Folic acid instability and bacterial metabolism combine to impact *C. elegans* development and ageing

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ABSTRACT

Folic acid supplementation is used to prevent folate deficiency, but it is also associated with negative effects on human health. Little is known about how gut bacteria interact with the uptake of synthetic supplements, such as folic acid, and the consequences for host health. Using the simplified *C. elegans- E. coli* host-microbe model system, we examine how folic acid impacts *E. coli* folate synthesis, and in turn, *C. elegans* health. We find that folic acid supplements contain a breakdown product that is taken up by the *E. coli* transporter AbgT, leading to increased bacterial folate levels. We show that this is the main route by which folic acid rescues a *C. elegans* developmental folate deficiency, but is also the route by which folic acid shortens adult lifespan. Together, this work shows how folic acid instability and bacterial uptake can combine to influence host health.

KEY WORDS

Folic acid; Microbiota; Ageing; *C. elegans*; bacterial metabolism

INTRODUCTION

Folic acid supplementation has been a major public health success, particularly in the prevention neural tube defects (Imbard et al., 2013), but there are numerous health concerns related to over-supplementation (Butterworth and Tamura, 1989; Cole et al., 2007; Kim, 2007; Marean et al., 2011; Milne et al., 1984; Pickell et al., 2011; Portal-Celhay and Blaser, 2012; Selhub et al., 2009). Folic acid is a synthetic oxidized folate. It can be absorbed in the gut in this form (Patanwala et al., 2014) but unlike bacterial and dietary folates, it must be converted into a reduced tetrahydrofolate (THF), before it can be taken up in peripheral cells by reduced folate carriers and used in metabolism (Ducker and Rabinowitz, 2017; Visentin et al., 2014). The routes of folic acid uptake and metabolism are not completely characterized and its interaction with gut microbes has not been well studied (Visentin et al., 2014).

Some bacteria can take up folates directly but many cannot. Instead they either make folate *de novo* or take up folate precursors such as para-amino benzoic acid (PABA, Figure 1, (Carter et al., 2007; LeBlanc et al., 2013; Magnusdottir et al., 2015). Folic acid and THFs are inherently unstable molecules, comprising of a central PABA moiety linked by a methylene bridge to a pteridine ring to form pteroic acid and by its carboxyl group to a L-glutamic acid residue by a peptide bond (Green and Matthews, 2007). The methylene bridge is prone to disassociation under several parameters, including low pH, to generate pteridine and PABA-glutamic acid (PABA-glu) (De Brouwer et al., 2007; Gazzali et al., 2016; Gregory, 1989; Hanson and Gregory, 2011; Maruyama et al., 1978), where PABA-glu can further dissociate to generate PABA (Der-Petrossian et al., 2007; Thiaville et al., 2016). Thus, the acidic microenvironments of the stomach and upper small intestine may cause folates to degrade to PABA-glu and PABA (Seyoum and Selhub, 1998). Indeed, PABA has been detected as a significant faecal excretory product following folic acid supplementation (Denko Cw Fau - Grundy et al.). Mammalian studies have shown that injection of labelled PABA into the cecum of rats (Rong et al., 1991) and piglets (Asrar and O'Connor, 2005) results in the incorporation of bacterially synthesized folate into host tissues. Human studies have shown that bacterial folate synthesis in both the human small (Camilo et al, 1996) and large intestine (Kim et al., 2004) can be incorporated into host tissues. It is not known, however, if this bacterial folate synthesis is enhanced by folic acid supplementation and if it is, whether there are any consequences for microbiome function and host health.

This study uses a simplified host-microbe model system to examine how folic acid affects bacterial folate synthesis and host health. The model organism, the nematode *Caenorhabditis elegans* is maintained on lab strains of *E. coli* for which it depends on for folate as well as for other vitamins (Yilmaz and Walhout, 2014).

Like several other Proteobacteria, *E. coli* is capable of *de novo* folate synthesis and the salvage of PABA and PABA-glu to make folate (Carter et al., 2007), but cannot take up intact folates (Nickerson and Webb, 1956; Webb, 1955)Figure 1). Here, we find that the major route of folic acid supplementation is an indirect route that this is dependent on the breakdown of folic acid into PABA-glu and subsequent uptake via the *E. coli* transporter, AbgT. The resultant increase in bacterial folate synthesis is beneficial for *C. elegans* during development, but has a detrimental impact on *C. elegans* ageing. This is consistent with our previous work, which has demonstrated that *E. coli* folate synthesis shortens *C. elegans* lifespan (Virk et al., 2012) most likely due to a bacterial-dependent chronic toxicity (Virk et al., 2016). Importantly, we also detect PABA-glu and PABA in three folic acid sources, with the highest quantities in a commercial folic acid supplement. Together, this work highlights an unappreciated bacterial-dependent route via which folic acid can have both positive and negative effects on host health, and thus has wider reaching implications for the importance of the microbiota in determining health outcomes following vitamin supplementation.

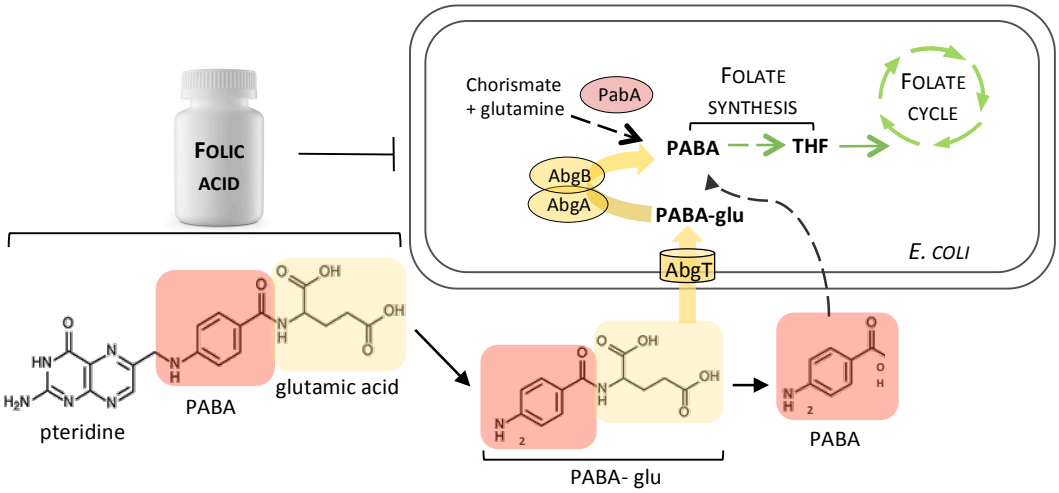

Figure 1. Schematic of the indirect uptake of folic acid by *E. coli.* Folic acid is composed of a central PABA moiety linked by a methylene bridge to a pteridine and to a single glutamic acid residue via a peptide bond. Folic acid is unstable and disassociates to generate PABA-glutamate (PABA-glu), which can further disassociate to PABA. *E. coli* is able to salvage PABA-glu via transport by the inner membrane protein, AbgT. PABA-glu is then cleaved intracellularly by the heterodimeric carboxypeptidase AbgA/B into PABA, where it can be used as a precursor for folate synthesis to generate the active form of folate, tetrahydrofolate (THF) which is used in the folate cycle. PABA can also diffuse across biological membranes. *E. coli* is also capable of *de novo* folate synthesis by generating PABA from chorismate and glutamine through the action of PabA, PabB and PabC. *E. coli* is unable to import folic acid directly.

RESULTS

Folic acid supplementation rescues *C. elegans* developmental folate deficiency via *E. coli abgT*

In order to test whether folic acid interacts with bacterial folate synthesis to supplement host folate in our model system, we used the previously characterized *C. elegans* folate uptake deletion mutant, *gcp-2.1 (ok1004)* (Virk et al., 2016). The *gcp-2.1* mutant strain lacks GCP-2.1, the *C. elegans* homologue of the GCPII glutamate carboxypeptidase enzyme that removes glutamates from polyglutamated folates, a prerequisite for folate absorption (Halsted et al., 1998). This strain exhibits a severe developmental folate-deficiency when maintained on *E. coli* treated with the sulfonamide drug sulfamethoxazole (SMX) (Virk et al., 2016). This growth defect is rescued with high concentrations of folic acid (Virk et al., 2016). It is not clear, however, whether this supplementation is direct (*C. elegans* uptake) or indirect (restoration of folate synthesis in *E. coli*). Here we show that the *C. elegans gcp-2.1* mutant grown on the *E. coli pabA* mutant on defined media (DM) has the same growth defect as these worms grown on wild type *E. coli* treated with 128 µg/ml SMX (Figure 2a). Folic acid was found to increase *gcp-2.1* mutant body length on the *pabA* mutant in a dose-dependent manner (Figure 2b). As *E. coli* cannot uptake intact folic acid, we examined the dependence of folic acid rescue on the expression of the *E. coli abgT* gene, which is responsible for the uptake of PABA-glu. A weaker response of the *gcp-2.1* mutant to folic acid was observed on the *abgTpabA* double mutant compared to worms on the *pabA* single mutant. In contrast, *gcp-2.1* mutants maintained on *pabA* with a plasmid overexpressing *abgT* (Carter et al., 2007) (*pabA (abgT OE))* were more responsive to folic acid (Figure 2b). Analysing the experiment by two-way ANOVA, 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. Mutation of *abgT* alone did not influence growth of the *gcp-2.1* (Supplementary Figure 1a). Rescue of *gcp-2.1* developmental folate deficiency was achieved at a 10-fold lower concentration and independently of *abgT* expression, with the relatively stable THF, folinic acid (Supplementary Figure 2), suggesting that *C. elegans* takes up folinic acid directly. These results suggest that the major route of folic acid uptake by the worm is through *E. coli* via uptake of the folic acid degradation product, PABA-glu by the AbgT transporter.

Folic acid supports *E. coli* growth via AbgT-dependent uptake of PABA-glu

The *abgT*-dependence of folic acid to rescue the *gcp-2.1* developmental folate-deficiency strongly suggests that PABA-glu is available to *E. coli* following folic acid supplementation. To test the relative ability of *E. coli* to take up PABA-glu and folic acid, we added these compounds to *pabA, abgTpabA* and *pabA (abgT OE) E. coli* and assessed growth on DM agar plates after 4 days incubation at 25°C. Under these conditions, we found that all strains containing the *pab*A mutant showed slower growth than wild type and *abgT* expression determined the response to folic acid and PABA-glu; 10 µM folic acid rescued growth of the

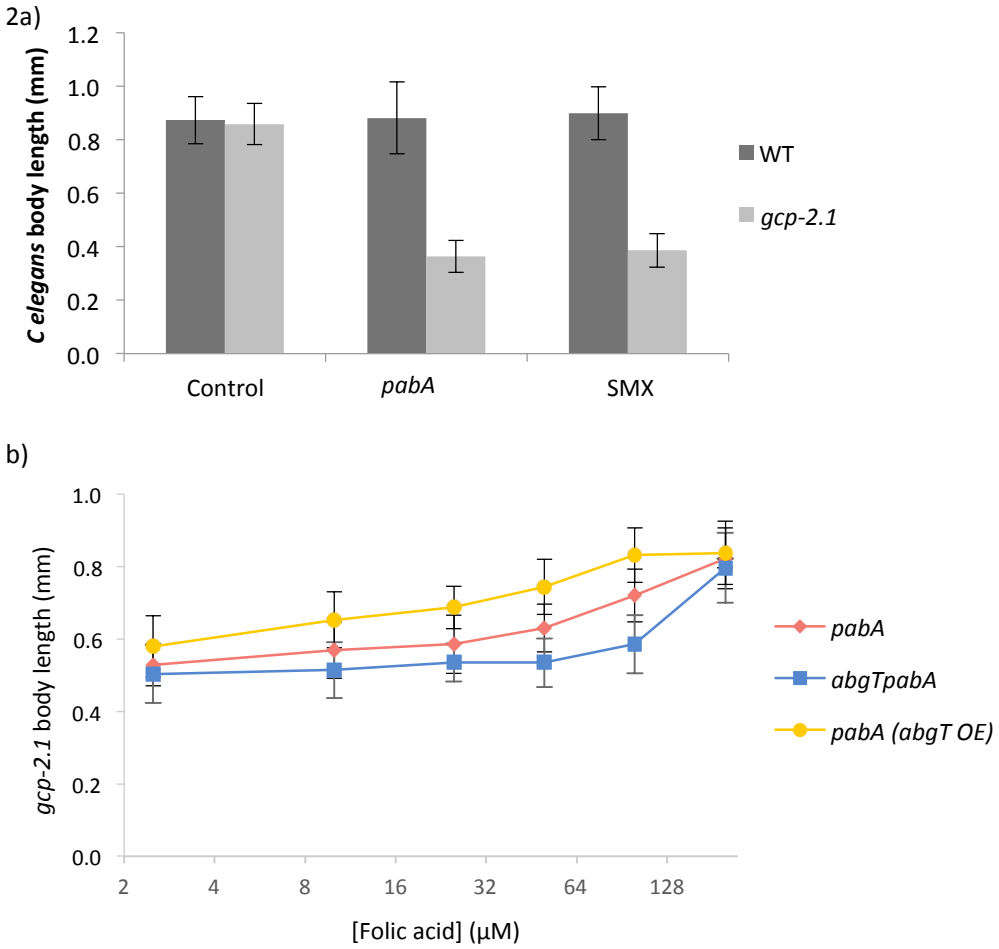

Figure 2. Folic acid supplements *C. elegans* via an *E. coli abgT*-dependent route during development a) body length of wild-type and *gcp-2.1* mutant *C. elegans* at L4 stage raised on DM agar plates seeded with WT *E. coli* (control), *pabA* mutant or WT *E. coli* treated with 128 µg/ml SMX b) body length of *gcp-2.1* mutant *C. elegans* at L4 stage raised on DM agar plates seeded with *pabA* mutant, *abgTpabA* double mutant or *pabA* mutant over-expressing *abgT* with increasing concentrations of folic acid. 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 is conferred by transformation with a high copy number plasmid, pJ128. *pabA* and *abgTpabA* strain are transformed with the empty vector, pUC19. Error bars represent standard deviation of *C. elegans* body length; n ≥40.

pabA mutant, whereas 100 µM was needed to achieve an equivalent rescue in the *abgT pabA* double mutant (Figure 3ai). 10 µM folic acid increased the growth of the *pabA* strain over-expressing AbgT above that of the *pabA* mutant alone or the wild type strain. Supplementation by PABA-glu had a similar effect but at a 10-fold lower concentration than folic acid (Figure 3aii). PABA, which can freely diffuse across biological membranes (Tran and Nichols, 1991), rescued bacterial growth was independent of *abgT* expression and achieved at nanomolar concentrations (Figure 3aiii). Overall, rescue of bacterial growth by folic acid at low concentrations can be explained by PABA-glu uptake by AbgT while low concentrations of PABA present in folic acid preparations may explain the ability of 100 µM folic acid to rescue *E. coli* growth and *C. elegans gcp-2.1* developmental folate-deficiency independently of *abgT* (Figure 2).

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

It order to verify that *E. coli* growth following folic acid supplementation is attributable to restored bacterial folate synthesis, we used LC-MS/MS to detect *E. coli* folate levels under the conditions used in the above experiment. Levels of the most detectable and thus likely the most abundant THF species, 5-methyl THF-glu_3, are presented in Figure 3b. In the absence of folic acid, folate levels in the *pabA* mutant strains were significantly lower than in WT extracts (Figure 3b, Supplementary Figure 3). Addition of folic acid increased folate levels, where the scale of increase was dependent on *abgT* expression; with 100 µM folic acid, folate species were highest in *pabA* (*abgT* OE) followed by WT, *pabA*, and finally lowest in the *abgT pabA* double mutant. Other THF species followed the same trend as 5-methyl THF-glu_3 and are presented in Supplementary Figure 3. In summary, folic acid supplementation increases *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 expected 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. We also tested the impact of the experimental conditions used here on folic acid breakdown 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) (Figure 3ci). 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) (Figure 3cii).

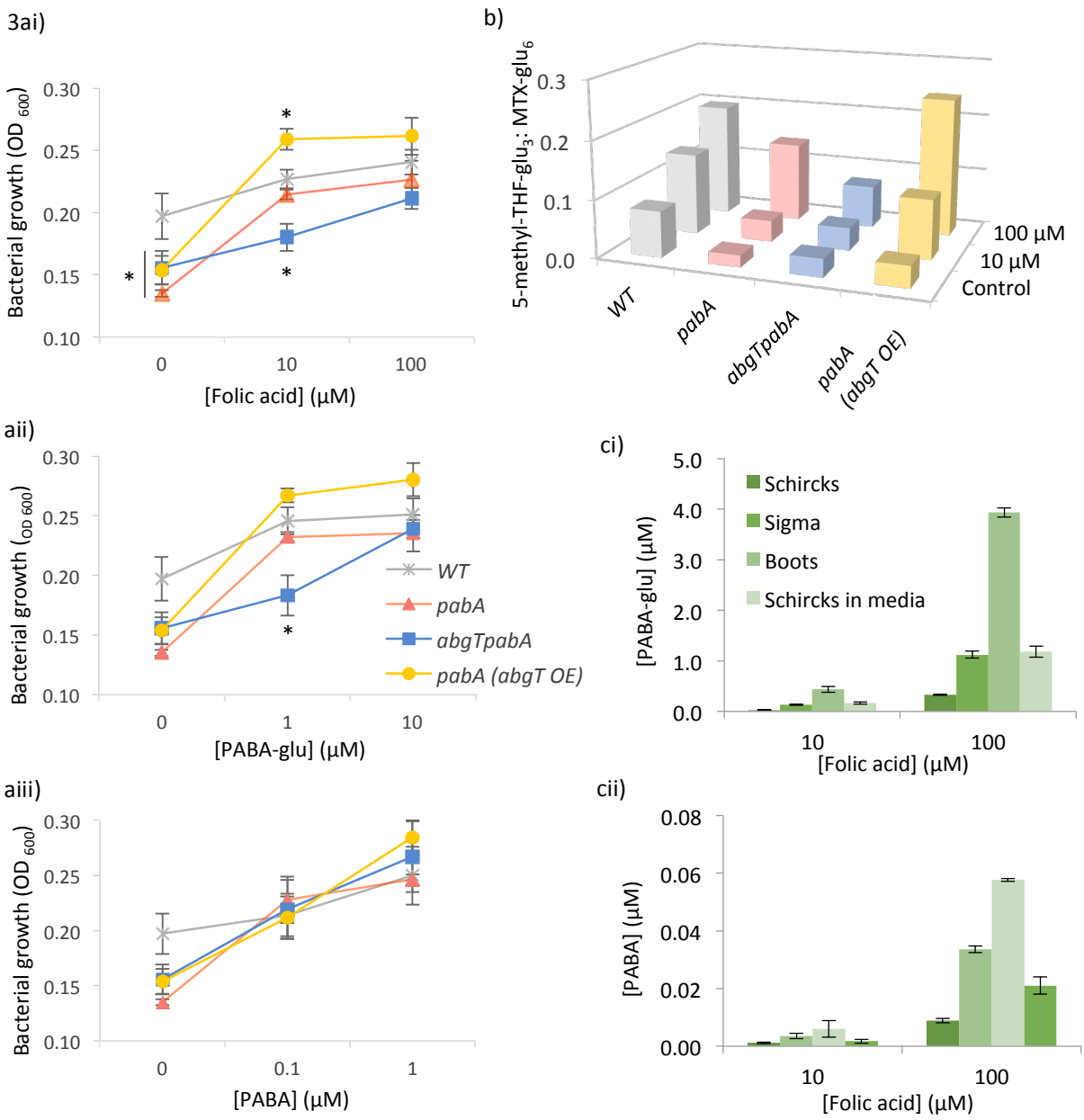

Figure 3. Folic acid breakdown supports bacterial growth and increases folate levels via AbgT-uptake of PABA-glu a) growth of *E. coli* WT, *pabA, abgTpabA* and *pabA* over-expressing *abgT (abgT* OE) on agar plates supplemented with i) folic acid ii) PABA-glu and iii) PABA as measured by OD_{600} after 4 days growth at 25°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<0.05 compared to WT growth on the same condition b) 5-methyl THF-glu3 levels in extracts of *E. coli* WT, *pabA, abgTpabA, pabA* (*abgT* OE) mutants supplemented with 10 µM and 100 µM folic acid. Folate counts from the LC-MS/MS are normalised by dividing by counts of an internal MTX-glu_6 spike. Extracts were made after 4 days of bacterial growth at 25°C on solid agar plates. See Supplementary Figure 3 for full folate analysis. c) Levels of i) PABA-glu and ii) PABA detected by LC-MS/MS in folic acid preparations from Schircks, Sigma, Boots and Schircks folic acid after addition to the agar media and incubation for 4 days at 25 ˚C. Error bars represent standard deviation over triplicate independent preparations.

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

Inhibiting bacterial folate synthesis, without affecting bacterial growth, is known to increase *C. elegans* lifespan (Virk et al., 2012; Virk et al., 2016). It was therefore hypothesized that factors that increase bacterial folate synthesis, such as folic acid (as shown here), may shorten *C. elegans* lifespan. Consistent with our previous findings (Virk et al., 2016), we find that *C. elegans* maintained on any *E. coli pabA* mutant are long-lived compared to *C. elegans* fed wild type *E. coli* (Figure 4, Supplementary Table 1), whereas the *abgT* mutation alone had no impact on *C. elegans* lifespan (P=0.4312, Supplementary Figure 1b). 10 µM folic acid was found to decrease *C. elegans* lifespan on *pabA* by 9.4% (P=0.0052), and decrease lifespan on the *pabA* mutant over-expressing *abgT* further (by 16.3%, P<0.0001, Figure 4a). In contrast, 10 µM folic acid had no effect on lifespan on the *abgTpabA* double mutant (P=0.1901, Figure 3a). 100 µM folic acid decreased lifespan on *pabA E. coli* by 23.9% (P<0.0001), whereas this concentration only shortened lifespan on the *abgT pabA* double mutant by 4.7% (P=0.0467, Figure 4a). 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 (Figure 4b). In contrast, PABA supplementation shortened *C. elegans* lifespan in all cases independently of *abgT* expression, consistent with its ability to diffuse across biological membranes (Figure 4c). Folic acid had no effect on the lifespan of *C. elegans* maintained on WT *E. coli* (Supplementary Figure 4). Together, these results suggest that folic acid shortens *C. elegans* lifespan on folate-deficient *E. coli* via AbgT-dependent uptake of PABA-glu during adulthood.

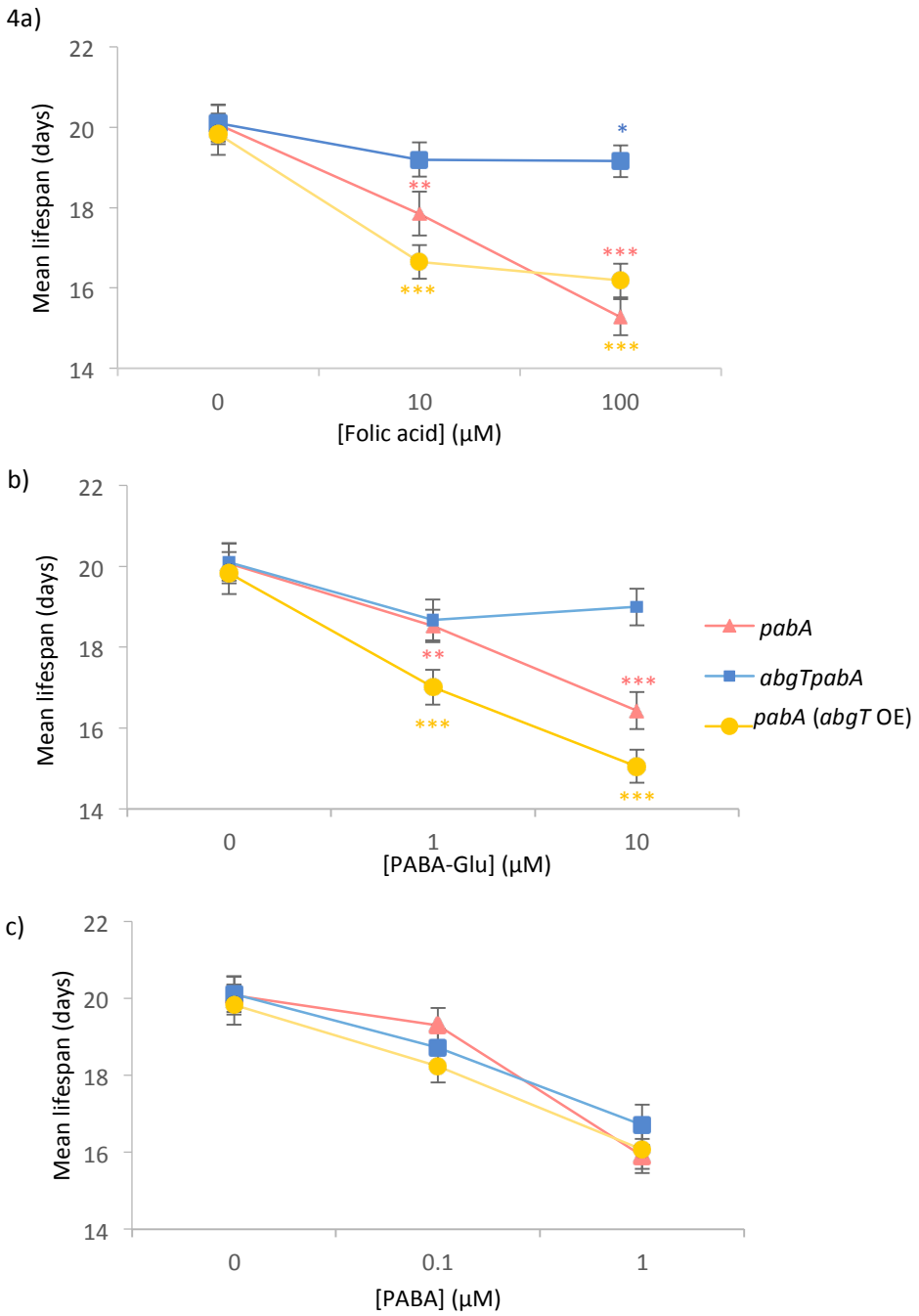

Figure 4. Folic acid shortens *C. elegans* lifespan via an *E. coli abgT*-dependent route during adulthood. Mean lifespan of WT *C. elegans* maintained from day 1 of adulthood on *pabA, abgTpabA,* or *pabA* (*abgT* OE) *E. coli* with supplementation of (a) folic acid (b) PABA-glu and (c) PABA. Error bars represent standard error. Asterisks denote the Log-rank non-parametric statistical test of survival, where: *P<0.05; **P<0.01; ***P<0.005 compared to lifespan on the non-supplemented condition of the same strain. Full lifespan data in Supplementary Table 1.

DISCUSSION

Folic acid supplementation generates a supply of folate in addition to that provided by the diet and gut microbiota. It is not clear, however, exactly how folic acid supplements host folate. Considering the conflicting reports of the safety of folic acid supplementation on host health, it is important to understand all possible routes of uptake. Using the established *C. elegans- E. coli* host-microbe model in which bacterial folate synthesis has been shown to influence host health (Chaudhari et al., 2016; Han et al., 2017; Virk et al., 2016), this study shows that folic acid can increase bacterial folate synthesis (Figure 3b) in a route that is dependent on its breakdown into PABA-glu (Figure 3c) and uptake by the bacterial transporter AbgT. We have shown that this route can have beneficial consequences in the case of *C. elegans* developmental folate-deficiency (Figure 2) but it can also have a negative impact on long-term host health, by shortening lifespan (Figure 4). Our previous work indicates that lifespan decrease is due to a bacterial folate-dependent toxicity. Together, we have uncovered a route by which folic acid interacts with bacterial metabolism to impact host health (Figure 5).

The detection of significant quantities of PABA-glu and PABA in three folic acid sources in this study, including in a commercial folic acid supplement (Figure 3c) indicates that this route may have implications for human folate supplementation. Indeed, investigations into folic acid supplements have reported significant failings of supplements to meet US (Hoag et al., 1997) or UK (Sculthorpe et al., 2001) standards for dissolution. In light of these issues with folic acid stability, manufacturers have adopted a policy of 'overages' in order to ensure sufficient vitamin is released (Andrews et al. 2017). As the *E. coli* PABA-glu transporter, AbgT, can influence *C. elegans* health following folic acid supplementation, depending on both bacterial and host genotype, it is hypothesized that supplement stability and gut microbiota composition may be two previously unexplored variables in understanding the health consequences of folic acid supplementation.

The AbgT protein is a member of a conserved family of over 13, 000 transporters, many of which are encoded for in the genomes of several pathogenic bacteria of the human gut microbiome, including *Enterobacter cloacae, N. gonorrhoeae, Salmonella enterica, Shigella boydii* and *Staphylococcus aureus* in addition to *E. coli.* Interestingly, several diseases are associated with an increased abundance of folate-synthesizing gut bacteria, such as inflammatory bowel disease (IBD(Shin et al., 2015) and small intestinal bacterial overgrowth (SIBO, (Camilo et al., 1996). Human trials are necessary to examine how microbial folate synthesis and microbial compositional changes in the human gut microbiota following folic acid supplementation. We hypothesize that there may be specific groups of patients where effects of folic acid

on microbial folate synthesis need to be considered. It may be necessary to design alternative strategies to prevent folate deficiency without increasing bacterial folate synthesis.

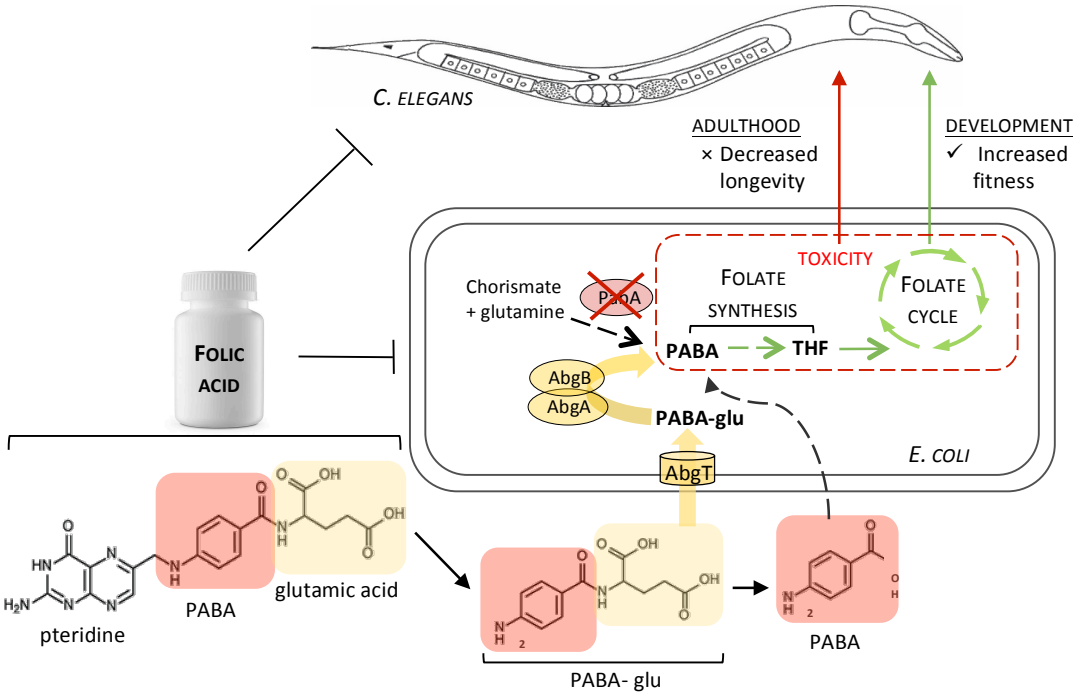

Figure 5. Schematic of the impact of folic acid supplementation on *C. elegans* via indirect uptake of breakdown products by *E. coli.* Folic acid is not taken up well by *C. elegans* directly. We find that the major uptake of folic acid by *C. elegans* is dependent on its breakdown into PABA-glu and uptake by the *E. coli* AbgT transporter. This route increases bacterial folate synthesis in both wild-type and *pabA* mutant *E. coli.* Under conditions of folate-deficiency (*pabA* mutant *E. coli*), increasing bacterial folate via this route is beneficial for *C. elegans* development. During *C. elegans* adulthood, this route has a negative impact on longevity as it promotes a bacterial folate-dependent toxicity.

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MATERIALS AND METHODS

Folates and related compounds

Folic acid, Folinic acid, PABA-Glu, 5-formylTHF-Glu_3 , 5-methylTHF-Glu_3, methotrexate-Glu_6 were obtained from Schircks, Switzerland. PABA, Vitamin B12 and folic acid were from Sigma Aldrich and folic acid supplement from Boots, UK.

Culture conditions

Defined media (DM) was prepared as described (Virk et al., 2016), except that 10 nM B12 was added. B12, folic acid and antibiotics are added post-autoclaving for agar plates. DM for liquid culture is filter-sterilised. 0.1 µM PABA added to the liquid DM media used to seed the plates in order to maintain bacterial growth (apart the growth experiments in Figure 3a). 30 µl of 3 ml fresh overnight LB culture is used to inoculate 5ml DM (15 ml Falcon tube). 25 µg/ml kanamycin (50 ug/ml ampicillin if necessary) added to both LB and DM pre-incubation. DM liquid cultures are incubated for 18 hr at 37°C, 220 RPM.

All strains derived from the Keio collection (Baba et al., 2006). See table. WT-Kan (Virk et al., 2016). *abgTpabA* double mutant was made using P1 transduction protocol as described in (Moore, 2011). The *abgT* over-expression plasmid (pJ128) (Carter et al., 2007) and empty vector (puc19) (Yanisch-Perron et al., 1985) were transformed into appropriate strains.

C. elegans strains used

SS104 *glp-4(bn2)*, UF208 (wild-type), and UF209 *gcp-2.1(ok1004)* (Virk et al., 2016).

E. coli preparation and growth assay

Table of _E. coli_ strains used in this study

Strain	Genotype	Plasmid	Characteristics	Source
BW25113/ pGreen 0029	WT	pGreen 0029	kan^r	Virk et al 2016
JW3323-1	*ΔpabA*	n/a	kan^r	Baba et al 2006
JW5822-1	*ΔabgT*	n/a	kan^r	Baba et al 2006
CM*abgTpabA*	*ΔabgT ΔpabA*	n/a	kan^r	This study
CM1	WT	pUC19‡, pGreen 0029	kan^r, amp^r	This study
CM2	*ΔpabA*	pUC19	kan^r, amp^r	This study
CM3	*ΔabgT ΔpabA*	pUC19	kan^r, amp^r	This study
CM4	WT *(abgT* OE)	pJ128‡, pGreen 0029	kan^r, amp^r	This study
CM5	*ΔpabA (abgT* OE)	pJ128	kan^r, amp^r	This study
CM6	*ΔabgT ΔpabA (abgT* OE)	pJ128	kan^r, amp^r	This study

E. coli was prepared as follows for all *E. coli* and *C. elegans* experiments. 30 µl of an overnight LB culture of *E. coli* was transferred into 5ml DM and incubated for 18 hr at 37 ◦C, 220 RPM. 100 µl 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 M9 onto the plate and a glass spreader 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. 100 µl was taken and diluted with 900 µl M9 in a cuvette. A spectrophotometer was used to read bacterial growth at 600 nm. Bacterial growth was calculated by multiplying OD_{600} by the volume of the sample (v).

E. coli folate extraction

Bacterial lawns were scraped from plates into micro centrifuge tubes using M9 solution and kept on ice. Volume (v), multiplied by the OD_{600} of the solution (diluted 1:5) gives a measure of the amount of material. Samples were concentrated in chilled 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 ($37.5 \times OD_{600} \times v$). FEB is spiked with 10 nM methotrexate-Glu_6 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-H_4PteGlu$_3$ (5-methylTHF-Glu$_3$) and 5/10-CHO-H_4PteGlu$_3$ (formyl THF$_3$) were optimised by infusion of standards into the instrument. The optimised conditions for –Glu$_3$ folates were applied to other higher folates using MRM transitions described by Lu *et al.*, 2007 (Lu et al., 2007). 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 µm, 2.1 x 100 mm) with a gradient of 0.1% 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 minutes, with a linear gradient to 100% B at 7 minutes, held for 1 minute, before a 7-minute re-equilibration step at 2% B that was 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 minutes to minimise instrument contamination. Folates 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 (Virk et al., 2012). *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 in Table S1. Statistical significance was determined using Log Rank and Wilcoxon tests of the Kaplan-Meier survival model.

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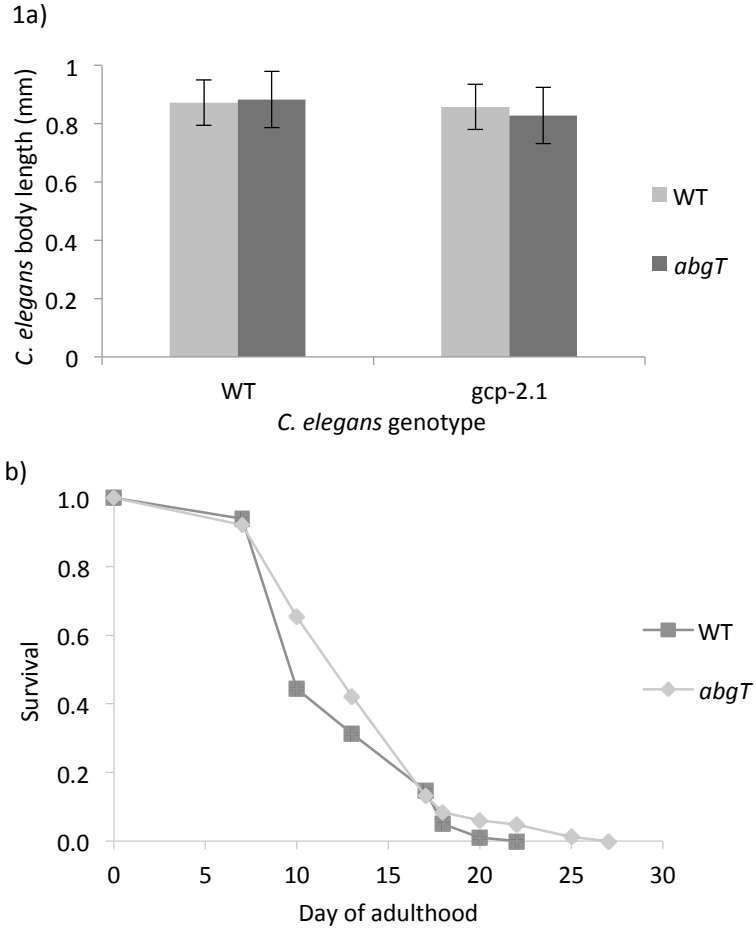
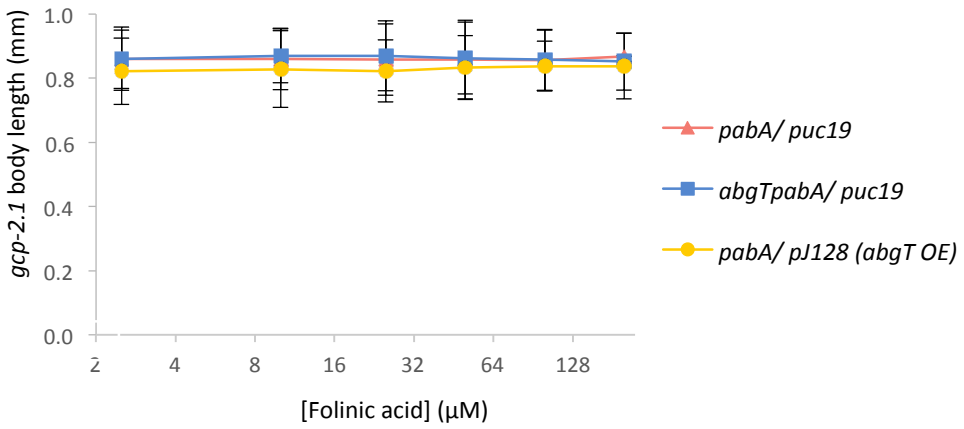
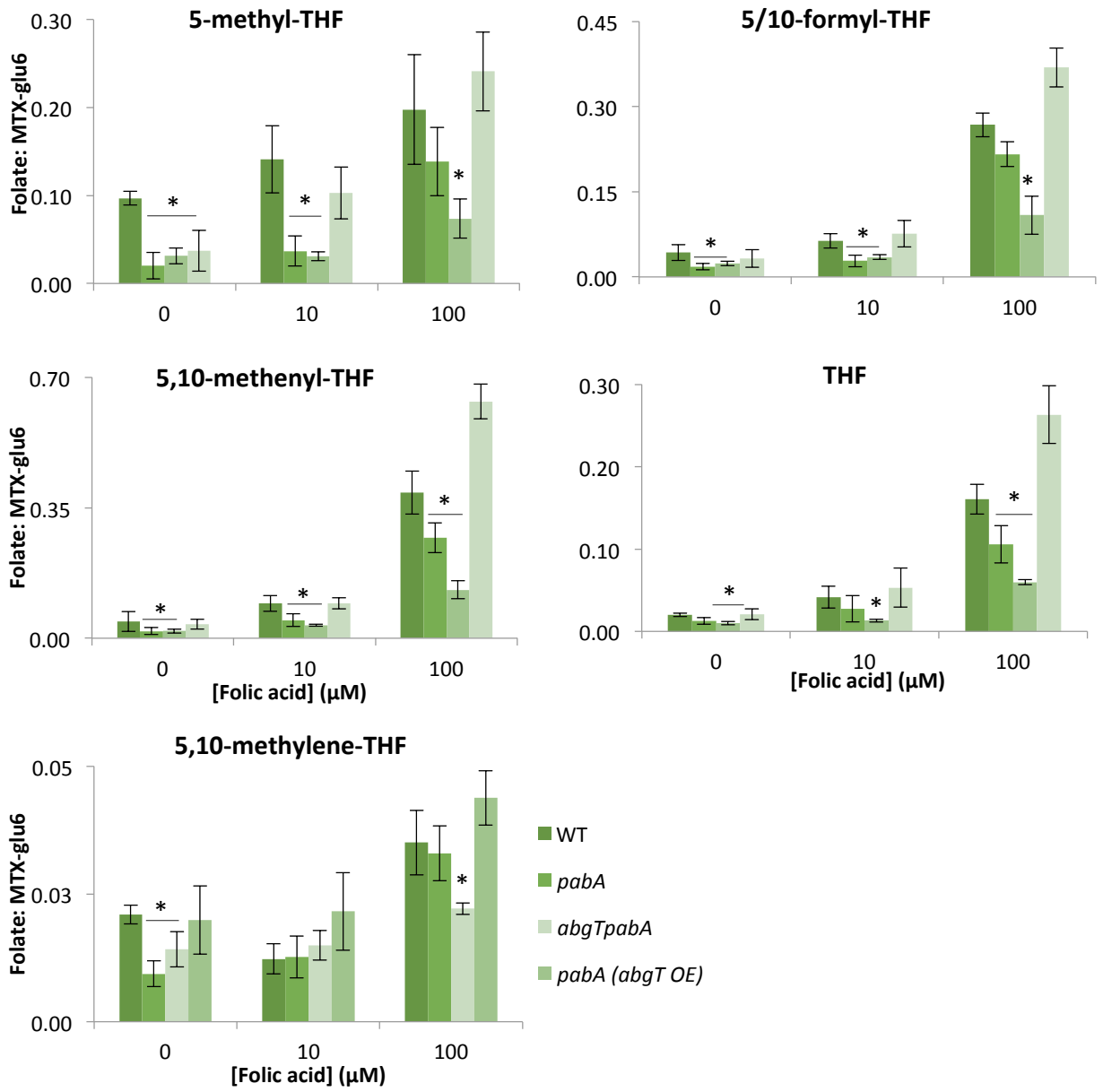
SUPPLEMENTARY INFORMATION



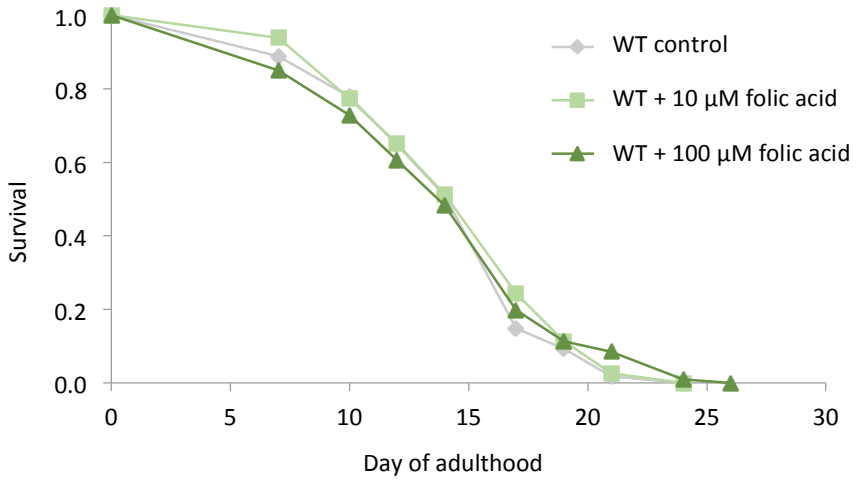
Supplementary Figure 1. The *E. coli abgT* deletion has no effect on *C. elegans* development and lifespan a) body length of wild-type and *gcp-2.1* mutant *C. elegans* at L4 stage raised on *abgT* mutant or wild-type *E. coli* (error bars represent standard deviation) b) survival curves of wild-type (*glp-4) C. elegans* on *E. coli* wild-type and *abgT* mutant. See Supplementary Table 1 for further details.



Supplementary Figure 2. Folinic acid restores growth of *C. elegans gcp-2.1* independently of *E. coli abgT.* Body length of *gcp-2.1* mutant *C. elegans* at L4 stage raised on DM agar plates seeded with *pabA* mutant, *abgTpabA* double mutant or *pabA* mutant over-expressing *abgT* with increasing concentrations of folinic acid. Over-expression is conferred by transformation with a high copy number plasmid, pJ128. *pabA* and *abgTpabA* strain are transformed with the empty vector, pUC19. Error bars represent standard deviation of *C. elegans* body length; n ≥40.



Supplementary Figure 3. LC-MS/MS detects lower levels of THFs in *pabA* mutants, where *abgT* determines response to folic acid. Levels of THFs detected in extracts of WT, *pabA, abgTpabA, pabA* (*abgT* OE) mutants displayed as a ratio with an internal MTX-glu$_6$ spike for normalization. Extracts were made after 4 days of bacterial growth at 25°C. Asterisks denote the test statistic from Student's *t* test comparison of means, where * = P<0.05. Error bars represent standard deviation over four replicates.



Supplementary Figure 4. The impact of folic acid supplementation on *C. elegans* lifespan on wild-type *E. coli.* Survival curves of *C. elegans* maintained from day 1 of adulthood on plates supplemented with 10 µM and 100 µM folic acid.

Supplementary Table 1: Summary of lifespan data

All lifespans conducted on defined media (DM) agar plates incubated at 25ºC

Fig.	Group name	n	Censor	Mean lifespan	Std. error	% change	p value (Log rank)	p value (Wilcoxon)	Supplement	Bacterial genotype	Plasmid	Antibiotics
5	WT	108	0	15.79	0.53	n/a	n/a	n/a	Alkaline control	CM1	pGreen 0029, pUC19	50 µg/ml carb, 25 µg/ ml kan
	pabA	94	3	20.08	0.52	Control	n/a	n/a	Alkaline control	CM2	pUC19	50 µg/ml carb, 25 µg/ ml kan
	pabA + 0.1 µM PABA	107	0	19.30	0.46	-3.89%	0.0973	0.3296	0.1 µM PABA	CM2	pUC19	50 µg/ml carb, 25 µg/ ml kan
	pabA + 1 µM PABA	118	0	15.91	0.45	-20.78%	<.0001*	<.0001*	1 µM PABA	CM2	pUC19	50 µg/ml carb, 25 µg/ ml kan
	pabA + 1 µM PABA-Glu	110	2	18.58	0.40	-7.49%	0.0030*	0.0277*	1 µM PABA-Glu	CM2	pUC19	50 µg/ml carb, 25 µg/ ml kan
	pabA + 10 µM PABA-Glu	125	10	16.43	0.46	-18.16%	<.0001*	<.0001*	10 µM PABA-Glu	CM2	pUC19	50 µg/ml carb, 25 µg/ ml kan
	pabA + 10 µM folic acid	93	5	18.20	0.55	-9.36%	0.0052*	0.0061*	10 µM Schircks folic acid	CM2	pUC19	50 µg/ml carb, 25 µg/ ml kan
	pabA + 100 µM folic acid	112	1	15.28	0.45	-23.93%	<.0001*	<.0001*	100 µM Schircks folic acid	CM2	pUC19	50 µg/ml carb, 25 µg/ ml kan
	abgTpabA	91	3	20.10	0.44	Control	n/a	n/a	Alkaline control	CM3	pUC19	50 µg/ml carb, 25 µg/ ml kan
	abgTpabA + 0.1 µM PABA	91	6	18.72	0.49	-6.88%	0.0631	0.0477*	0.1 µM pABA	CM3	pUC19	50 µg/ml carb, 25 µg/ ml kan
	abgTpabA + 1 µM PABA	102	1	16.71	0.45	-16.86%	<.0001*	<.0001*	1 µM pABA	CM3	pUC19	50 µg/ml carb, 25 µg/ ml kan
	abgTpabA + 1 PABA-Glu	101	11	18.11	0.52	-9.94%	0.1733	0.0076*	1 µM PABA-Glu	CM3	pUC19	50 µg/ml carb, 25 µg/ ml kan
	abgTpabA + 10 µM PABA-Glu	106	4	19.00	0.46	-5.49%	0.1817	0.0512	10 µM PABA-Glu	CM3	pUC19	50 µg/ml carb, 25 µg/ ml kan
	abgTpabA + 10 µM folic acid	110	1	19.20	0.43	-4.49%	0.1901	0.114	10 µM Schircks folic acid	CM3	pUC19	50 µg/ml carb, 25 µg/ ml kan
	abgTpabA + 100 µM folic acid	100	3	19.16	0.40	-4.67%	0.0476*	0.0743	100 µM Schircks folic acid	CM3	pUC19	50 µg/ml carb, 25 µg/ ml kan
	pabA (abgT OE)	62	10	19.90	0.52	Contol	n/a	n/a	Alkaline control	CM5	pJ128	50 µg/ml carb, 25 µg/ ml kan
	pabA (abgT OE) + 0.1 µM PABA	121	1	18.55	0.45	-6.72%	0.0049*	0.0184*	0.1 µM PABA	CM5	pJ128	50 µg/ml carb, 25 µg/ ml kan
	pabA (abgT OE) + 1 µM PABA	104	2	16.07	0.50	-19.19%	<.0001*	<.0001*	1 µM PABA	CM5	pJ128	50 µg/ml carb, 25 µg/ ml kan
	pabA (abgT OE) + 1 µM PABA-Glu	104	1	17.01	0.43	-14.47%	<.0001*	<.0001*	1 µM PABA-Glu	CM5	pJ128	50 µg/ml carb, 25 µg/ ml kan
	pabA (abgT OE) + 10 µM PABA-Glu	117	0	15.05	0.41	-24.33%	<.0001*	<.0001*	10 µM PABA-Glu	CM5	pJ128	50 µg/ml carb, 25 µg/ ml kan
	pabA (abgT OE) + 10 µM folic acid	119	0	16.65	0.42	-16.30%	<.0001*	<.0001*	10 µM Schircks folic acid	CM5	pJ128	50 µg/ml carb, 25 µg/ ml kan
	pabA (abgT OE) + 100 µM folic acid	117	0	16.19	0.42	-18.61%	<.0001*	<.0001*	100 µM Schircks folic acid	CM5	pJ128	50 µg/ml carb, 25 µg/ ml kan
Sup. 1	WT	91	9	13.85	0.44	Control	n/a	n/a	n/a	WT	pGreen 0029	25 µg/ml kan
	abgT	114	3	12.68	0.35	-8.48%	0.4312	0.0963	n/a	JW5822-1	n/a	25 µg/ml kan
Sup. 2	WT	108	0	14.58	0.40	Control	n/a	n/a	Alkaline control	WT	pGreen 0029	25 µg/ ml kan
	WT + 10 µM folic acid	115	0	15.00	0.39	2.86%	0.4448	0.4588	10 µM Schircks folic acid	WT	pGreen 0029	25 µg/ ml kan
	WT + 100 µM folic acid	106	1	14.57	0.48	-0.10%	0.566	0.844	100 µM Schircks folic acid	WT	pGreen 0029	25 µg/ ml kan
Repeat expt. (not shown)	WT	102	9	15.96	0.48	Control	n/a	n/a	Alkaline control	CM1	pGreen 0029, pUC19	50 µg/ml carb, 25 µg/ ml kan
	WT + 10 µM folic acid	98	12	15.96	0.58	0.03%	0.8268	0.4283	10 µM Schircks folic acid	CM1	pGreen 0029, pUC19	50 µg/ml carb, 25 µg/ ml kan
	WT + 100 µM folic acid	102	18	16.01	0.48	0.36%	0.866	0.7792	100 µM Schircks folic acid	CM1	pGreen 0029, pUC19	50 µg/ml carb, 25 µg/ ml kan
	pabA	113	10	18.35	0.56	Control	n/a	n/a	Alkaline control	CM2	pUC19	50 µg/ml carb, 25 µg/ ml kan
	pabA + 10 µM folic acid	90	11	17.44	0.64	-4.95%	0.2527	0.2605	10 µM Schircks folic acid	CM2	pUC19	50 µg/ml carb, 25 µg/ ml kan
	pabA + 100 µM folic acid	122	3	15.86	0.40	-13.57%	<.0001*	0.0019*	100 µM Schircks folic acid	CM2	pUC19	50 µg/ml carb, 25 µg/ ml kan
	abgTpabA	136	14	18.93	0.50	Control	n/a	n/a	Alkaline control	CM3	pUC19	50 µg/ml carb, 25 µg/ ml kan
	abgTpabA + 10 µM folic acid	115	26	19.22	0.52	1.51%	0.6416	0.5684	10 µM Schircks folic acid	CM3	pUC19	50 µg/ml carb, 25 µg/ ml kan
	abgTpabA + 100 µM folic acid	93	11	19.26	0.60	1.71%	0.5868	0.4482	100 µM Schircks folic acid	CM3	pUC19	50 µg/ml carb, 25 µg/ ml kan
	pabA (abgT OE)	101	23	18.42	0.56	Control	n/a	n/a	Alkaline control	CM5	pJ128	50 µg/ml carb, 25 µg/ ml kan
	pabA (abgT OE) + 10 µM folic acid	124	0	15.84	0.47	-14.00%	0.0005*	0.0035*	10 µM Schircks folic acid	CM5	pJ128	50 µg/ml carb, 25 µg/ ml kan
	pabA (abgT OE) + 100 µM folic acid	105	0	15.77	0.50	-14.36%	0.0003*	0.0041*	100 µM Schircks folic acid	CM5	pJ128	50 µg/ml carb, 25 µg/ ml kan
	WT *(abgT* OE)	110	9	16.11	0.50	Control	n/a	n/a	Alkaline control	CM4	pGreen 0029, pJ128	50 µg/ml carb, 25 µg/ ml kan
	WT *(abgT* OE) + 10 µM folic acid	99	4	16.05	0.57	-0.37%	0.9479	0.8905	10 µM Schircks folic acid	CM4	pGreen 0029, pJ128	50 µg/ml carb, 25 µg/ ml kan
	WT *(abgT* OE) + 100 µM folic acid	127	4	15.78	0.39	-2.02%	0.3698	0.8168	100 µM Schircks folic acid	CM4	pGreen 0029, pJ128	50 µg/ml carb, 25 µg/ ml kan