Yasir *et al. Annals of Microbiology* (2024) 74:15
https://doi.org/10.1186/s13213-024-01760-z

Annals of Microbiology

UNIVERSITÀ DEGLI STUDI DI MILANO

SHORT COMMUNICATION

Open Access

Overflow metabolism provides a selective advantage to *Escherichia coli* in mixed cultures

Muhammad Yasir[1†], Nicholas M. Thomson[1†], A. Keith Turner[1], Mark A. Webber[1,2*] and Ian G. Charles[1,2]

Abstract

Purpose It has previously been shown that organic acids produced by *Escherichia coli* suppress the growth of *Pseudomonas aeruginosa* in co-cultures under conditions of glucose excess, due to overflow metabolism. Inactivation of genes involved in central carbon metabolism favours fermentation of glucose over respiration and therefore increases production of organic acid by-products such as acetate and lactate. We sought to extend and refine the list of genes known to contribute to the metabolic balance between respiration and fermentation, to better understand the role of overflow metabolism in competitive survival of *E. coli*.

Methods We confirmed the previous finding that *E. coli* excludes *P. aeruginosa* from co-cultures by producing organic acids in the presence of glucose. Using a genome-wide transposon screen we identified *E. coli* genes that are important for survival in co-cultures with *P. aeruginosa*, both with and without glucose supplementation.

Results Central carbon metabolism was the dominant gene function under selection in our experimental conditions, indicating that the observed inhibition is a side-effect of overflow metabolism adopted by *E. coli* as a response to high glucose concentrations. The presence of a competing species increased the selective pressure for central carbon metabolism genes, with 31 important for growth in the presence of *P. aeruginosa* and glucose, while only 9 were significant for pure *E. coli* cultures grown with glucose. In our experiments, each transposon mutant was competed against all others in the pool, suggesting that overflow metabolism provides benefits to individual *E. coli* cells in addition to competitive inhibition derived from acidification of the growth medium.

Conclusion Co-culture assays using transposon mutant libraries can provide insight into the selective pressures present in mixed species competition. This work demonstrates central carbon metabolism is the dominant gene function under selection in *E. coli* for aerobic growth in glucose and a side-effect of this is overflow metabolism which can inhibit growth of bystander species.

Keywords TraDIS-*Xpress*, Mixed acid fermentation, Competition, Overflow metabolism, Warburg effect

Introduction

Overflow metabolism, also known as the Warburg effect (Warburg 1956) or the Crabtree effect (Crabtree 1929; Deken 1966), is the tendency for cells to utilise a mixture of respiratory and fermentative metabolism under aerobic conditions when carbon sources are in plentiful supply (Holms 1996). This strategy is commonly observed in cells from all domains of life but a well-studied example is the bacterium *Escherichia coli*, which employs a partly fermentative metabolic strategy at high growth rates,

†Muhammad Yasir and Nicholas M. Thomson contributed equally to this work.

*Correspondence:
Mark A. Webber
mark.webber@quadram.ac.uk
[1] Quadram Institute Bioscience, Norwich, UK
[2] Norwich Medical School, University of East Anglia, Norwich, UK

leading to the formation of acetate and other organic acids (Wolfe 2005; Eiteman and Altman 2006).

Respiration maximises the yield of chemical energy, in the form of NADH and ATP, by oxidising every C-C bond in a carbon source. However, the ubiquity of overflow metabolism shows that absolute efficiency is not the only factor affecting the metabolic balance of cells. Models to explain and predict the seemingly sub-optimal mixture of metabolic pathways in overflow metabolism therefore continue to be suggested. These include the effects of cytoplasmic molecular crowding (Vazquez and Oltvai 2016), the idea that fermentative pathways place fewer demands on the cells' protein expression machinery (Basan et al. 2015), the limited surface area of the inner membrane for respiratory enzymes (Szenk et al. 2017), ATP dynamics (Lin and Jacobs-Wagner 2022), and combinations of these and other factors (Swain and Fagan 2019; de Groot et al. 2020; Regueira et al. 2021). However, a full understanding of the causes and regulation of overflow metabolism remains elusive.

E. coli is a pioneer coloniser of the infant human gut (Mackie et al. 1999), and certain probiotic strains like *E. coli* Nissle 1917 can help to maintain a healthy balance of species and outcompete pathogenic bacteria through regulated processes such as the production of microcins and signalling compounds including indole (Sassone-Corsi et al. 2016; Zarkan et al. 2020). One effect of the production of organic acids during overflow metabolism is that *E. coli* is able to inhibit the growth of other bacteria such as *Pseudomonas aeruginosa* (Christofi et al. 2019) and *Vibrio cholerae* (Sengupta et al. 2017; Nag et al. 2018). This inhibition, therefore, is essentially a side-effect of overflow metabolism as a response to high-glucose environments, in which *E. coli* is better able to tolerate the consequent acidic conditions (Christofi et al. 2019).

P. aeruginosa is a versatile coloniser of habitats ranging from natural settings including soil, fresh water and marine environments (Kidd et al. 2012; Diggle and Whiteley 2020) to built environments such as drains, sewers, and surfaces within households and hospitals (Bédard et al. 2016; Gilbert and Stephens 2018; Crone et al. 2020). In humans, *P. aeruginosa* is rarely pathogenic for healthy carriers but is a prodigious opportunistic pathogen of immunocompromised people. In some cases, the gut is thought to act as a reservoir of infection that facilitates migration to the respiratory and urinary tracts (Kerr and Snelling 2009; Streeter and Katouli 2016; Cornforth et al. 2018; Wheatley et al. 2022).

Christofi et al (2019) used a panel of defined knockout mutants to investigate the inhibition of *P. aeruginosa* by organic acids produced by *E. coli*. Each mutant was competed individually against *P. aeruginosa*. While this is useful for identifying the effects of individual genes, their approach does not allow for genetic heterogeneity within a population. It is also unclear whether other mechanisms are important for *E. coli* survival and competition against *P. aeruginosa* under the same conditions.

We used a library of more than 800,000 *E. coli* transposon-insertion mutants to assay the effects of mutations in every *E. coli* gene on overflow metabolism in the presence of glucose. In this Transposon-Directed Insertion Sequencing with Expression (TraDIS-*Xpress*) library, the transposon encodes an outward-transcribing, inducible promoter to modulate gene expression in addition to conventional inactivation. Importantly, this allows the interrogation of all genes, including essential genes that would otherwise not be assayed (Yasir et al. 2020). We grew co-cultures consisting of the transposon mutant library and a single strain of *P. aeruginosa*, with and without glucose. This approach, in which all mutants are competed against each other and *P. aeruginosa* simultaneously, effectively rules out the contribution of an individual mutant population towards the accumulation of organic acids in the medium because all cells experience the same conditions no matter their contribution to establishing their environment. Our hypothesis was that using TraDIS-*Xpress* would allow us to broaden the panel of genes identified by Christofi et al., with a focus on the intracellular mechanisms required to thrive in high-glucose environments and in complex competitive situations.

Materials and methods

Strains and growth conditions

The TraDIS-*Xpress* mutant library, described previously by Yasir et al. (2020), was constructed by electroporating a customised mini-Tn*5* transposon into *E. coli* strain BW25113, with selection of consequent transposon mutants on Miller's formulation of lysogeny broth agar (LBA) containing kanamycin (30 $\mu g.mL^{-1}$). Aliquots of the transposon library were recovered from storage at − 70 °C and approximately 10^7 cells (estimated by OD_{600} with 1 OD_{600} unit = 10^9 CFU) were inoculated into 5 mL of Miller's lysogeny broth (LB) in a screw-capped 50 mL tube. For co-cultures, an equal number of *P. aeruginosa* strain PA14 cells from an overnight culture in LB were added. An identical co-culture was grown in parallel, except that sterile glucose was added to the medium at 4% (w/v). Cultures consisting of only the *E. coli* transposon library were also grown under the same conditions, in LB with and without glucose. Each culture was grown in duplicate. All cultures were incubated for 24 h at 37 °C with shaking at 200 rpm. Cultures reached stationary phase at a density of approximately 3×10^9 cells. mL^{-1} having experienced approximately 10 generations.

Quantifying numbers of each species

A single colony of *P. aeruginosa* strain PA14 or approximately 10^7 cells from the *E. coli* strain BW25113 TraDIS-*Xpress* library were inoculated into 5 mL of LB and grown overnight to reach stationary phase. Equal numbers of cells from each overnight culture were then transferred into fresh LB, with or without 4% glucose, as described above. Pure cultures of *E. coli* and *P. aeruginosa* and co-cultures of both species were grown in parallel. After 24 h growth at 37 °C with shaking at 200 rpm, tenfold serial dilutions were performed on each culture and 100 µL of the 10^{-6} and 10^{-7} dilutions were spread on LBA plates containing kanamycin (for selective growth of *E. coli*) and trimethoprim (for selective growth of *P. aeruginosa*). The plates were incubated overnight at 37 °C and number of colony-forming units (CFU) were determined from plates with approximately 10 – 100 colonies. Each culture was grown and enumerated in triplicate.

Sequencing of transposon insert sites

DNA was extracted from the cell pellets of each culture using a Quick-DNA fungal/bacterial 96 extraction kit (Zymo Research). Transposon-containing DNA fragments were prepared for sequencing using a Nextera DNA library preparation kit (Illumina) with custom i5 primers Yasir et al (2020). The sequencing libraries were selected for a size range of 300 – 500 bp and sequenced using the Illumina NextSeq 500 platform with NextSeq 500/550 high output v2 kit for 75 cycles as described by Yasir et al (2020).

TraDIS-*Xpress* data analysis

Nucleotide sequence data were converted to fastq format using bcl2fastq v. 2.20 (Illumina) then reads were mapped to the *E. coli* strain BW25113 reference genome sequence (CP009273) using the Burrows-Wheeler Aligner (Li and Durbin 2009) in Bio-TraDIS v. 1.4.1 (Barquist et al. 2016) to generate maps of insertion site frequency. Frequency of insertions for test conditions (in duplicate) were compared to the *E. coli*-only control using AlbaTraDIS v. 0.0.5 (Page et al. 2020), generating measures for $\log_2$ fold-change ($\log_2$FC) in sequence reads (as a proxy for number of cells containing each mutation in test conditions versus the control) and a p-value of statistical significance adjusted for the false discovery rate (q). Significantly changed mutants were defined as those with $-1 > \log_2 FC > 1$ and $q < 0.01$. The full output of AlbaTraDIS is provided in Supplementary Tables 1, 2 and 3.

Determination of pH and organic acid concentrations in culture supernatants

Cultures were centrifuged and then filtered (0.2 µm cellulose acetate) to remove cells. The supernatant pH was measured using Whatman Panpeha universal indicator paper strips, with a pH range of 0 – 14 and sensitivity of 0.5 pH units.

Samples of supernatant (100 µL) were then mixed with internal standard (100 µL of 100 $\mu g.mL^{-1}$ D5-propionic acid in methanol) and derivatised to the nitrophenylhydrazine by addition of 50 µL each of 50 mM 3-nitrophenylhydrazine hydrochloride in 80% methanol, 50 mM dimethylaminopropyl)-N-ethylcarbodiimide hydrochloride in 80% methanol and 7% pyridine in methanol. The mixture was shaken and heated at 40 °C for 30 min before terminating the reaction by addition of 0.5% aqueous hexanoic acid (250 µL). Liquid chromatography coupled to mass spectrometry (LC–MS/MS) analysis was conducted using a Waters Acquity UPLC coupled to a TQ-Absolute triple quadrupole mass spectrometer. The analytical column was a Waters Premiere HSS T3 C18 100×2.1 mm×1.7 µm at 35 °C. Mobile phases were solvent A: water with 0.1% formic acid and solvent B: acetonitrile with 0.1% formic acid with a constant flow of 0.5 $mL.min^{-1}$. A gradient of solvent B was applied as 20% at injection for 1 min, 55% at 7 min, 100% at 7.5 min and held for 2 min, then back to initial conditions for 3 min. Sample injections were 0.2 µl. Samples were undiluted for measurements of butyric acid, formic acid, lactic acid and propionic acid, and diluted 50-fold for measurement of acetic acid. Quantification was made against standard preparations of target compounds (5 – 100 $\mu g.mL^{-1}$). Results for formic acid are reported as concentrations above the background level of approximately 20 $\mu g.mL^{-1}$.

Determination of pyocyanin concentrations in culture supernatant

Pyocyanin concentrations were measured spectrophotometrically using a modified version of the method of Essar et al. (1990). Centrifuged, filtered supernatants (2.5 mL) were vortex mixed with chloroform (1.5 mL) then centrifuged at 8,000×g for 5 min. The chloroform layer was taken (1.4 mL) and added to 0.5 mL of 0.2 N HCl then re-extracted by vortex mixing and centrifuged again. The acid fraction (0.45 mL) was mixed with a further 0.45 mL of 0.2 N HCl and the absorbance was measured at 520 nm. The concentration, measured as $\mu g.mL^{-1}$ was calculated by $A_{520} \times 34.144$.

Table 1 Significant loci for the growth of *E. coli* with 4% glucose

Name	Annotation	Log_2FC	q
ftsN	Essential cell division protein	1.121	6.71E-21
sucA	2-oxoglutarate decarboxylase; thiamine triphosphate-binding	1.568	1.20E-14
prc	Carboxy-terminal protease for penicillin-binding protein 3	-1.011	6.70E-12
atpA	F1 sector of membrane-bound ATP synthase; alpha subunit	1.316	4.10E-11
nuoG	NADH:ubiquinone oxidoreductase; chain G	1.215	8.32E-09
dapF	Diaminopimelate epimerase	1.507	5.24E-08
nuoC	NADH:ubiquinone oxidoreductase; fused CD subunit	1.266	7.09E-07
atpG	F1 sector of membrane-bound ATP synthase; gamma subunit	1.652	0.0002
nuoF	NADH:ubiquinone oxidoreductase; chain F	1.230	0.0016
atpD	F1 sector of membrane-bound ATP synthase; beta subunit	1.053	0.0021
nuoI	NADH:ubiquinone oxidoreductase; chain I	2.023	0.0066
nuoB	NADH:ubiquinone oxidoreductase; chain B	1.198	0.0098

Significance was determined by $\log_2$ fold change in abundance of mutants ($\log_2FC$) above 1 and below − 1, and q-value less than 0.01

Results

Disruption of respiration pathways facilitates survival during overflow metabolism

Following growth of the transposon mutant library in LB containing 4% glucose, 12 genes were identified where the abundance of transposon insertions significantly changed ($-1 > \log_2FC > 1$, and $q < 0.01$) compared to growth in LB without glucose (Table 1). Nine of these 12 genes code for respiratory metabolic functions, and contained significantly more mutants within the gene. These mutations are likely to shift metabolism towards fermentation under aerobic conditions. Therefore, under high glucose concentrations, a selective advantage was conferred by mutations that hinder or prevent respiration, even without additional competition with *P. aeruginosa*. The remaining 3 genes encode functions in amino acid metabolism (*dapF*) and re-structuring of the cell envelope during cell division (*ftsN* and *prc*).

E. coli inhibition of *P. aeruginosa* requires high concentrations of glucose

Co-cultures of *E. coli* and *P. aeruginosa* grown in LB without glucose permitted growth of both species to approximately the same density as when they were grown in isolation (Fig. 1). During overnight growth, the co-culture became a blue-green colour due to production of the antimicrobial compound pyocyanin by *P. aeruginosa* (Lau et al. 2004). Pyocyanin is known to inhibit *E. coli* growth (Christofi et al. 2019) but did not significantly reduce the number of *E. coli* in co-cultures without glucose. *P. aeruginosa* produced the highest concentration of pyocyanin (9.1 µg/mL) when grown alone without glucose (Fig. 2). Pyocyanin production decreased to 4.4 µg/mL when glucose was added, and dropped to 2.0 µg/mL when *P. aeruginosa* was competed against *E. coli* without glucose. When glucose was introduced to the competitive culture, pyocyanin production could not be detected. Previous reports have suggested a MIC of pyocyanin against *E. coli* of at least 20 µg/mL (Shouman et al. 2023).

In co-cultures grown in LB + 4% glucose, *E. coli* grew to a higher density and inhibited the growth of *P. aeruginosa* nearly 40-fold (Fig. 1), from 2.66×10^{10} CFU/mL when grown alone to 7.11×10^{8} CFU/mL when grown together (mean values; $n = 3$). Pyocyanin production was also no longer visible. The inclusion of glucose led to a reduction of *E. coli* cell density at 24 h (Fig. 1) in both pure and co-cultures. This is likely to be due to acidification of the culture medium as the abundant glucose was fermented to short-chain organic acids (El-Mansi 2004). All conditions without glucose had a final supernatant pH of 7.5. With 4% glucose, pure *P. aeruginosa* culture supernatant pH was 7.0, indicating a small amount of acid production, while *E. coli* with 4% glucose, either alone or in competition, decreased the supernatant pH to 4.5. This drop in pH was caused primarily by production of acetic acid (Fig. 2). Small quantities of formic acid were produced in all cultures, while butyric acid, lactic acid and propionic acid were also detected in some cultures.

Disruptions to respiration provide a competitive advantage for *E. coli* against *P. aeruginosa*, but only in the presence of glucose

The TraDIS-*Xpress* screen identified 57 loci in which insertional inactivation led to significantly changed abundances ($-1 > \log_2FC > 1$ and $q < 0.01$) of *E. coli* mutants in co-cultures with *P. aeruginosa* compared to the *E. coli* mutants grown alone (Fig. 3). Both with and without glucose, the gene with the largest $\log_2FC$ and most significant q-values was *tolC* (Tables 2 and 3), which encodes

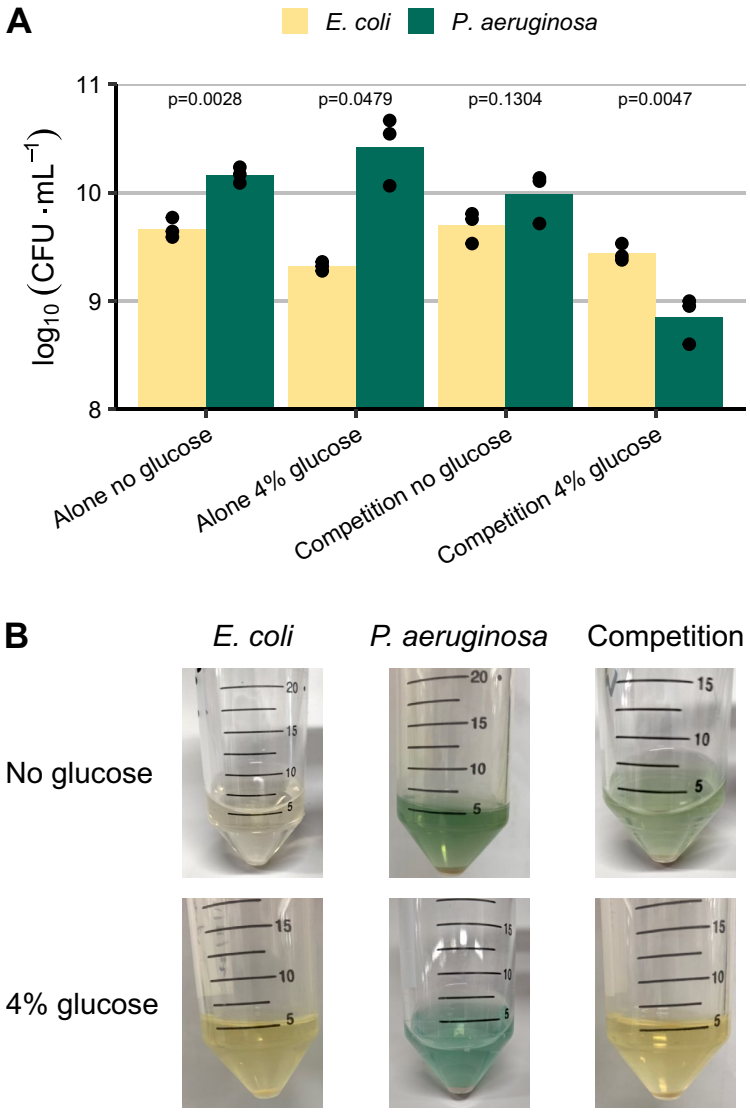


Fig. 1 Comparison between growth of *E. coli* BW25113 TraDIS-Xpress library and *P. aeruginosa* PA14, either alone or in co-cultures and in the presence or absence of 4% glucose. **A** Cultures ($n = 3$) were grown in LB for 24 h, as described in Materials and Methods then diluted and spread on LBA plates supplemented with appropriate antibiotics. Colonies were counted after overnight incubation at 37 °C. Bars indicate mean values and points indicate individual data. The p-value result of a two-way T-test comparing average CFU/mL of *E. coli* and *P. aeruginosa* under each condition is given above each pair of bars. **B** The colour of a culture after 24 h incubation provide an approximate indication of relative *P. aeruginosa* cell numbers. Cultures dominated by *E. coli* remained yellow-beige, while those with higher numbers of *P. aeruginosa* became blue-green due to production of pyocyanin

an outer membrane channel used by several export systems. Insertional inactivation of *tolC* was deleterious to survival irrespective of glucose addition, which suggests that a competitive factor of *P. aeruginosa* inhibits *E. coli* but is at least partly counteracted by a TolC-dependent export process. The most probable identity for this factor is pyocyanin as we established that high levels of pyocyanin were produced when *P. aeruginosa* dominated the co-cultures. Mutants of *E. coli* lacking TolC exhibit increased sensitivity to pyocyanin and decreased metabolic output in glucose minimal medium so would be expected to be less competitive against *P. aeruginosa* (Sulavik et al. 2001; Dhamdhere and Zgurskaya 2010).

In co-cultures without glucose, 14 loci with a range of functions (Fig. 3 B) were important for *E. coli* competition with *P. aeruginosa* (Table 2, Fig. 3B). Three of these loci led to reduced fitness when they were disrupted (negative $\log_2$FC), whilst disruption to 11 loci increased fitness. This included 3 repetitive extragenic palindromic elements, which are referred to here by their base pair

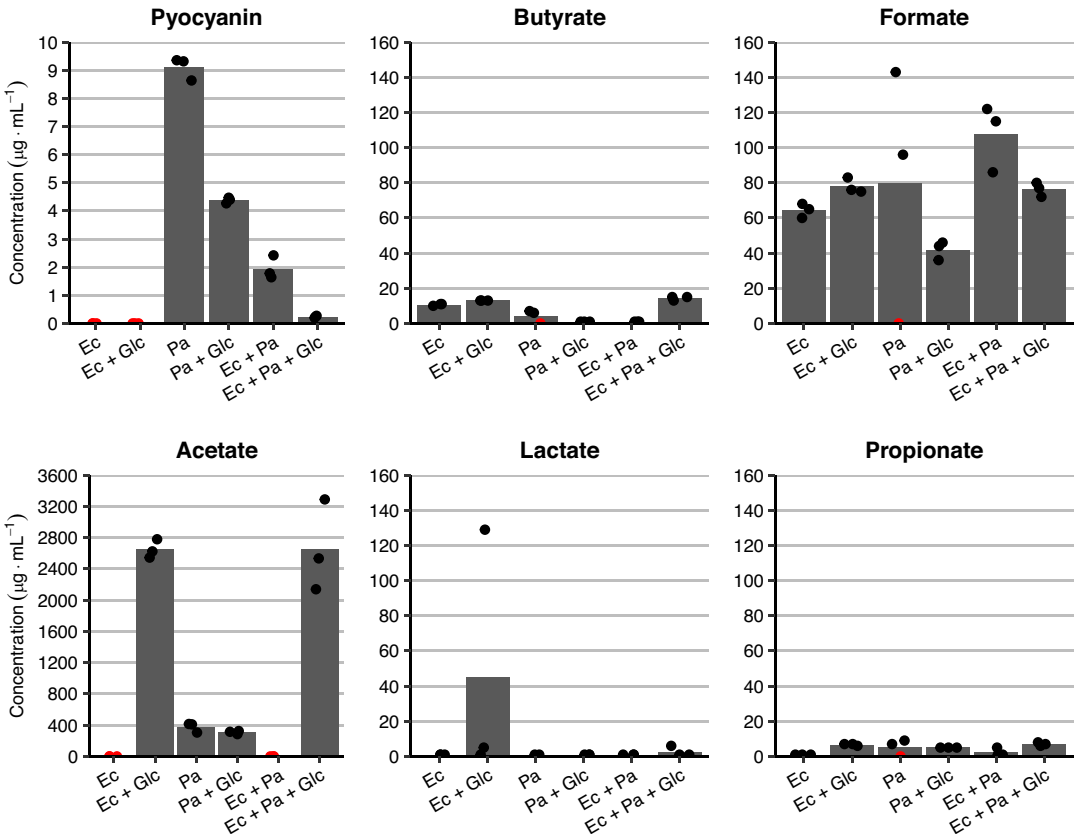


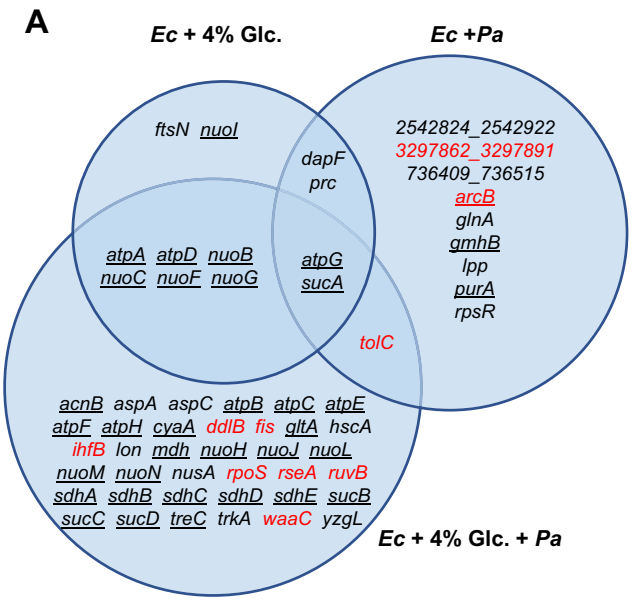
Fig. 2 Concentrations of pyocyanin and organic acids in culture supernatants after 24 h growth. *E. coli* BW25113 and *P. aeruginosa* PA14 were grown in LB either alone or in co-culture, and with or without 4% glucose. Bars indicate mean values ($n = 3$) and points indicate individual data. Red points indicate samples in which no pyocyanin or acid was detected. Concentrations of acids below 5 μg.mL^{-1} were recorded as trace amounts

coordinates in the reference genome (Fig. 3A). The function of such elements is not understood fully, but they can regulate gene expression when they occur within 15 bp downstream of a stop codon (Liang et al. 2015). Only the region between bp 736409 – 736515 met this criterion out of those identified in this study. It is situated 15 bp downstream of *dtpD* on the reverse strand, which encodes a proton-dependent dipeptide symporter and was not itself identified as significant. However, the small size (approximately 100 bp) of these palindromic elements could lead to increased experimental variation as they contain fewer unique transposon insertions than typical coding regions.

For the co-culture of *E. coli* and *P. aeruginosa* with 4% glucose, TraDIS-*Xpress* identified 46 genes where disruptive transposon insertions led to a significant fold change in abundance, although only 3 genes were shared with the LB co-culture without glucose (Fig. 3). Of these 46 genes, a competitive disadvantage was conferred by 8, while mutations in the other 38 genes were advantageous. Notably, the most frequently identified gene function under either growth condition was central carbon metabolism (CCM). However, while only 5 CCM genes (36%) were significant without glucose, 31 CCM genes (67%) were significant with glucose. The much larger number of significant genes when glucose was included demonstrates a clear competitive advantage for these mutants in the presence of abundant glucose.

From all the genes identified as significant in any condition (Fig. 3A), 34 were involved in aerobic CCM, and insertional inactivation conferred a selective advantage in all but *arcB* (*E. coli* monoculture without glucose, $\log_2 FC = -1.01$; $q = 8.98 \times 10^{-49}$), which encodes the kinase of the ArcAB two-component system that, under anaerobic conditions, represses transcription of genes coding for aerobic metabolism (Iuchi et al. 1989). The fact that *arcB* mutants are selected against highlights the importance of the ability to suppress aerobic respiration and so promote overflow metabolism to facilitate competition in these conditions.

In co-cultures with glucose, mutants of genes encoding TCA cycle and oxidative phosphorylation functions were over-represented, with 29 significant genes including citrate synthase (*gltA*), aconitate hydratase



B

Gene Function	*Ec* + 4% Glc.	*Ec* + *Pa*	*Ec* + 4% Glc. + *Pa*
Amino acid metabolism	1	2	3
Cell structure/division	2	1	1
Central metabolism	9	5	31
Efflux	0	1	2
Transcription	0	0	5
Translation	0	1	2
Other/unknown	0	4	2
Total	**12**	**14**	**46**

Fig. 3 Overview of significant loci identified for *E. coli* BW25113 grown with 4% glucose, and during competition with *P. aeruginosa* PA14 with and without 4% glucose. **A** Venn diagram containing names of all significant genes ($-1 > \log_2 FC > 1$; $q < 0.01$) under each condition. Red gene names indicate those for which transposon insertions within the coding region reduced competitive fitness (negative $\log_2 FC$). Underlined genes are involved in central carbon metabolism. Three unnamed short repeat regions were identified in the no glucose condition, which are represented by the range of their base pair position in the genome. **B** Number of significant genetic loci for each of the important gene functions identified for each condition

Table 2 Significant genetic loci for competition of *E. coli* with *P. aeruginosa* in the absence of glucose

Name	Annotation	$\log_2 FC$	q
tolC	**Transport channel**	**-1.575**	**9.22E-119**
arcB	Aerobic respiration control sensor histidine protein kinase cognate to two-component response regulators ArcA and RssB	-1.014	8.98E-49
prc	Carboxy-terminal protease for penicillin-binding protein 3	1.029	1.39E-27
glnA	Glutamine synthetase	1.078	2.81E-09
736409_736515	Repetitive extragenic palindromic element	2.377	1.61E-08
sucA	**2-oxoglutarate decarboxylase; thiamine triphosphate-binding**	**1.072**	**2.62E-06**
dapF	Diaminopimelate epimerase	1.065	6.34E-06
lpp	Murein lipoprotein	1.337	9.78E-05
purA	Adenylosuccinate synthetase	1.497	0.0016
3297862_3297891	Repetitive extragenic palindromic element	-1.637	0.0032
atpG	**F1 sector of membrane-bound ATP synthase gamma subunit**	**1.056**	**0.0040**
rpsR	30S ribosomal subunit protein S18	1.051	0.0043
2542824_2542922	Repetitive extragenic palindromic element	1.448	0.0061
gmhB	D,D-heptose 1,7-bisphosphate phosphatase for LPS core synthesis	1.128	0.0073

Significance was determined by $\log_2$ fold change in abundance of mutants ($\log_2 FC$) above 1 and below – 1, and q-value less than 0.01. Loci in bold type were identified both with and without glucose

2/2-methylisocitrate dehydratase (*acnB*), 2-oxoglutarate dehydrogenase (*sucAB*), succinyl-CoA synthetase (*sucCD*), succinate:quinone oxidoreductase (*sdhA-E*) and malate dehydrogenase (*mdh*) from the TCA cycle; *aspA* and *aspC,* which code for functions that derive energy from L-glutamate by generating TCA cycle intermediates; and NADH:quinone oxidoreductase (*nuoBC,F–H,J,L-M*) and proton translocating F_0F_1 ATP synthase (*atpA-H*) from oxidative phosphorylation (Fig. 4). Disruptions to all these genes are likely to increase metabolic

Table 3 Significant genetic loci for competition of *E. coli* with *P. aeruginosa* in the presence of 4% glucose

Name	Annotation	log_2FC	q
tolC	**Transport channel**	**-2.612**	**1.90E-268**
aspA	Aspartate ammonia-lyase	1.669	3.45E-88
gltA	Citrate synthase	1.431	3.12E-76
cyaA	Adenylate cyclase	1.105	2.78E-71
sucA	**2-oxoglutarate decarboxylase; thiamine triphosphate-binding**	**2.235**	**7.40E-54**
sdhA	Succinate dehydrogenase; flavoprotein subunit	1.758	5.98E-51
atpA	F1 sector of membrane-bound ATP synthase alpha subunit	1.615	9.95E-49
treC	Trehalose-6-P hydrolase	1.051	8.17E-47
sucC	Succinyl-CoA synthetase beta subunit	1.318	1.29E-41
mdh	Malate dehydrogenase; NAD(P)-binding	1.601	1.33E-37
sucD	Succinyl-CoA synthetase; NAD(P)-binding; alpha subunit	1.399	9.95E-33
aspC	Aspartate aminotransferase; PLP-dependent	1.147	3.08E-31
rpoS	RNA polymerase sigma S (sigma 38) factor	-1.376	7.68E-31
nuoG	NADH:ubiquinone oxidoreductase chain G	1.479	3.69E-29
atpD	F1 sector of membrane-bound ATP synthase beta subunit	1.724	7.65E-29
nuoL	NADH:ubiquinone oxidoreductase membrane subunit L	1.461	1.09E-28
trkA	NAD-binding component of TrK potassium transporter	1.086	1.10E-28
acnB	Bifunctional aconitate hydratase 2/2-methylisocitrate dehydratase	1.426	2.37E-26
nuoF	NADH:ubiquinone oxidoreductase chain F	1.887	4.37E-23
nuoC	NADH:ubiquinone oxidoreductase; fused CD subunit	1.629	2.42E-22
sdhB	Succinate dehydrogenase FeS subunit	1.439	3.13E-21
nuoJ	NADH:ubiquinone oxidoreductase; membrane subunit J	1.836	3.15E-21
lon	DNA-binding ATP-dependent protease La	1.061	5.30E-20
nuoN	NADH:ubiquinone oxidoreductase; membrane subunit N	1.652	1.38E-18
nuoB	NADH:ubiquinone oxidoreductase; chain B	1.847	1.38E-18
nuoM	NADH:ubiquinone oxidoreductase; membrane subunit M	1.685	1.00E-15
atpB	F0 sector of membrane-bound ATP synthase subunit a	1.812	5.82E-12
waaC	ADP-heptose:LPS heptosyl transferase I	-1.099	1.49E-09
nuoH	NADH:ubiquinone oxidoreductase; membrane subunit H	1.749	7.08E-09
sdhC	Succinate dehydrogenase; membrane subunit; binds cytochrome b556	1.566	1.57E-08
atpG	**F1 sector of membrane-bound ATP synthase; gamma subunit**	**1.611**	**2.13E-08**
atpC	F1 sector of membrane-bound ATP synthase; epsilon subunit	1.434	2.28E-08
sdhD	Succinate dehydrogenase; membrane subunit; binds cytochrome b556	1.525	9.15E-08
rseA	Anti-sigma factor	-1.021	5.24E-07
atpF	F0 component of proton translocating ATPase subunit b	1.416	8.52E-07
nusA	Transcription termination/antitermination L factor	1.279	2.19E-06
sdhE	Flavinator of succinate dehydrogenase; antitoxin of CptAB toxin-antitoxin pair	1.333	7.82E-06
ihfB	Integration host factor (IHF); DNA-binding protein; beta subunit	-1.117	9.13E-06
fis	Global DNA-binding transcriptional dual regulator	-1.099	2.03E-05
ruvB	ATP-dependent DNA helicase; component of RuvABC resolvasome	-1.132	8.19E-05
hscA	DnaK-like molecular chaperone specific for IscU	1.205	0.0001
atpE	F0 sector of membrane-bound ATP synthase; subunit c	1.110	0.0003
ddlB	D-alanine:D-alanine ligase	-1.071	0.0003
sucB	Dihydrolipoyltranssuccinase	1.089	0.0004
atpH	F1 sector of membrane-bound ATP synthase; delta subunit	1.351	0.0008
yzgL	Putative uncharacterised protein	1.025	0.0060

Significance was determined by log_2 fold change in abundance of mutants (log_2FC) above 1 and below – 1, and q-value less than 0.01. Loci in bold type were identified both with and without glucose

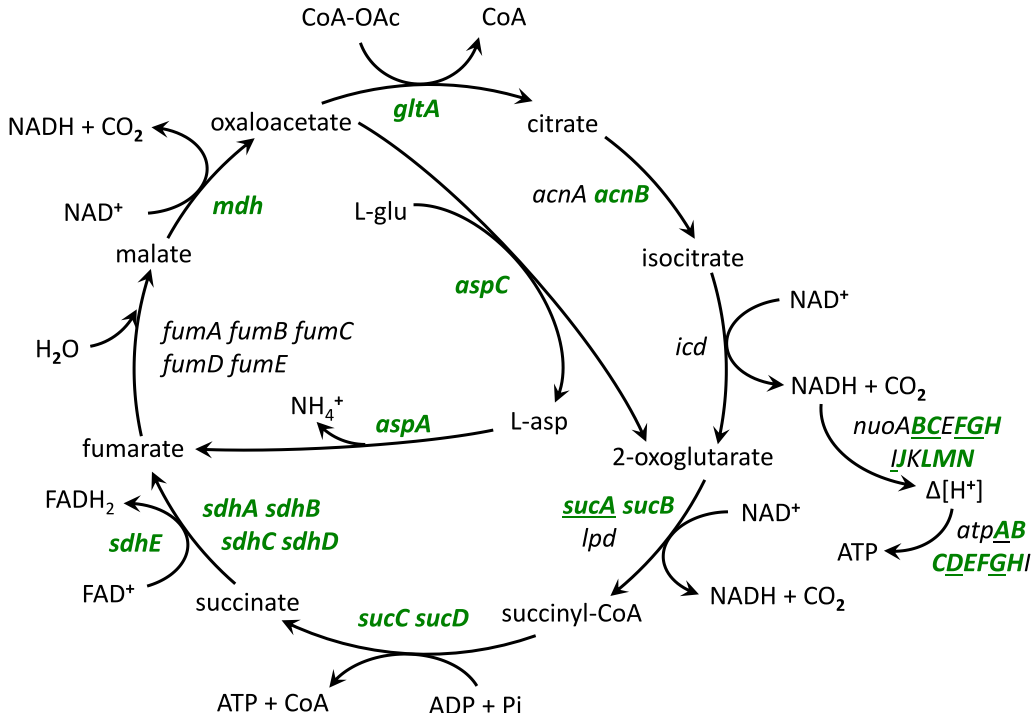


Fig. 4 Genes coding for enzymes of the tricarboxylic acid cycle, NADH:quinone oxidoreductase and proton translocating F_OF_1 ATP synthase, which are all involved in respiratory metabolism. Insertional inactivation of the 31 genes shown in bold, green typeface conferred a selective advantage in the presence of *P. aeruginosa* PA14 and 4% glucose. Insertional inactivation of the 9 underlined genes also conferred a selective advantage when *E. coli* was grown alone with 4% glucose

flux towards the fermentative mixed organic acid pathways.

The other significant loci were divided between multiple functions, although no other functions had more than 5 significant loci for either condition and there were no obvious links between those functions and competition with *P. aeruginosa*. We did not identify any regulatory genes other than global gene/protein regulators such as *lon* and *fis*. This suggests that no specific regulatory or sensing mechanisms are important for the production of organic acids in response to *P. aeruginosa*. We also did not identify genes involved in production or resistance to other potentially inhibitory chemicals such as indole.

Discussion

Taken together, our results both support and extend the findings of Christofi et al. (2019). However, while Christofi et al. grew each mutant of interest individually in competition with *P. aeruginosa* with and without glucose, our TraDIS-*Xpress* assay included an ensemble of all mutants in the entire library, thus competing each against the pool and against *P. aeruginosa*. Our approach more closely resembles the likely conditions encountered in nature by a spontaneous mutant arising within a population of bacteria, in which the mutant must compete not only with clonal siblings but also with its' parent strain, other mutants and, probably, other species. The probability of any mutant multiplying to form a significant subpopulation is proportional to its relative fitness in those conditions; from a deleterious mutation that is likely to die out to a highly advantageous mutation that might quickly become the dominant strain. Therefore, TraDIS-*Xpress* co-culture assays could provide fresh insight into the evolution of overflow metabolism and many other processes.

Crucially, in our screen each mutant constitutes only a tiny minority of the population—probably too few for individual CCM mutants to have a discernible effect on the acid concentration of the culture, and their increased acid production (if it has any effect) should benefit all the *E. coli* mutants present by repressing growth of *P. aeruginosa*. Correspondingly, mutants that significantly decrease acid production also make up only a small proportion of the population. They are, however, able to benefit from the *P. aeruginosa*-inhibiting effect of acids produced by most of the *E. coli* and therefore might not experience a significant negative selective pressure. The overall environmental conditions are determined by the majority of cells, which do not have transposon insertions that significantly affect acid production or survival.

Under our experimental design, these majority cells may have contributed a proportion of their acid production through anaerobic respiration or fermentation in the absence of oxygen, rather than overflow metabolism sensu stricto, at higher cell densities. However, due to their specific transposon insertions, mutants that significantly increased in abundance possessed a selective advantage in these conditions. Therefore, while screening mutants in isolation (*c.f.* Christofi et al. 2019) identified genes involved in mixed acid fermentation, our TraDIS-*Xpress* screen identified mutants with altered fitness for the complex mixture of stresses imposed by the acidic environment and growth within a heterogeneous population.

Transposon insertions in 12 *E. coli* genetic loci conferred a selective advantage during monoculture growth in 4% glucose. 9 of these genes code for either the NADH oxidoreductase or ATP synthase complexes, in which the mutants are expected to be deficient in aerobic and anaerobic respiration. This suggests that fermentation under these conditions confers a selective advantage over respiring *E. coli* cells. Since an abundant supply of glucose, as in our experiment, will promote overflow metabolism, it may be the case that the CCM mutants identified here shift the metabolic balance further towards fermentation (even completely preventing respiration) and thus confer a slight survival advantage over cells that retain wild-type metabolism due to the limitations of cellular physiology mentioned in the introduction (de Groot et al. 2020). Therefore, while bulk production of organic acids benefits the *E. coli* population as a whole, mutants that are genetically 'primed' for overflow metabolism gain an additional individual selective advantage. When 4% glucose and *P. aeruginosa* were present, as well as additional genes coding for NADH oxidoreductase or ATP synthase, many mutations in genes coding for TCA cycle functions were identified as advantageous. Therefore, although *P. aeruginosa* growth was supressed, it did exert a competitive stress upon the *E. coli* mutants, potentiating the selective pressure for CCM mutants that favour fermentation. Since the same pathways were identified with and without *P. aeruginosa*, the benefits of overflow metabolism are not simply due to the accumulation of organic acids in the medium.

The overall small $\log_2$FC values (Tables 2 and 3) indicate that the fitness advantages experienced by these mutants were slight. Nevertheless, the data were highly reproducible between duplicates and indicated extremely high levels of significance for many of the genes. TraDIS-*Xpress* results can comprise data from tens to hundreds of transposon inserts per gene, all of which are assayed simultaneously. This provides a degree of internal validation and increases the resolution of the assay compared to those approaches using single gene knockouts. Small, but statistically significant,phenotypic changes, such as we observed here, may not be observable by other, less sensitive, assay methods. In this experiment, we observed that mutations in genes coding for aerobic respiration possess a selective advantage under the test conditions. We hypothesise, therefore, that inactivating mutations in the suppressor of aerobic respiration functions, *arcB*, would be detrimental under these test conditions. The data indeed show that *arcB* mutants do have a significant disadvantage. Thus, the TraDIS-*Xpress* data are very robust and often include their own validating data, precluding the need for additional testing of defined mutants.

Mixed acid fermentation is a common trait within *E. coli* and a wide variety of other bacteria, so it is likely that, under appropriate conditions, many members of the mammalian gut microbiome will be able to outcompete *P. aeruginosa* and other species in a similar manner. However, due to the wide range of habitats occupied by *P. aeruginosa*, adaptation and evolution for specific environmental conditions have been observed. For example, total cell growth varies for *P. aeruginosa* strains isolated from different locations, even when grown under the same in vitro conditions (Diaz et al. 2018). Therefore, *P. aeruginosa* strain source and history might affect their susceptibility to lactic and acetic acids. Acetic acid has been suggested previously as an antibiotic-free decontamination method for *P. aeruginosa* biofilms in hospital drains (Stjärne Aspelund et al. 2016) and for burn wounds (Halstead et al. 2015). This raises the tempting prospect of using probiotic bacteria to modulate the microbiome for therapeutic purposes by production of organic acids, which may be more resilient than antibiotics against the development of resistance.

Conclusions

Our results enrich our understanding of overflow metabolism in *E. coli*, its impact on individuals in a population, and its role in competition with *P. aeruginosa.* We identified CCM genes that contribute towards the balance between respiration and fermentation during overflow metabolism and highlighted the importance of *tolC* for survival in the presence of pyocyanin produced by *P. aeruginosa*. The TraDIS-*Xpress* data adds a further quantitative dimension to the importance of genes and sensitivity in their detection. The density of transposon insertions across the genome for all the mutants combined means that it is likely that 99% of the *E. coli* genome was screened. Therefore, it is unlikely that any other gene functions are involved under the experimental conditions employed.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13213-024-01760-z.

Supplementary Material 1.

Supplementary Material 2.

Supplementary Material 3.

Acknowledgements
The authors would like to thank Mark Philo (Quadram Institute) for his help with measuring organic acid concentrations by liquid chromatography coupled to mass spectrometry.

Authors' contributions
MY conceived and designed the work; MY and NMT acquired and analysed the data; all authors contributed to interpreting the data; NMT drafted the manuscript and all authors revised the work and approved the submitted manuscript.

Funding
This work was funded by the Biotechnology and Biological Sciences Research Council Institute Strategic Programme Microbes in the Food Chain BB/R012504/1 and its constituent project BBS/E/F/000PR10349.

Availability of data and materials
The datasets generated and analysed during the current study are available in the Array Express repository under accession number E-MTAB-13391 (https://www.ebi.ac.uk/biostudies/arrayexpress).

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

Received: 4 October 2023 Accepted: 3 April 2024
Published online: 15 April 2024

References

Barquist L, Mayho M, Cummins C et al (2016) The TraDIS toolkit: sequencing and analysis for dense transposon mutant libraries. Bioinformatics 32:1109–1111. https://doi.org/10.1093/bioinformatics/btw022
Basan M, Hui S, Okano H et al (2015) Overflow metabolism in Escherichia coli results from efficient proteome allocation. Nature 528:99–104. https://doi.org/10.1038/nature15765
Bédard E, Prévost M, Déziel E (2016) Pseudomonas aeruginosa in premise plumbing of large buildings. Microbiologyopen 5:937–956. https://doi.org/10.1002/mbo3.391
Christofi T, Panayidou S, Dieronitou I et al (2019) Metabolic output defines Escherichia coli as a health-promoting microbe against intestinal Pseudomonas aeruginosa. Sci Rep 9:1–13. https://doi.org/10.1038/s41598-019-51058-3
Cornforth DM, Dees JL, Ibberson CB et al (2018) Pseudomonas aeruginosa transcriptome during human infection. Proc Natl Acad Sci U S A 115. https://doi.org/10.1073/pnas.1717525115
Crabtree HG (1929) Observations on the carbohydrate metabolism of tumours. Biochem J 23:536–545. https://doi.org/10.1042/bj0230536
Crone S, Vives-Flórez M, Kvich L et al (2020) The environmental occurrence of Pseudomonas aeruginosa. APMIS 128:220–231. https://doi.org/10.1111/apm.13010
de Groot DH, Lischke J, Muolo R et al (2020) The common message of constraint-based optimization approaches: overflow metabolism is caused by two growth-limiting constraints. Cell Mol Life Sci 77:441–453. https://doi.org/10.1007/s00018-019-03380-2
Deken RH (1966) The crabtree effect: a regulatory system in yeast. J Gen Microbiol. 44:149–156
Dhamdhere G, Zgurskaya HI (2010) Metabolic shutdown in Escherichia coli cells lacking the outer membrane channel TolC. Mol Microbiol 77:743–754. https://doi.org/10.1111/j.1365-2958.2010.07245.x
Diaz KE, Remold SK, Onyiri O et al (2018) Generalized growth of estuarine, household and clinical isolates of Pseudomonas aeruginosa. Front Microbiol 9:1–8. https://doi.org/10.3389/fmicb.2018.00305
Diggle SP, Whiteley M (2020) Microbe profile: Pseudomonas aeruginosa: opportunistic pathogen and lab rat. Microbiol (united Kingdom) 166:30–33. https://doi.org/10.1099/mic.0.000860
Eiteman MA, Altman E (2006) Overcoming acetate in Escherichia coli recombinant protein fermentations. Trends Biotechnol 24:530–536. https://doi.org/10.1016/j.tibtech.2006.09.001
El-Mansi M (2004) Flux to acetate and lactate excretions in industrial fermentations: physiological and biochemical implications. J Ind Microbiol Biotechnol 31:295–300. https://doi.org/10.1007/s10295-004-0149-2
Essar DW, Eberly L, Hadero A, Crawford IP (1990) Identification and characterization of genes for a second anthranilate synthase in Pseudomonas aeruginosa: interchangeability of the two anthranilate synthase and evolutionary implications. J Bacteriol 172:884–900. https://doi.org/10.1128/jb.172.2.884-900.1990
Gilbert JA, Stephens B (2018) Microbiology of the built environment. Nat Rev Microbiol 16:661–670. https://doi.org/10.1038/s41579-018-0065-5
Halstead FD, Rauf M, Moiemen NS et al (2015) The antibacterial activity of acetic acid against biofilm-producing pathogens of relevance to burns patients. PLoS One 10:1–15. https://doi.org/10.1371/journal.pone.0136190
Holms H (1996) Flux analysis and control of the central metabolic pathways in Escherichia coli. FEMS Microbiol Rev 19:85–116. https://doi.org/10.1016/S0168-6445(96)00026-5
Iuchi S, Cameron DC, Lin EC (1989) A second global regulator gene (arcB) mediating repression of enzymes in aerobic pathways of Escherichia coli. J Bacteriol 171:868–873. https://doi.org/10.1128/jb.171.2.868-873.1989
Kerr KG, Snelling AM (2009) Pseudomonas aeruginosa: a formidable and ever-present adversary. J Hosp Infect 73:338–344. https://doi.org/10.1016/j.jhin.2009.04.020
Kidd TJ, Ritchie SR, Ramsay KA et al (2012) Pseudomonas aeruginosa exhibits frequent recombination, but only a limited association between genotype and ecological setting. PLoS One 7. https://doi.org/10.1371/journal.pone.0044199
Lau GW, Hassett DJ, Ran H, Kong F (2004) The role of pyocyanin in Pseudomonas aeruginosa infection. Trends Mol Med 10:599–606. https://doi.org/10.1016/j.molmed.2004.10.002
Li H, Durbin R (2009) Fast and accurate short read alignment with Burrows-Wheeler transform. Bioinformatics 25:1754–1760. https://doi.org/10.1093/bioinformatics/btp324
Liang W, Rudd KE, Deutscher MP (2015) A role for REP sequences in regulating translation. Mol Cell 58:431–439. https://doi.org/10.1016/j.molcel.2015.03.019
Lin WH, Jacobs-Wagner C (2022) Connecting single-cell ATP dynamics to overflow metabolism, cell growth, and the cell cycle in Escherichia coli. Curr Biol 32:3911–3924.e4. https://doi.org/10.1016/j.cub.2022.07.035
Mackie RI, Sghir A, Gaskins HR (1999) Developmental microbial ecology of the neonatal gastrointestinal tract. Am J Clin Nutr 69. https://doi.org/10.1093/ajcn/69.5.1035s
Nag D, Breen P, Raychaudhuri S, Withey JH (2018) crossm glucose metabolism by Escherichia coli inhibits vibrio cholerae. Infect Immun 86:1–13
Page AJ, Bastkowski S, Yasir M et al (2020) AlbaTraDIS: comparative analysis of large datasets from parallel transposon mutagenesis experiments. PLoS Comput Biol 16:1–13. https://doi.org/10.1371/journal.pcbi.1007980
Regueira A, Lema JM, Mauricio-Iglesias M (2021) Microbial inefficient substrate use through the perspective of resource allocation models. Curr Opin Biotechnol 67:130–140. https://doi.org/10.1016/j.copbio.2021.01.015

Sassone-Corsi M, Nuccio SP, Liu H et al (2016) Microcins mediate competition among Enterobacteriaceae in the inflamed gut. Nature 540:280–283. https://doi.org/10.1038/nature20557
Sengupta C, Ekka M, Arora S et al (2017) Cross feeding of glucose metabolism byproducts of Escherichia coli human gut isolates and probiotic strains affect survival of Vibrio cholerae. Gut Pathog 9:1–4. https://doi.org/10.1186/s13099-016-0153-x
Shouman H, Said HS, Kenawy HI et al (2023) Molecular and biological characterization of pyocyanin from clinical and environmental Pseudomonas aeruginosa. Microb Cell Fact 22:166 https://doi.org/10.1186/s12934-023-02169-0
Stjärne Aspelund A, Sjöström K, Olsson Liljequist B et al (2016) Acetic acid as a decontamination method for sink drains in a nosocomial outbreak of metallo-β-lactamase-producing Pseudomonas aeruginosa. J Hosp Infect 94:13–20. https://doi.org/10.1016/j.jhin.2016.05.009
Streeter K, Katouli M (2016) Pseudomonas aeruginosa: a review of their pathogenesis and prevalence in clinical settings and the environment. Infect Epidemiol Med. 2:25–32. https://doi.org/10.18869/modares.iem.2.1.25
Sulavik MC, Houseweart C, Cramer C et al (2001) Antibiotic susceptibility profiles of Escherichia coli strains lacking multidrug efflux pump genes. Antimicrob Agents Chemother 45:1126–1136. https://doi.org/10.1128/AAC.45.4.1126-1136.2001
Swain A, Fagan WF (2019) A mathematical model of the Warburg effect: effects of cell size, shape and substrate availability on growth and metabolism in bacteria. Math Biosci Eng 16:168–186. https://doi.org/10.3934/mbe.2019009
Szenk M, Dill KA, de Graff AMR (2017) Why do fast-growing bacteria enter overflow metabolism? Testing the membrane real estate hypothesis. Cell Syst 5:95–104. https://doi.org/10.1016/j.cels.2017.06.005
Vazquez A, Oltvai ZN (2016) Macromolecular crowding explains overflow metabolism in cells. Sci Rep 6:1–7. https://doi.org/10.1038/srep31007
Warburg O (1956) On the origin of cancer cells. Science (80). 123:309–314. https://doi.org/10.1126/science.123.3191.309
Wheatley RM, Caballero JD, van der Schalk TE et al (2022) Gut to lung translocation and antibiotic mediated selection shape the dynamics of Pseudomonas aeruginosa in an ICU patient. Nat Commun 13. https://doi.org/10.1038/s41467-022-34101-2
Wolfe AJ (2005) The acetate awitch 69:12–50. https://doi.org/10.1128/MMBR.69.1.12
Yasir M, Keith Turner A, Bastkowski S et al (2020) TRADIS-XPress: a high-resolution whole-genome assay identifies novel mechanisms of triclosan action and resistance. Genome Res 30:239–249. https://doi.org/10.1101/gr.254391.119
Zarkan A, Liu J, Matuszewska M et al (2020) Local and universal action: the paradoxes of indole signalling in bacteria. Trends Microbiol xx:1–12. https://doi.org/10.1016/j.tim.2020.02.007

Publisher's Note