

RESEARCH ARTICLE

Open Access



A bacterial route for folic acid supplementation

Claire Maynard¹, Ian Cummins¹, Jacalyn Green² and David Weinkove^{1,3*} 

Abstract

Background: To prevent folate deficiencies, many countries supplement various foodstuffs with folic acid. This compound is a synthetic oxidised folate that differs from naturally occurring reduced folates in its metabolism and uptake. Notably, safety reviews of folic acid supplementation have not considered interactions with gut bacteria. Here, we use the *Caenorhabditis elegans* – *Escherichia coli* animal–microbe model to examine a possible bacterial route for folic acid uptake. It has been assumed that supplements are taken up directly by the worm, especially because *E. coli* is unable to take up folates. However, *E. coli*, like many other bacteria, can transport the folate breakdown product, para-aminobenzoate-glutamate (PABA-glu), via AbgT and use it for bacterial folate synthesis. This pathway may impact host health because inhibition of bacterial folate synthesis increases *C. elegans* lifespan.

Results: Folic acid supplementation was found to rescue a *C. elegans* developmental folate-deficient mutant; however, a much higher concentration was required compared to folinic acid, a reduced folate. Unlike folinic acid, the effectiveness of folic acid supplementation was dependent on the *E. coli* gene, *abgT*, suggesting a bacterial route with PABA-glu uptake by *E. coli* as a first step. Surprisingly, we found up to 4% PABA-glu in folic acid preparations, including in a commercial supplement. Via breakdown to PABA-glu, folic acid increases *E. coli* folate synthesis. This pathway restores folate synthesis in a bacterial mutant defective in PABA synthesis, reversing the ability of this mutant to increase *C. elegans* lifespan.

Conclusions: Folic acid supplementation in *C. elegans* occurs chiefly indirectly via bacterial uptake of breakdown products via *E. coli* AbgT, and can impact *C. elegans* development and longevity. Examining how folic acid supplementation affects bacterial folate synthesis in the human gut may help us to better understand the safety of folic acid supplementation.

Background

The folate cycle involves a series of essential biosynthetic reactions known as one-carbon metabolism [1]. Folates are a family of molecules composed of three common elements: a central aromatic core derived from para-amino benzoic acid (PABA), a pterin ring that can be modified and a chain of one or more glutamates [2]. At each step of the folate cycle, an enzyme mediates a modification of the pterin ring of the bound folate, allowing the transfer of a chemical group containing one carbon atom (methyl, formyl, etc.) to or from the compound being synthesised [1]. This cofactor role results in

folate molecules being recycled and thus they are only required in very small amounts.

Animals cannot synthesise folates and must acquire them from their diet or gut microbes. When these sources are insufficient, folate deficiency can lead to neural tube defects during human embryonic development [1]. The rate of these defects can be lowered by preconception supplementation with folic acid, an oxidised form of folate not found in nature. Mandatory fortification of flour with folic acid has successfully decreased the incidence of birth defects in many countries, including the US and Canada [3]. However, there are concerns that folic acid supplementation may have adverse effects on health, especially in older people [4–7], and there are many unknowns about the efficacy of uptake and biological utilisation of folic acid [8]. Despite these uncertainties, recent reviews of the evidence by

* Correspondence: david.weinkove@durham.ac.uk

¹Department of Biosciences, Durham University, Stockton Road, Durham DH1 3LE, UK

³Biophysical Sciences Institute, Durham University, South Road, Durham DH1 3LE, UK

Full list of author information is available at the end of the article



experts acting for government public health bodies have concluded that the risks are minimal and have recommended the fortification of flour or other food products as a beneficial intervention [3, 9, 10].

None of the above safety reviews mention potential interactions with gut bacteria. Bacteria also require folates for biosynthesis. Many make their own folates, but folates can also be taken up from the environment. Little is known about how bacterial folate biochemistry affects the host. Inhibiting *Escherichia coli* folate biosynthesis, either by treatment with the drug sulfamethoxazole (SMX) or mutation of the PABA synthesis pathway (e.g., a *pabA* or *pabB* mutant), extends the lifespan of the nematode *Caenorhabditis elegans* that feeds on it [11–13]. While these interventions decrease the folate levels in both *E. coli* and *C. elegans*, there remains sufficient folate available to support normal growth of both organisms [12]. We have suggested that bacterial folate, at higher levels than need for growth, enables a bacterial activity that shortens the life of *C. elegans* and is independent of the folate status of *C. elegans* [13]. Thus, the folate status of gut bacteria may be important for host health.

To understand the interaction between bacterial and animal folates, we have developed a *C. elegans* folate deficiency model, a mutant in *gcp-2.1* and homologue of human GCPII, which is required for uptake of dietary polyglutamated folates [13]. The only way we have found of decreasing *C. elegans* folate enough to slow growth is by raising the *C. elegans gcp-2.1* mutant on SMX-treated *E. coli*. Under these conditions, worms show delayed development and infertility [13]. This phenotype can be prevented with 1–10 μM folinic acid, a reduced folate which can be absorbed directly by *C. elegans*, demonstrating that this defect is due to folate

deficiency. In contrast, prevention with folic acid requires much higher concentrations (100 μM) [13]. We also discovered that, at high concentrations, folic acid can partially reverse the lifespan increase caused by inhibiting *E. coli* folate synthesis [12]. A possible explanation for this result might be the restoration of *E. coli* folate levels, but that would be a surprising conclusion because *E. coli* does not possess a folate transporter [14, 15]. However, it is known that *E. coli* can take up the folic acid breakdown product PABA-glu through the transporter AbgT and catabolise it to PABA [16]. PABA can also diffuse across *E. coli* membranes. Increases in bacterial PABA levels from either source can be used to synthesise folate. In this study, we examine whether these routes might explain the lifespan effects of folic acid.

Here, we use a system in which the external media, microbe and animal can be carefully controlled, to ask whether folic acid is taken up directly by *C. elegans* or uptake of folic acid breakdown products by *E. coli* represents an important alternative route (Fig. 1). We show that the prevention of developmental defects of the *C. elegans* folate deficiency model by adding folic acid depends on the *E. coli* AbgT transporter, demonstrating that bacterial uptake is the major route of supplementation. Consistent with this route, we show folic acid increases *E. coli* folate levels through a pathway that involves uptake of PABA-glu by the AbgT transporter and that PABA-glu is present in folic acid preparations. We also find that this pathway can reverse the lifespan increase caused by inhibiting *E. coli* folate synthesis. In summary, we have uncovered an unappreciated breakdown product in folic acid supplements and a bacterial route of uptake that can increase host folate

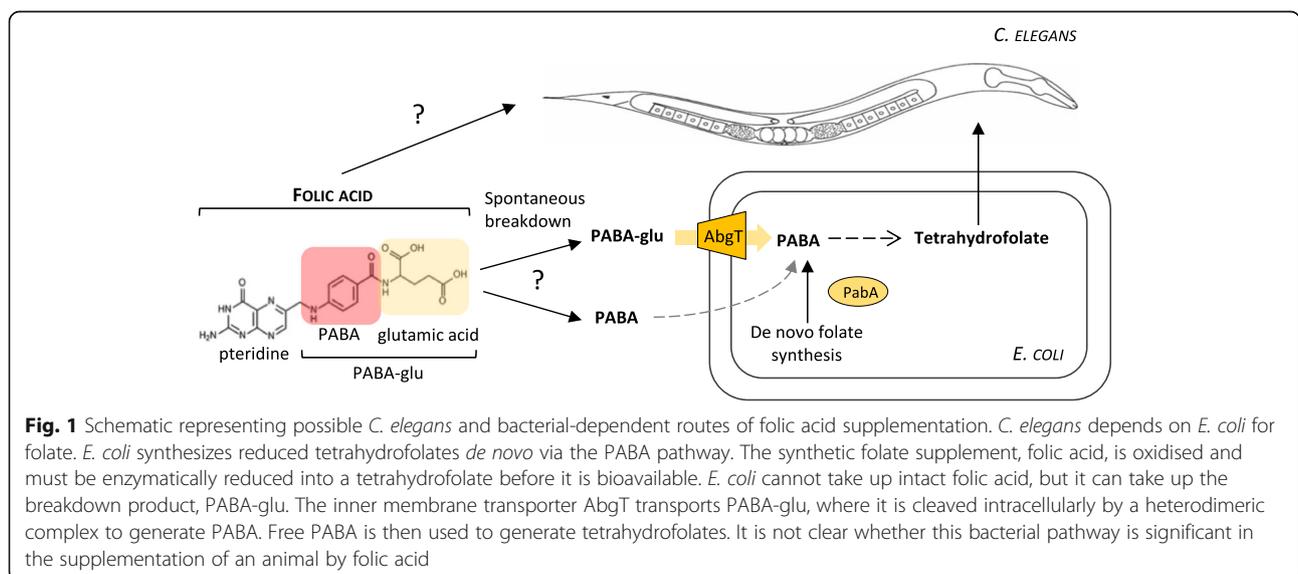


Fig. 1 Schematic representing possible *C. elegans* and bacterial-dependent routes of folic acid supplementation. *C. elegans* depends on *E. coli* for folate. *E. coli* synthesizes reduced tetrahydrofolates *de novo* via the PABA pathway. The synthetic folate supplement, folic acid, is oxidised and must be enzymatically reduced into a tetrahydrofolate before it is bioavailable. *E. coli* cannot take up intact folic acid, but it can take up the breakdown product, PABA-glu. The inner membrane transporter AbgT transports PABA-glu, where it is cleaved intracellularly by a heterodimeric complex to generate PABA. Free PABA is then used to generate tetrahydrofolates. It is not clear whether this bacterial pathway is significant in the supplementation of an animal by folic acid

levels indirectly but might also increase bacterial toxicity, accelerating ageing.

Results

***E. coli* is required for folic acid supplementation to prevent a *C. elegans* folate deficiency**

In order to examine if *E. coli* is required for folic acid to prevent developmental defects in our *C. elegans* folate deficiency model, we tested whether the *E. coli abgT* genotype influenced the outcome of supplementation. Our folate deficiency model [13] is based on the measurable growth defect of the *C. elegans gcp-2.1(ok1004)* mutant during larval development, when it is grown on OP50 *E. coli* treated with 128 µg/mL SMX (Fig. 2a). In this study, we found a similar growth phenotype (Fig. 2a) when we grew this *C. elegans* mutant on an *E. coli ΔpabA* mutant (which cannot make its own folate because it

cannot make the folate precursor PABA). To eliminate exogenous PABA, we grew the bacteria on a defined media (DM) containing only amino acids, salts, cholesterol and trace metals [13]. Mutation of *E. coli abgT* alone did not influence the growth of the *C. elegans gcp-2.1* mutant (Fig. 2a), because *de novo* folate synthesis is unaffected by the *ΔabgT* mutant. To examine the role of AbgT in the absence of endogenous folate synthesis, an *E. coli ΔabgT ΔpabA* double mutant strain was constructed. Developmental growth of *C. elegans gcp-2.1* mutants on the *ΔabgT ΔpabA E. coli* was delayed just as it was on the *ΔpabA* mutant. In response to folic acid supplementation over a 100-fold concentration range, a dose-dependent increase in body length was observed for *gcp-2.1* mutants on the *E. coli ΔpabA* mutant, which was not observed on the *ΔabgT ΔpabA* mutant (Fig. 2bi, asterisks indicate significant difference between *ΔpabA* and *ΔabgT ΔpabA*). In contrast to

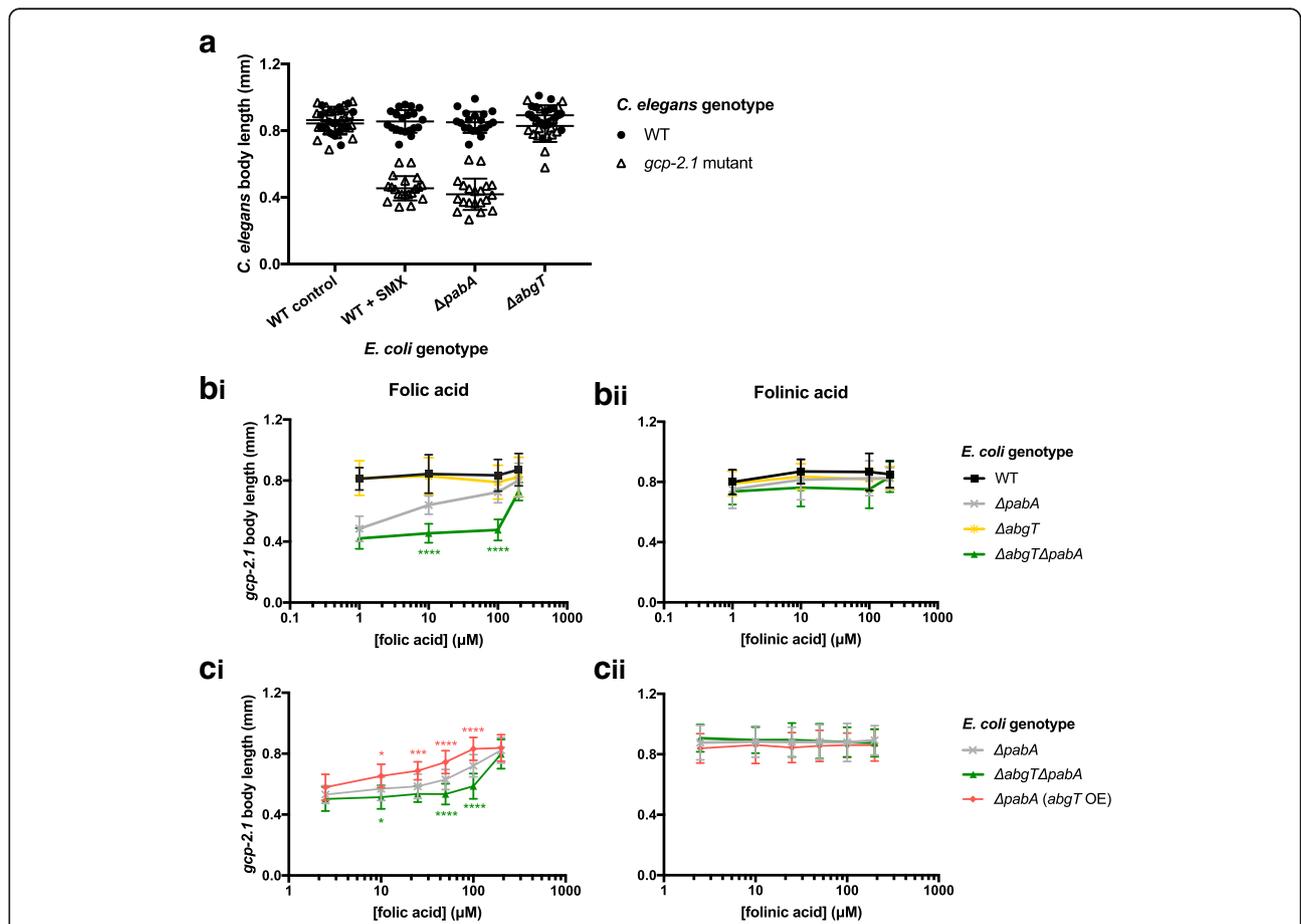


Fig. 2 Folic acid supplementation prevents developmental growth defect of a *C. elegans* folate deficiency model via an *E. coli abgT*-dependent route. Body length of WT and *gcp-2.1* mutant *C. elegans* at L4 developmental stage raised on DM agar plates seeded with (a) WT *E. coli* (control), WT *E. coli* treated with 128 µg/mL SMX, *ΔpabA* mutant, and *ΔabgT* mutant, (b) *ΔpabA* mutant, *ΔabgT ΔpabA* double mutant, *ΔpabA (abgT OE)*, and supplemented with increasing concentrations of folic and (c) folinic acid. Error bars represent standard deviation of *C. elegans* body length, where $n \geq 40$. Asterisks (*) indicate test statistic of unpaired non-parametric Mann-Whitney *t* tests, where **** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$. By two-way ANOVA analyses, we find that there is a significant interaction effect of strain type ($F = 102.67, p < 0.0001$) and folic acid concentration ($F = 123.55, p < 0.0001$) on *C. elegans gcp-2.1* body length. Over-expression of *abgT* is conferred by transformation with a high copy number plasmid, pJ128. *ΔpabA* and *ΔabgT ΔpabA* strains are transformed with the empty vector, pUC19

the 200 μM folic acid required to completely rescue growth of the *gcp-2.1* mutant worms on *E. coli* $\Delta pabA$ and $\Delta abgT \Delta pabA$, only 1 μM folinic acid was required, and supplementation was independent of *E. coli* genotype (Fig. 2bii), consistent with a direct route to the worm for folinic acid.

Overexpression of *abgT* in the *E. coli* $\Delta pabA$ mutant ($\Delta pabA$ (*abgT* OE)) increased *gcp-2.1* mutant body length at lower concentrations of folic acid, with a complete rescue achieved at 100 μM folic acid (Fig. 2ci). Consistent with the previous experiment, folinic acid rescued *gcp-2.1* mutant growth at a 100-fold lower concentration and independently of *abgT* expression (Fig. 2cii). Analysing the experiment by two-way ANOVA, we find that there is a significant interaction effect of *abgT* genotype ($F = 102.67$, $p < 0.0001$) and folic acid concentration ($F = 123.55$, $p < 0.0001$) on *C. elegans gcp-2.1* body length (Fig. 2ci). These results are consistent with folinic acid being taken up directly by the worm [17], and the major route of folic acid uptake requiring *E. coli* and the *E. coli* AbgT transporter.

Folic acid supports growth of *E. coli pabA* mutants via *abgT*-dependent uptake of PABA-glu

As AbgT is known to transport the folate breakdown product PABA-glu, the results above suggest that PABA-glu is available to *E. coli* following folic acid supplementation. To test this assertion, we conducted growth experiments using the *E. coli* $\Delta pabA$ mutant under conditions in which there was insufficient exogenous PABA available to support growth, namely growth on DM agar plates when seeded after several generations of growth in liquid DM. We assessed how the growth of the $\Delta pabA$, $\Delta abgT \Delta pabA$ and $\Delta pabA$ (*abgT* OE) strains could be restored with folic acid, PABA-glu or PABA when added to the media. We found that the growth restored by folic acid or PABA-glu depended on *abgT* expression; 10 μM folic acid rescued the growth of the

$\Delta pabA$ mutant, whereas 100 μM was needed to achieve an equivalent rescue in the $\Delta abgT \Delta pabA$ double mutant (Fig. 3). In the presence of 10 μM folic acid, growth of the $\Delta pabA$ strain over-expressing AbgT was greater than that of the $\Delta pabA$ mutant alone. Supplementation by PABA-glu had a similar effect to folic acid but at a 10-fold lower concentration (Fig. 3), consistent with results of experiments in liquid at 37 $^{\circ}\text{C}$ [16]. PABA, which can diffuse across biological membranes [18], rescued bacterial growth at nanomolar concentrations independently of *abgT* expression (Fig. 3). Overall, restoration of bacterial growth by folic acid can be largely explained by PABA-glu uptake by AbgT, while low concentrations of PABA present in folic acid preparations may explain the ability of high concentrations of folic acid to rescue the growth of the *E. coli* $\Delta abgT \Delta pabA$ double mutant.

Folic acid increases *E. coli* folate levels in an AbgT-dependent mechanism

In order to verify that *E. coli* growth following folic acid supplementation is attributable to restored bacterial folate synthesis, we used LC-MS/MS (see Methods) to detect levels of individual *E. coli* tetrahydrofolates (THFs) under the conditions used in the above experiment. Levels of the most detectable, and thus likely most abundant, THF species (5-methyl THF-glu₃, 5/10-formyl THF-glu₃, THF-glu₃ and 5,10-methenyl THF-glu₃) are presented in Fig. 4. With supplementation of 10 μM folic acid, folate levels in $\Delta pabA$ and $\Delta abgT \Delta pabA$ were not significantly increased compared to their non-supplemented controls. In contrast, in wild-type (WT) and $\Delta pabA$ (*abgT* OE) strains, addition of 10 μM folic acid resulted in significantly higher levels of folates. In response to 100 μM folic acid, folate levels increased in all strains compared to their non-supplemented controls, where the scale of increase was dependent on *abgT* expression (Fig. 4). At 100 μM folic acid, folate levels were highest in $\Delta pabA$ (*abgT* OE) followed by WT, $\Delta pabA$, and finally lowest in the $\Delta abgT \Delta pabA$ double mutant (Fig. 4). The

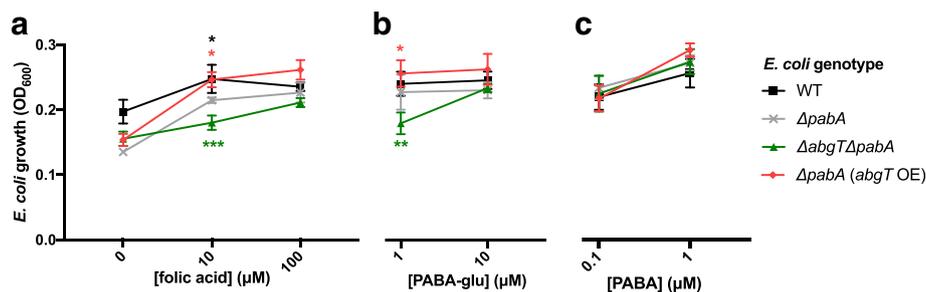


Fig. 3 Folic acid supplement breakdown supports *E. coli* growth. Bacterial lawn growth, under PABA-depleted conditions, of *E. coli* WT, $\Delta pabA$, $\Delta abgT \Delta pabA$ and $\Delta pabA$ (*abgT* OE) on DM plates supplemented with (a) folic acid, (b) PABA-glu and (c) PABA as measured by OD₆₀₀ after 4 days growth at 25 $^{\circ}\text{C}$ (see Methods). Each data point is the average of 8 plates. Error bars represent standard deviation. Asterisks denote the test statistic from Student's *t* test comparison of means, where *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$ compared to $\Delta pabA$ growth on the comparable condition

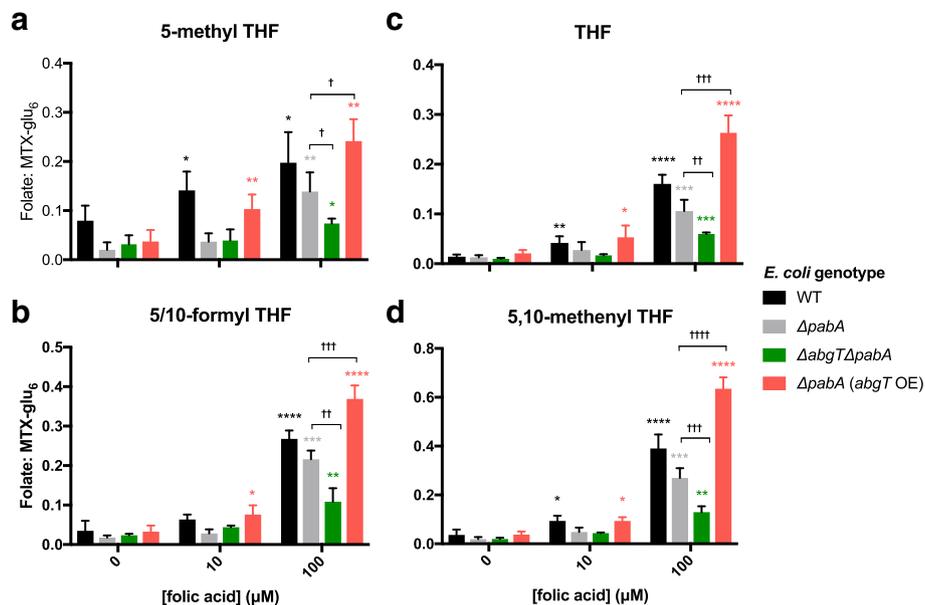


Fig. 4 Folic acid increases *E. coli* folate levels via uptake of PABA-glu by AbgT. Levels of (a) 5-methyl THF-glu₃, (b) 5/10-formyl THF-glu₃, (c) THF-glu₃ and (d) 5,10-methenyl THF-glu₃ in extracts of *E. coli* WT, $\Delta pabA$, $\Delta abgT \Delta pabA$ and $\Delta pabA$ (*abgT* OE) mutants supplemented with 10 μ M and 100 μ M folic acid. Extracts were made after 4 days of bacterial growth at 25 °C on solid agar plates. Folate counts from the LC-MS/MS were normalised by dividing by counts of an internal MTX-glu₆ spike. Error bars represent standard error of the mean of at least four replicate samples per data point (Data in Additional file 3: Table S2). Asterisks indicate test statistic of unpaired parametric *t* tests with Welch's correction, where *****p* < 0.0001, ****p* < 0.001, ***p* < 0.01, **p* < 0.05. Pairwise comparisons between $\Delta pabA$ and $\Delta abgT \Delta pabA$, $\Delta pabA$ (*abgT* OE) at 100 μ M folic acid are indicated by †

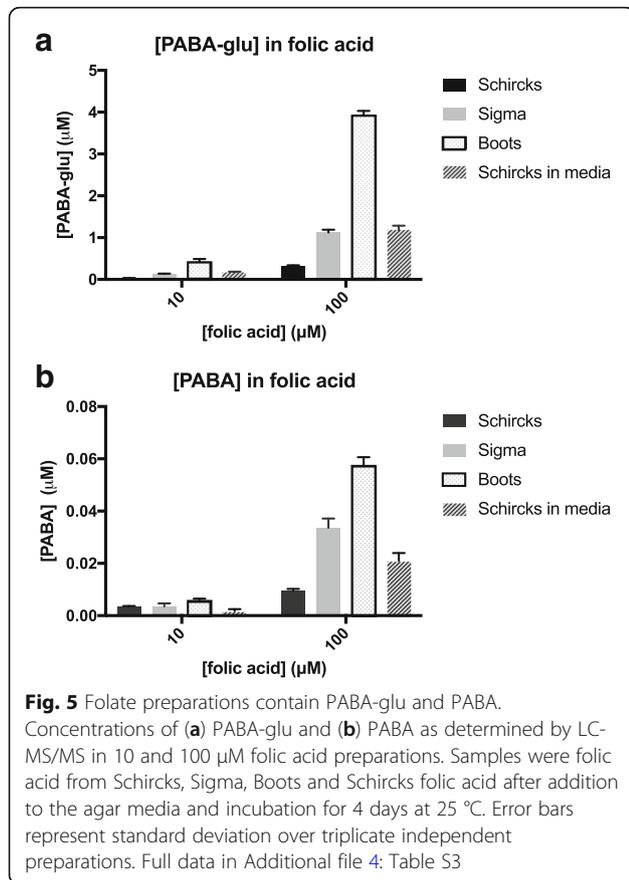
abgT genotype had a strong effect on the folate levels of the $\Delta pabA$ strains at 100 μ M folic acid (Fig. 4, pair-wise comparisons between $\Delta pabA$ and the $\Delta abgT \Delta pabA$ and $\Delta pabA$ (*abgT* OE) strains are indicated by crosses). In summary, folic acid supplementation was found to increase *E. coli* folate levels in an *abgT*-dependent mechanism.

Folic acid preparations contain PABA-glu and PABA

Together, the data presented here indicate that the main route of *C. elegans* folic acid supplementation is indirect via *E. coli* uptake of PABA-glu and PABA. We used LC-MS/MS to test for the presence of these breakdown products in folic acid preparations from Schircks (used in all other experiments in this study), Sigma Aldrich, and Boots, a UK retailer of supplements. We also tested for further folic acid breakdown under the experimental conditions used here by analysing extracts from agar media supplemented with Schircks folic acid and incubated at 25 °C for 4 days. We detected PABA-glu in all three folic acid sources at between 0.3% (Schircks) and 4% (Boots, Fig. 5). Under the conditions used for *C. elegans* experiments, PABA-glu increased to 1.18%, suggesting further breakdown. PABA was found at between 0.01% (Schircks) and 0.06% (Boots, Fig. 5); this level of PABA in folic acid preparations may explain why folic acid can increase folates in a $\Delta abgT \Delta pabA$ double mutant.

Folic acid shortens *C. elegans* lifespan via AbgT-dependent uptake of PABA-glu

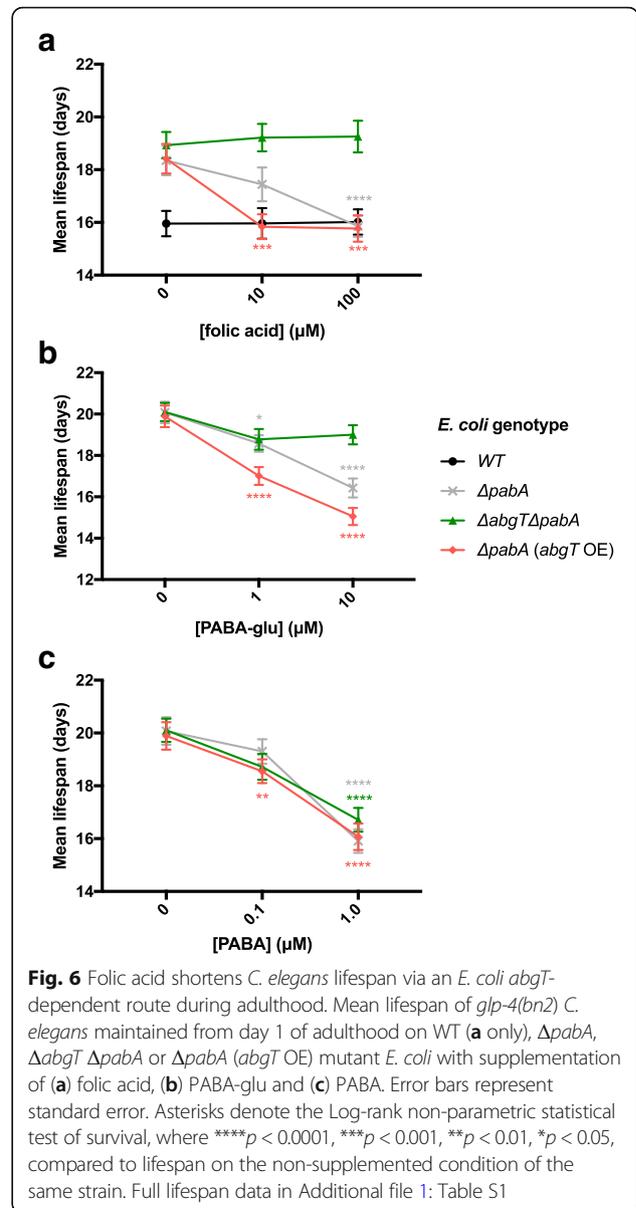
Inhibiting bacterial folate synthesis, without affecting bacterial growth, is known to increase *C. elegans* lifespan [12, 13]. It was therefore hypothesized that, if folic acid increased bacterial folate synthesis, it may shorten *C. elegans* lifespan under conditions where bacterial folate synthesis was inhibited. Consistent with our previous findings [13], we find that *C. elegans* maintained on any *E. coli* $\Delta pabA$ mutant are long-lived compared to *C. elegans* fed WT *E. coli* (Fig. 6, Additional file 1: Table S1), whereas the $\Delta abgT$ mutation alone had no impact on *C. elegans* lifespan (*p* = 0.4312, Additional file 2: Figure S1b). Further, 10 μ M folic acid was found to decrease *C. elegans* lifespan on $\Delta pabA$ *E. coli* by 9.4% (*p* = 0.0052), and to decrease it even further on $\Delta pabA$ *E. coli* over-expressing *abgT* (by 16.3%, *p* < 0.0001, Fig. 6a), whereas it had no effect on lifespan on the $\Delta abgT \Delta pabA$ double mutant (*p* = 0.1901, Fig. 6a). In contrast, 100 μ M folic acid decreased the lifespan on $\Delta pabA$ *E. coli* by 23.9% (*p* < 0.0001), whereas this concentration only shortened the lifespan on the $\Delta abgT \Delta pabA$ double mutant by 4.7% (*p* = 0.0467, Fig. 6a). Lifespans on media supplemented with PABA-glu showed an *abgT*-dependent response similar to that observed with folic acid supplementation, but at a 10-fold lower concentration (Fig. 6b). In contrast,



PABA supplementation shortened *C. elegans* lifespan in all cases independently of *abgT* expression (Fig. 6c), consistent with the ability of PABA to rescue *E. coli* folate production in a $\Delta abgT \Delta pabA$ double mutant. Together, these results suggest that folic acid shortens *C. elegans* lifespan on folate-depleted *E. coli* via AbgT-dependent uptake of PABA-glu.

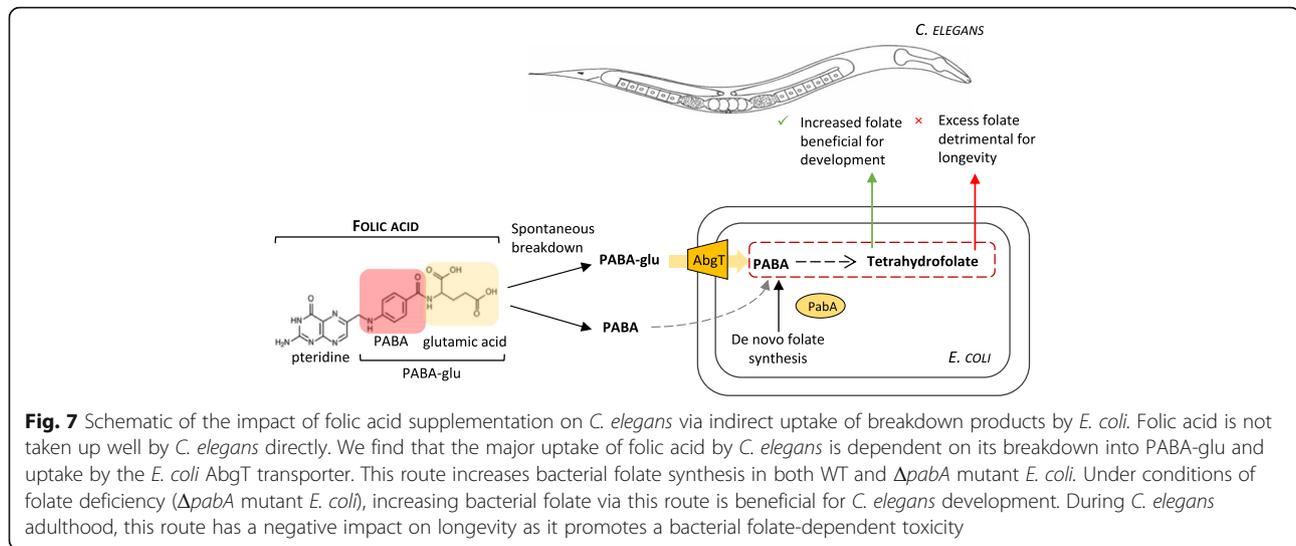
Discussion

In this study we have found that a major route of uptake for the synthetic supplement folic acid by *C. elegans* is via *E. coli* rather than directly via the worm. Why is folic acid not taken up directly by *C. elegans* with the same affinity as folinic acid? One possible explanation is that FOLT-1, the only characterised *C. elegans* folate transporter, is a reduced folate carrier with specificity for folinic acid [17]. In humans, folic acid is also taken up in a different manner to natural reduced folates, with the major route thought to be via the protein-coupled folate transporter in the small intestine [19]. A *C. elegans* PCFT homologue has been identified, but its affinity for folic acid has not been characterized [20]. In addition, a homologue of the human folate receptor has been characterised in *C. elegans* [21]. This study does not rule out



that direct routes for folic acid exist in *C. elegans*, but indicates that they are inferior to the *E. coli* route.

We have found that *E. coli* uptake of folic acid is dependent on its spontaneous breakdown into PABA-glu, which can be taken up by the *E. coli* PABA-glu transporter, AbgT. This transporter, which as far as we know is the only *E. coli* transporter for PABA-glu, has evolved to salvage PABA-glu from the breakdown of natural folates, and is found in many bacteria in the human gut microbiota [22]. To our knowledge, this is the first study to show that this bacterial gene, and its level of expression, can influence the biology of the host, both by providing a route for folic acid to prevent host folate deficiency (Fig. 2), but also because it can lead folic acid to shorten the *C. elegans* lifespan when worms are cultured on folate-depleted *E. coli* (Fig. 6). The



increase in folate synthesis caused by folic acid supplementation leads to a bacterial activity/toxicity that is harmful to the worm over the long term [13] (Fig. 7).

It is possible that this bacterial route for folic acid exists in humans. As far as we are aware, no other studies have tested folic acid supplements for the presence of PABA-glu or PABA, but several studies have reported issues with the stability and dissolution of commercial folic acid supplements [23, 24]. In light of these issues, manufacturers have adopted a policy of ‘overages’ in order to ensure sufficient folic acid is released in the small intestine following ingestion [25]. The presence of PABA-glu and PABA in a commercially available folic acid source (Fig. 6), combined with the instability of folic acid at the low pH conditions of the stomach [26–28], makes it likely that PABA-glu and PABA will be available to the gut microbiota following supplementation. PABA has been identified as a human faecal excretory product after ingestion of folic acid [29]. Furthermore, studies in rodents [30] and piglets [31] have demonstrated that infusion of labelled PABA into the cecum results in the incorporation of bacterially synthesized folate in host tissues. Thus, the literature suggests that the components for such a route exist in humans, but the importance of this route is yet to be determined. We detected this route in *C. elegans* because of the poor bioavailability of folic acid in our folate deficiency model. Folic acid is taken up well by humans and leads to increases in serum folate levels, but circulating folic acid is often found to have retained its oxidised form, suggesting that it is not always bioavailable even when absorbed by the host [3, 8, 32]. The bacterial route increases bioavailability.

Further studies are required in order to determine whether folic acid supplementation affects the folate status

of human gut microbes and whether this in turn impacts host health. Interestingly, there are several diseases associated with an increased abundance of folate-synthesizing gut bacteria, such as inflammatory bowel disease [33] and small intestinal bacterial overgrowth [34], but a causal relationship between bacterial folate and disease has not been established. The *abgT* gene is found in the genomes of several enteric pathogens, including *Enterobacter cloacae*, *Neisseria gonorrhoeae*, *Salmonella enterica*, *Shigella boydii* and *Staphylococcus aureus*, in addition to *E. coli*. Whilst there is much consideration about the consequences of folic acid supplementation [3, 9, 10], our work here indicates that folic acid supplement instability and bacterial metabolism are previously unexplored variables that may impact human health and thus warrant consideration in future reports and studies.

Conclusions

The main route of uptake of the synthetic supplement folic acid by *C. elegans* is via *E. coli*, with a dependence on breakdown of folic acid. Folic acid supplements contain breakdown products, raising the possibility that this route could occur in humans. This route shortens the lifespan of *C. elegans* on folate-depleted *E. coli*, suggesting that there are circumstances in which this bacterial route could have negative consequences for human health.

Methods

Folates and related compounds

Folic acid, folinic acid, PABA-Glu, 5-formylTHF-Glu₃, 5-methylTHF-Glu₃ and methotrexate-Glu₆ were obtained from Schircks, Switzerland. PABA, vitamin B12 and folic acid were purchased from Sigma Aldrich and folic acid supplement from Boots, UK.

Culture conditions

DM was prepared as previously described [13], except that 10 nM vitamin B12 was added. Vitamin B12, folic acid and antibiotics were added post-autoclaving for agar plates. DM for liquid culture was filter sterilised. Following addition of 0.1 μ M PABA to the liquid DM media, this was used to seed the plates in order to maintain bacterial growth (apart from growth experiments in Fig. 2). Then, 30 μ L of 3 mL fresh overnight LB culture were used to inoculate 5 mL of DM (in 15 mL Falcon tubes). Kanamycin (25 μ g/mL and 50 μ g/mL of ampicillin if necessary) were added to both LB and DM pre-incubation. DM liquid cultures were incubated for 18 h at 37 °C and 220 RPM.

All strains were derived from the Keio collection [35] (Table 1). The $\Delta abgT \Delta pabA$ double mutant was made using the P1 transduction protocol as described in [36]. The *abgT* over-expression plasmid (pJ128) [16] and empty vector (puc19) [37] were transformed into appropriate strains.

C. elegans strains used

SS104 *glp-4(bn2)*, UF208 (WT) and UF209 *gc p-2.1(ok1004)* [13].

E. coli preparation and growth assay

E. coli was prepared as follows for all *E. coli* and *C. elegans* experiments. A 30 μ L aliquot of an overnight LB culture of *E. coli* was transferred into 5 mL of DM and incubated for 18 h at 37 °C and 220 RPM. Then, 100 μ L of the DM culture was seeded onto DM agar plates and incubated at 25 °C for 4 days. *E. coli* was removed by pipetting 1 mL of M9 medium onto the plate and a glass spreader was used to scrape off the bacterial lawn. The bacterial suspension was pipetted into a 1.5 mL Eppendorf and the volume was recorded (v). Tubes were vortexed vigorously to obtain a homogenised solution. Finally, 100 μ L were taken and diluted with 900 μ L of M9

in a cuvette. A spectrophotometer was used to read bacterial growth at 600 nm. Bacterial growth was calculated by multiplying OD₆₀₀ by the volume of the sample (v).

E. coli folate extraction

Bacterial lawns were scraped from plates into microcentrifuge tubes using M9 solution and kept on ice. Volume (v) multiplied by the OD₆₀₀ of the solution (diluted 1:5) gives a measure of the amount of material. Samples were concentrated in a cooled microcentrifuge and pellets were snap frozen in liquid nitrogen. Pellets were thawed and resuspended in a volume of ice-cold 90% methanol: 10% folate extraction buffer (FEB: 50 mM HEPES, 50 mM CHES, 0.5% w/v ascorbic acid, 0.2 M DTT, pH 7.85 with NaOH) in proportion to bacterial content (37.5 \times OD₆₀₀ \times v). FEB was spiked with 10 nM methotrexate-Glu₆ as an internal standard. Samples were vortexed vigorously and left on ice for 15 min before centrifugation in a cooled microcentrifuge for 15 min at full speed. Supernatants were used for analysis.

Folate LC-MS/MS analysis

Folates were detected by multiple reaction monitoring (MRM) analysis using a SCIEX QTRAP 6500 instrument. MRM conditions for folic acid, PABA, PABA-Glu, 5-Me-H₄PteGlu₃ (5-methylTHF-Glu₃) and 5/10-CHO-H₄PteGlu₃ (formyl THF₃) were optimised by infusion of standards into the instrument. The optimised conditions for -Glu₃ folates were applied to other higher folates using MRM transitions as described by Lu et al. [38]. Further confirmation of identity for folates of interest was achieved by performing enhanced product ion scans and comparing the fragment spectra with known standards.

The QTRAP 6500 was operated in ESI+ mode and was interfaced with a Shimadzu Nexera UHPLC system. Samples were separated using a Thermo PA2 C18 column (2.2 μ m, 2.1 \times 100 mm) with a gradient of 0.1%

Table 1 *E. coli* strains used in this study

Strain	Genotype	Plasmid	Characteristics	Source
BW25113/pGreen 0029	WT	pGreen 0029	kan ^r	Virk et al. 2016 [13]
JW3323-1	$\Delta pabA$	n/a	kan ^r	Baba et al. 2006 [25]
JW5822-1	$\Delta abgT$	n/a	kan ^r	Baba et al. 2006 [25]
CMabgTpabA	$\Delta abgT \Delta pabA$	n/a	kan ^r	This study
CM1	WT	pUC19, pGreen 0029	kan ^r , amp ^r	This study
CM2	$\Delta pabA$	pUC19	kan ^r , amp ^r	This study
CM3	$\Delta abgT \Delta pabA$	pUC19	kan ^r , amp ^r	This study
CM4	WT (<i>abgT</i> OE)	pJ128, pGreen 0029	kan ^r , amp ^r	This study
CM5	$\Delta pabA$ (<i>abgT</i> OE)	pJ128	kan ^r , amp ^r	This study
CM6	$\Delta abgT \Delta pabA$ (<i>abgT</i> OE)	pJ128	kan ^r , amp ^r	This study

r= resistance

formic acid in water (mobile phase A) and acetonitrile (mobile phase B). Samples were maintained at 4 °C and 2 µL aliquots were injected. The column was maintained at 40 °C with a flow rate of 200 µL/min, starting at 2% B, held for 2 min, with a linear gradient to 100% B at 7 min, held for 1 min, before a 7 min re-equilibration step at 2% B necessary for consistent retention times. The column eluate flow to the MS was controlled via the QTRAP switching valve, allowing analysis between 4 and 8 min to minimise instrument contamination. Foliates were quantified with reference to external standards and matrix effects were assessed by spiking of standards into extracted samples.

Lifespan analysis

Survival analyses were performed as described [12]. *glp-4(bn2)* worms were maintained at 15 °C and shifted to 25 °C at the L3 stage. At the L4/young adult stage, animals were placed on bacteria under the experimental conditions. All lifespan data is provided in Additional file 1: Table S1. Statistical significance was determined using Log Rank and Wilcoxon tests of the Kaplan–Meier survival model.

Additional files

Additional file 1: Table S1. Full analysis of lifespan conditions including statistical analysis. (XLSX 46 kb)

Additional file 2: Figure S1. The *E. coli* $\Delta abgT$ deletion has no effect on *C. elegans* lifespan survival curves of *C. elegans glp-4(bn2)* on WT *E. coli* and $\Delta abgT$ mutant. See Additional file 1: Table S1 for further details. (PDF 72 kb)

Additional file 3: Table S2. Data corresponding to Fig. 4 in the main text. (XLSX 50 kb)

Additional file 4: Table S3. Data corresponding to Fig. 5 in the main text. (XLSX 10 kb)

Acknowledgements

We thank the *C. elegans* Genetics Center, the *C. elegans* Knockout Consortium, and NBRP-*E. coli* at NIG for strains and we thank Sushmita Maitra and John Mathers for useful comments on the manuscript.

Funding

This work was supported by the Biotechnology and Biological Sciences Research Council (BBSRC) (grant number BB/J014516/1).

Availability of data and materials

Data corresponding to all lifespan analyses can be found in Additional file 1: Table S1. Data corresponding to LC-MS/MS measurements of folate levels (Fig. 4) and folic acid breakdown (Fig. 5) can be found in Additional file 3: Table S2 and Additional file 4: Table S3, respectively.

Authors' contributions

Conceptualization: CM, DW. Methodology: CM, DW, IC. Investigation: CM, IC. Data curation: CM, IC. Writing: CM, DW, IC, JG. Supervision: DW. Funding acquisition: DW. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Publisher's Note

Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Author details

¹Department of Biosciences, Durham University, Stockton Road, Durham DH1 3LE, UK. ²Midwestern University, Illinois, Downers Grove, IL 60515, USA.

³Biophysical Sciences Institute, Durham University, South Road, Durham DH1 3LE, UK.

Received: 22 April 2018 Accepted: 16 May 2018

Published online: 15 June 2018

References

- Ducker GS, Rabinowitz JD. One-carbon metabolism in health and disease. *Cell Metab.* 2017;25(1):27–42.
- Green J, Matthews R. 2007. Folate Biosynthesis, Reduction, and Polyglutamylation and the Interconversion of Folate Derivatives, *EcoSal Plus*. 2007; <https://doi.org/10.1128/ecosalplus.3.6.3.6>.
- Bailey LB, Stover PJ, McNulty H, Fenech MF, Gregory JF, Mills JL, et al. Biomarkers of nutrition for development—folate review. *J Nutr.* 2015;145(7):1636S–80S.
- Kim Y-I. Folate and colorectal cancer: An evidence-based critical review. *Mol Nutr Food Res.* 2007;51(3):267–92.
- Marean A, Graf A, Zhang Y, Niswander L. Folic acid supplementation can adversely affect murine neural tube closure and embryonic survival. *Hum Mol Genet.* 2011;20(18):3678–83.
- Pickell L, Brown K, Li D, Wang XL, Deng L, Wu Q, et al. High intake of folic acid disrupts embryonic development in mice. *Birth Defects Res A Clin Mol Teratol.* 2011;91(1):8–19.
- Smith AD, Kim Y-I, Refsum H. Is folic acid good for everyone? *Am J Clin Nutr.* 2008;87(3):517–33.
- Gregory JF, Quinlivan EP, Davis SR. Integrating the issues of folate bioavailability, intake and metabolism in the era of fortification. *Trends Food Sci Technol.* 2005;16(6):229–40.
- Boyles AL, Yetley EA, Thayer KA, Coates PM. Safe use of high intakes of folic acid: research challenges and paths forward. *Nutr Rev.* 2016;74(7):469–74.
- Public Health England. Folic acid: updated SACN recommendations. 2017. Retrieved 25 May 2018. https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/637111/SACN_Update_on_folic_acid.pdf.
- Han B, Sivaramakrishnan P, Lin CJ, Neve IAA, He J, Tay LWR, et al. Microbial genetic composition tunes host longevity. *Cell.* 2017;169(7):1249–62. e13
- Virk B, Correia G, Dixon DP, Feyst I, Jia J, Oberleitner N, et al. Excessive folate synthesis limits lifespan in the *C. elegans*: *E. coli* aging model. *BMC Biol.* 2012;10:67.
- Virk B, Jia J, Maynard CA, Raimundo A, Lefebvre J, Richards SA, et al. Folate acts in *E. coli* to accelerate *C. elegans* aging independently of bacterial biosynthesis. *Cell Rep.* 2016;14(7):1611–20.
- Nickerson WJ, Webb M. Effect of folic acid analogues on growth and cell division of nonexacting microorganisms. *J Bacteriol.* 1956;71(2):129–39.
- Webb M. Inactivation of analogues of folic acid by certain non-exacting bacteria. *Biochim Biophys Acta.* 1955;17(2):212–25.
- Carter EL, Jager L, Gardner L, Hall CC, Willis S, Green JM. *Escherichia coli* abg genes enable uptake and cleavage of the folate catabolite p-aminobenzoyl-glutamate. *J Bacteriol.* 2007;189(9):3329–34.
- Balamurugan K, Ashokkumar B, Moussaif M, Sze JY, Said HM. Cloning and functional characterization of a folate transporter from the nematode *Caenorhabditis elegans*. *Am J Physiol Cell Physiol.* 2007;293(2):C670–81.
- Tran PV, Nichols BP. Expression of *Escherichia coli* *pabA*. *J Bacteriol.* 1991;173(12):3680–7.
- Qiu A, Jansen M, Sakaris A, Min SH, Chattopadhyay S, Tsai E, et al. Identification of an intestinal folate transporter and the molecular basis for hereditary folate malabsorption. *Cell.* 2006;127(5):917–28.
- Ortbauer M, Ripper D, Fuhrmann T, Lassi M, Auernigg-Haselmaier S, Stiegler C, et al. Folate deficiency and over-supplementation causes impaired folate metabolism: Regulation and adaptation mechanisms in *Caenorhabditis elegans*. *Mol Nutr Food Res.* 2016;60(4):949–56.
- Chaudhari SN, Mukherjee M, Vagasi AS, Bi G, Rahman MM, Nguyen CQ, et al. Bacterial folates provide an exogenous signal for *C. elegans* germline stem cell proliferation. *Dev Cell.* 2016;38(1):33–46.

22. Delmar JA, Yu EW. The AbgT family: a novel class of antimetabolite transporters. *Protein Sci.* 2016;25(2):322–37.
23. Hoag SW, Ramachandruni H, Shangraw RF. Failure of prescription prenatal vitamin products to meet USP standards for folic acid dissolution. *J Am Pharm Assoc (Wash).* 1997;NS37(4):397–400.
24. Sculthorpe NF, Davies B, Ashton T, Allison S, McGuire DN, Malhi JS. Commercially available folic acid supplements and their compliance with the British Pharmacopoeia test for dissolution. *J Public Health Med.* 2001; 23(3):195–7.
25. Andrews KW, Roseland JM, Gusev PA, Palachuvattil J, Dang PT, Savarala S, et al. Analytical ingredient content and variability of adult multivitamin/mineral products: national estimates for the Dietary Supplement Ingredient Database. *Am J Clin Nutr.* 2017;105(2):526–39.
26. De Brouwer V, Zhang GF, Storozhenko S, Straeten DV, Lambert WE. pH stability of individual folates during critical sample preparation steps in prevision of the analysis of plant folates. *Phytochem Anal.* 2007;18(6):496–508.
27. Gazzali AM, Lobry M, Colombbeau L, Acherar S, Azais H, Mordon S, et al. Stability of folic acid under several parameters. *Eur J Pharm Sci.* 2016;93: 419–30.
28. Seyoum E, Selhub J. Properties of food folates determined by stability and susceptibility to intestinal pteroylpolyglutamate hydrolase action. *J Nutr.* 1998;128(11):1956–60.
29. Denko CW, Grundy WE, Wheeler NC, Henderson CR, Berryman, GH, Friedemann, TE, Youmans, JB. The excretion of B-complex vitamins by normal adults on a restricted intake. *Arch Biochem.* 1946;11:109–17.
30. Rong N, Selhub J, Goldin BR, Rosenberg IH. Bacterially synthesized folate in rat large intestine is incorporated into host tissue folyl polyglutamates. *J Nutr.* 1991;121(12):1955–9.
31. Asrar FM, O'Connor DL. Bacterially synthesized folate and supplemental folic acid are absorbed across the large intestine of piglets. *J Nutr Biochem.* 2005;16(10):587–93.
32. Patanwala I, King MJ, Barrett DA, Rose J, Jackson R, Hudson M, et al. Folic acid handling by the human gut: implications for food fortification and supplementation. *Am J Clin Nutr.* 2014;100(2):593–9.
33. Shin NR, Whon TW, Bae JW. Proteobacteria: microbial signature of dysbiosis in gut microbiota. *Trends Biotechnol.* 2015;33(9):496–503.
34. Camilo E, Zimmerman J, Mason JB, Golner B, Russell R, Selhub J, et al. Folate synthesized by bacteria in the human upper small intestine is assimilated by the host. *Gastroenterology.* 1996;110(4):991–8.
35. Baba T, Ara T, Hasegawa M, Takai Y, Okumura Y, Baba M, et al. Construction of *Escherichia coli* K-12 in-frame, single-gene knockout mutants: the Keio collection. *Mol Syst Biol.* 2006;2:2006.0008.
36. Moore SD. Assembling New *Escherichia coli* Strains by Transduction Using Phage P1. In: Williams JA, editor. *Strain Engineering. Methods in Molecular Biology.* New York (USA): Humana Press; 2011. p. 155–69.
37. Yanisch-Perron C, Vieira J, Messing J. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene.* 1985;33(1):103–19.
38. Lu W, Kwon YK, Rabinowitz JD. Isotope ratio-based profiling of microbial folates. *J Am Soc Mass Spectrom.* 2007;18(5):898–909.

Ready to submit your research? Choose BMC and benefit from:

- fast, convenient online submission
- thorough peer review by experienced researchers in your field
- rapid publication on acceptance
- support for research data, including large and complex data types
- gold Open Access which fosters wider collaboration and increased citations
- maximum visibility for your research: over 100M website views per year

At BMC, research is always in progress.

Learn more biomedcentral.com/submissions

