is consistent with the somewhat higher glucose uptake rate in this strain (the blue curve in the right column of Figure 5).

Despite some small differences between strains, the data in Figure 5 reveal that the pattern of uptake and secretion of carbon compounds by the cell is not significantly perturbed by the addition of large concentrations of acetate to the growth medium. A similar observation can be made for the changes in extracellular pH. In none of the three strains there is a noticeable difference in extracellular pH at a given OD in strains growing in medium with and without added acetate (Supplementary Figure S5). Moreover, the extracellular pH curves are quite similar for the wild-type and mutant strains. Using the uptake and secretion patterns as proxies for the functioning of central metabolic pathways, this suggests that addition of acetate indeed does not entail a profound reorganization of...
metabolism, apart from a global rescaling of fluxes due to the reduced growth rate.

In order to further probe this conclusion, we used a genome-scale reconstruction of *E. coli* metabolism [23]. From the data in Figure 5 we computed the glucose uptake rate and the secretion rates of acetate, ethanol, formate, lactate, and pyruvate, which were integrated into the model to constrain the exchange fluxes. Moreover, the biomass reaction in the model was set equal to the measured growth rate. We also formulated a limited number of additional uptake and reversibility constraints that are directly motivated by the composition of the growth medium and the utilization of glucose as the sole carbon source (*Materials and Methods* and Supplementary File 1).

Using this model, we performed a metabolic flux analysis [1, 39] to obtain the flux distribution minimizing the difference between the predicted and measured fluxes, including the biomass production rate [35, 54]. We used a Monte Carlo sampling approach to characterize the space of possible solutions, focusing on the reactions in central carbon metabolism (*Materials and methods*). As shown in Figure 6, when scaling the fluxes in each condition by the growth rate, the distribution of internal metabolic fluxes is the same in the absence or presence of acetate. The results of this computational analysis are in agreement with the conclusions drawn from inspection of Figure 5: the inhibitory effect of acetate does not profoundly change the global functioning of metabolism.

As explained above, when 128 mM acetate is added to the medium, quantification of the acetate uptake or secretion rates is unreliable. Therefore, in this case, we did not constrain the corresponding reaction in the metabolic flux analysis. Interestingly, the exchange fluxes predicted by the approach correspond well with the $^{13}$C flux measurements carried out by Enjalbert *et al.* [20]. In the case of the wild-type strain, the direction of the acetate exchange flux inverts upon the supply of acetate (uptake of 3.5 mmol gDW$^{-1}$ h$^{-1}$ for 0 mM acetate and secretion of 2.7 mmol gDW$^{-1}$ h$^{-1}$ for 128 mM acetate), whereas the deletion of the Pta-AckA pathway prevents this inversion (uptake of 0.7 mmol gDW$^{-1}$ h$^{-1}$ for 0 mM acetate and uptake of 3.8 mmol gDW$^{-1}$ h$^{-1}$ for 128 mM acetate).

24
The question which molecular mechanisms underlie growth inhibition of *Escherichia coli* cultures by excess acetate in the growth medium is of fundamental interest for understanding the physiology of this bacterium, but may also have important implications for applications in food preservation and biotechnology. The potential mechanisms explaining the observed growth inhibition are debated since many decades [21]. The classical hypotheses put forward to explaining growth inhibition by acetate are the uncoupling effect of weak acids and the perturbation of the anion concentration caused by the accumulation of acetate anions in the cell [2, 15, 44, 45, 48, 51]. Recent work has shown that acetate, when present at high concentrations in the medium, can be assimilated by *E. coli* even when growing on glucose [20]. This suggests that a net uptake of acetate through the AckA-Pta pathway could perturb the fluxes in central metabolism necessary for sustaining maximal growth. Moreover, it could affect the concentration of Ac∼P, an intermediate of the AckA-Pta pathway known to assume a wide range of regulatory functions in the cell [60, 61].

In this work, we have focused on the latter hypothesis. In order to investigate the role of acetate metabolism in growth inhibition by acetate, we have developed a series of *E. coli* mutant strains that probe relevant parts of the metabolic pathways of acetate synthesis and consumption. In particular, we constructed mutant strains that prevent external acetate from being metabolized by the cell by deleting both the Acs and the Pta-AckA pathways. Within the Pta-AckA pathway, we can allow the production of Ac∼P by deleting just one of the genes, or prevent all synthesis of Ac∼P by deleting both genes, thus probing potential regulatory roles of this metabolite. For several of the mutants we have measured growth rates and extracellular concentrations of a number of by-products of central carbon metabolism, known to accumulate in the growth medium in wild-type *E. coli* strains and strains with deletion of the *pta*, *ackA*, or *acs* genes. All measurements have been carried out in carefully controlled reference conditions, which allows crossing and comparing the results obtained for the different *E. coli* strains. Some care should be
exercised though in generalizing the results to other organisms and conditions.

By means of these strains we tested the hypothesis that the influx of excess acetate in
the medium “overloads” the metabolic pathways of acetate utilization and thus perturbs
central carbon metabolism. This hypothesis is clearly not supported by our data: (i) the
deletion of both pta and acs prevents acetate utilization by the bacteria, but in no way
relieves growth inhibition (Figure 2), and (ii) the functioning of central carbon metabolism
in the wild type and ∆acs pta mutant are not greatly perturbed by the addition of acetate
to the growth medium (Figure 5). These conclusions are corroborated by integrating the
data with a genome-scale model of E. coli metabolism to predict the possible intracellular
flux distributions consistent with the measured uptake and secretion rates and growth
rate. The analysis indicates that, when a scaling factor due to the growth-rate difference
is accounted for, the predicted distributions of internal fluxes before and after acetate
addition are essentially the same. This suggests that the net influx of excess acetate into
central carbon metabolism does not produce a suboptimal flux distribution responsible
for the reduced growth rate.

As explained above, the utilization of acetate may also perturb metabolic functioning in
a different way, by the phosphorylation of acetate to Ac~P by AckA and the Ac~P-
mediated modification of enzyme activity. Ac~P contains a high-energy bond between
phosphate and the acetyl-moiety and can therefore transfer either the phosphate group
to an appropriate acceptor, in this case two-component response regulators, or the acetyl
group to lysines of target proteins. Since the number of targets of both regulatory mech-
anisms are in the hundreds or thousands [7, 34, 60, 61], we can not individually assess all
these interactions. However, we can measure the global effect on growth rate by prevent-
ing the production of Ac~P. Our results show that in the triple mutant ∆acs pta ackA, a
strain devoid of Ac~P, the addition of acetate has a slightly weaker effect on growth. We
therefore conclude that part of the growth-inhibitory effect of acetate seems to involve
the perturbation of Ac~P levels in the cell, thus interfering with the regulatory role of
this signaling metabolite. From our data we estimate that this accounts for about 20%
of the observed reduction in growth rate.
The question that immediately comes up is what accounts for the remaining 80% of the reduction in growth rate. Two commonly advanced hypotheses were mentioned in the introduction. First, the classical explanation of growth inhibition by acetate and other weak acids is “uncoupling” [2, 29, 51]. Acetic acid HAc diffuses into the cell where it dissociates into acetate Ac\(^{-}\) and a proton H\(^{+}\). In order to maintain the membrane potential, the protons need to be pumped out of the cells, which costs ATP and thus draws away energy from growth. Another explanatory hypothesis involves the perturbation of the anion composition of the cell, leading to the inhibition of enzyme activity by the accumulating Ac\(^{-}\) anions themselves or by the replacement of pools of other anions regulating enzyme activity. It has been shown previously that acetate inhibits methionine biosynthesis, and more particularly the activity of the MetE enzyme [38, 45]. While there is no evidence that acetate acts directly on the enzyme, it is very well possible that enzyme inhibition is mediated by a change in concentration of another anion following acetate addition to the medium. Moreover, acetate may act on the transcription of enzymatic genes, as found in a recent study [40].

Our study does not provide a definite answer to the question which of the two effects identified above is (mainly) responsible for growth inhibition by acetate. However, some of our observations argue against the uncoupling hypothesis. First, if uncoupling played an important role, one would expect the biomass yield to be significantly lower in cultures growing in the presence of high concentrations of acetate in the medium, reflecting the energy-spilling activity of the proton pumps [47]. Estimating the yields from the data in Figure 5 gives a somewhat different result. While biomass yields in the presence of acetate are slightly higher than in the absence of acetate, in the sense that the measurements are located below the diagonal of the scatterplot in Figure 7, the differences are too small (< 15%) to be statistically significant for the given measurement uncertainties and their effect seems too weak to account for the strong reduction in growth rate observed in our conditions. These observations are consistent with previous reports [20, 47].

Second, it should be emphasized that the uncoupling hypothesis posits a futile cycle in which not only H\(^{+}\) but also Ac\(^{-}\) molecules are pumped out of the cell following the
diffusion of HAc into the cell [2]. This necessarily involves active transport of acetate. Two acetate transporters have been reported in the literature, SatP and ActP, and the deletion of either of these was shown to halve the acetate uptake rate [49]. Given the high rate of acetate secretion necessary for obtaining a significant reduction in growth rate, one would expect that deleting either SatP or ActP would reduce the flux through the futile cycle and thus energy spilling and growth inhibition. The results reported in the section ∆ackA mutant partly relieves growth inhibition by acetate and in Supplementary Figure S4 do not confirm this. Growth inhibition is as strong in the mutants as in the wild-type.

The above arguments are suggestive, but need to be supported by a quantitative characterization of the membrane potential and other energetic variables as well as a precise carbon balance in order to unambiguously rule out an important role for uncoupling.

Previous studies have shown the occurrence of an anion imbalance in the presence of high concentrations of Ac\(^-\) in the cytoplasm, for example a six-fold decrease in glutamate concentration [44]. We found that in our conditions (partial) relief of growth inhibition by methionine is small (Supplementary Figure S2), contrary to previous studies [44], although this does not exclude that the perturbed anion balance affects other enzymes, as discussed above. While the cumulative effect of a modified anion distribution on specific reactions may well constitute a major factor of growth inhibition by acetate, only global metabolic studies correlating flux distributions with anion concentrations will be capable of identifying all reactions that are sensitive to specific anions. Although our study therefore does not provide a definite answer to the question what causes growth inhibition by acetate and other weak acids in bacteria, it does uncover a new regulatory effect by Ac\(^-\)P and identifies promising directions to further investigate other possible explanations.

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### Table 1: Oligonucleotides used in this study

Note that ackA-Right-PCR-verif and ackA-pta-Left-PCR-verif were used to verify the *ackA* deletion, pta-Left-PCR-verif and ackA-pta-Right-PCR-verif were used to verify the *pta* deletion, and ackA-pta-Right-PCR-verif and ackA-compl-verif for verifying the *ackA* complementation.

| Primer name                  | Sequence (5’ to 3’)                                                                 | Purpose         |
|------------------------------|------------------------------------------------------------------------------------|-----------------|
| acs1P1                       | GTTACCGACT CCGATCGGCA AATTGTGGGT TACGATGGCA TCGCGATAGC ATTCGCGGGA TCCGTCGACC | construction   |
| acs2P2                       | AAGCCTTATAG CCACATATTA TAAACATCC ACAAAGGAGAA CAAAAGCATG TGTAGGCTGG AAGCTGCCTTG | construction   |
| acs-Right-PCR-verif           | AAAAATGCCCA ATACCCCT                                                                  | verification   |
| acs-Left-PCR-verif            | TTTTAAATCC CGCTCCCT                                                                | verification   |
| ackA-pta-Left-Primer         | TGCTCCTCGT ACGTTTTTTTTT AGCCACGTAT CAATTATAGG TACTTCCATG ATTCGCGGGA TCCGTCGACC | construction   |
| ackA-pta-Right-Primer        | GAGCGCCAAA GCCTGCGGATGT AGTACGAGAT TACTGCTGCT GTGCGAGCTGT GTGAGGCTGG AAGCTGCCTTG | construction   |
| ackA-pta-Left-PCR-verif      | CCCTGACGTT TTTTAAAGCC                                                              | verification   |
| ackA-pta-Right-PCR-verif     | CAGCGCAGTT AAGCAAGA                                                               | verification   |
| ackA-Right-PCR-verif         | TATCCTCCTTT CGTTACCGCC                                                            | verification   |
| pta-Left-PCR-verif           | GCCGGAACGAAAGAGGA                                                                | verification   |
| poxB-seq-Rev                 | CTCTTTTCTC TCCCATCCC                                                             | verification   |
| poxB-seq-Fwd                 | TTAAAGCTGTT CCCAACC                                                               | verification   |
| pta-CCDB1                    | CTTTCTAGAG AATAGGAACCT TCGAACTGCA GTCGACGGGA TCCCCGGAAT TTATATTCC CAGAAACATCA GG | construction   |
| ackA-KN1                     | TGGCTCCTCGT ACGTTTTTTTT AGCCACGTAT CAATTATAGG TACTTCCATg ATAGGAACCT CAAGATCC      | construction   |
| Y2-ACKA                      | CTTTCTAGAG AATAGGAACCT CGAAGTCCAG GTGCGACGGAT CCCCGGAATc acGGTTTTAC CTCTTTCTGT  | construction   |
| ackA compl verif             | CGCAAAATGG CATAGACTCA A                                                         | verification   |
| Strain                        | Origin           |
|------------------------------|------------------|
| BW25113                      | Keio collection  |
| BW25113 $\Delta ackA$       | this study       |
| BW25113 $\Delta pta$        | this study       |
| BW25113 $\Delta pta ackA$   | this study       |
| BW25113 $\Delta acs$        | this study       |
| BW25113 $\Delta acs pta$    | this study       |
| BW25113 $\Delta acs pta ackA$ | this study    |
| BW25113 $\Delta acs pta ackA :: ackA_{wt}$ | this study |
| BW25113 $\Delta actP$       | Keio collection  |
| BW25113 $\Delta satP$       | Keio collection  |

Table 2: **Strains used to dissect different mechanisms of growth inhibition by acetate.** All strains are derived from the wild-type strain of the Keio collection, BW25113 [3]. This strain will be called wild type. All deletions developed for this study were constructed without leaving antibiotic resistance cassettes on the chromosome. Notice that $\Delta pta ackA$ is an abbreviation for the double mutant $\Delta pta \Delta ackA$ (a similar notation is used for the other strains). The last strain, $\Delta acs pta ackA :: ackA_{wt}$, restoring the original $ackA$ gene, is used as a control.
Figure 1: **Schematic representation of the major metabolic pathways of acetate metabolism.** Acetate can be generated directly from pyruvate by decarboxylation (using the enzyme pyruvate oxidase, PoxB) or from acetyl-CoA via the intermediate acetyl-phosphate (Ac∼P) (reactions catalyzed by the enzymes phospho-trans-acetylase, Pta, and acetate kinase, AckA). Acetate can freely diffuse across the cell membrane in the protonated form [26], HAc, or as an acetate ion Ac\(^-\), via the transporters ActP or SatP [49]. Protons that enter the cell in the form of HAc can be expelled at the expense of energy. The enzyme acetyl-CoA synthetase, Acs, efficiently converts intracellular acetate into acetyl-CoA. Acetate is also involved in several metabolic pathways. For example, the biosynthesis of methionine is inhibited by acetate. Excess intracellular acetate perturbs the anion balance in the cell and could thus inhibit other metabolic reactions [45]. The pool of the major intracellular anion, glutamate, is strongly reduced when the intracellular concentration of acetate is high.
Figure 2: **Growth inhibition by acetate.** The inoculated culture is split and cultured in separate shake flasks containing minimal medium with glucose at pH 7.4. A small volume of minimal medium with acetate is added to one culture (red) and an identical volume of minimal medium without acetate to the second, control culture (blue). The moment of acetate addition is indicated by the black arrow. The optical density measurements represent the mean of three experiments. The error bars (mostly smaller than, and therefore hidden by the circles showing the data-points) are two times the standard error of the mean. The data shown correspond to the wild-type strain and a final concentration of acetate of 128 mM.
Figure 3: **Growth inhibition by different concentrations of acetate and different pH levels.** Experiments as in Figure 2 were carried out for different concentrations of acetate at pH 7.4 (blue dots) and pH 6.4 (red dots). Each data point shows the mean of the growth rate of three or four independent experiments, as well as an error bar equal to twice the standard error of the mean. An exponential function with baseline 0.3 h\(^{-1}\) was fit to the data (blue and red curves). The baseline approaches the growth rate in minimal medium with 128 mM of acetate as the sole carbon source (green dashed line). For reference, the growth rate on 3 g L\(^{-1}\) of acetate, corresponding to 50 mM, is also shown (green dot). Notice that for some measurements the error bar is so small as to coincide with the measurement dot.
Figure 4: Growth rate of mutant strains in the absence of acetate and in the presence of 128 mM acetate. The growth rate of all mutant strains was measured in a standard shake flask culture as in Figure 2 and computed from the data as described in the Materials and Methods. We report the mean of at least three independent experiments. The error bars represent twice the standard error of the mean. The growth rates without and with acetate are shown in (a) and (b), respectively, and the inhibition index in (c). The asterisks * and ** in (b) and (c) indicate growth rates and inhibition indices for mutant strains that are significantly different from the growth rate and inhibition index of the wild-type strain, for significance thresholds of 0.06 and 0.01, respectively (Materials and methods).
Figure 5: Caption on next page
Figure 5 (prev. page): Measurement of metabolites taken up and excreted by *E. coli*. Bacteria were grown in a shake flask, as described in Figure 2, and samples were removed at regular time intervals and analyzed for the different metabolites (*Materials and methods*). The left column shows cultures grown on glucose alone. 128 mM acetate was added to the cultures in the right column after three hours of growth (indicated by the dashed, vertical line). The metabolite measured is indicated on top of each row. The measurements were carried out in quadruplicate in the wild-type strain (green), the Δacs pta mutant (red), and the Δacs pta ackA mutant (blue). The error bars indicate twice the standard error of the mean. For reference, OD₆₀₀ curves are shown as dashed lines. Note that the ordinate scale is smaller for the metabolites in the bottom four rows and the ordinate scale of acetate measurements with added acetate is much larger than the others.
Figure 6: Caption on next page
Changes in metabolic fluxes in central carbon metabolism predicted by a genome-scale model of *E. coli*. The measurements of extracellular metabolites in Figure 5 were used to compute uptake and secretion rates in each of the conditions, as outlined in the Materials and methods. These rates were used to constrain the exchange fluxes of a genome-scale model of *E. coli* metabolism [23], while the rate of the biomass reaction was set to the experimentally-determined growth rate. We used metabolic flux analysis to define a space of solutions consistent with the measured fluxes and the stoichiometry structure of the metabolic network. This solution space was sampled in a random and unbiased manner using a Monte-Carlo approach (Materials and methods). (a) Scatter plot of the predicted fluxes for 64 reactions in central carbon metabolism of the wild-type *E. coli* strain growing on glucose, in the absence and presence of acetate. In each condition, the fluxes have been normalized by the growth rate. The scatter plot shows a very strong correlation between the predicted flux distributions in the absence and presence of acetate, with all reactions clustered around the diagonal ($R^2 = 0.89$). (b) Idem for the ∆acs pta strain ($R^2 = 0.94$). (c) Idem for the ∆acs pta ackA strain ($R^2 = 0.94$).
Figure 7: Biomass yield of wild-type mutant strains in the absence of acetate and in the presence of 128 mM acetate. The biomass yields of the wild-type strain (green), the Δacspta mutant (red), and the Δacspta ackA mutant (blue), in the absence of acetate and in the presence of 128 mM acetate, were measured as described in the Materials and Methods section. We report the mean of four independent experiments. The error bars represent twice the standard error of the mean. The diagonal corresponds to identical biomass yields in the presence and absence of acetate. Although the measurement means are located below the diagonal, suggesting a lower biomass yield in the presence of acetate, the pairwise differences in yield with and without acetate are not statistically significant, at confidence levels 0.01 and 0.06 (Materials and methods).