Interspecies Systems Biology Uncovers Metabolites Affecting *C. elegans* Gene Expression and Life History Traits

Emma Watson,[1,2] Lesley T. MacNeil,[1,2] Ashlyn D. Ritter,[1,2] L. Safak Yilmaz,[1,2] Adam P. Rosebrock,[3,4] Amy A. Caudy,[3,4] and Albertha J.M. Walhout[1,2,*]

[1]Program in Systems Biology
[2]Program in Molecular Medicine
University of Massachusetts Medical School, Worcester, MA 01605, USA
[3]Donnelly Centre for Cellular and Biomolecular Research
[4]Department of Molecular Genetics
University of Toronto, Toronto M5S 3E1, Canada
*Correspondence: marian.walhout@umassmed.edu
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SUMMARY

Diet greatly influences gene expression and physiology. In mammals, elucidating the effects and mechanisms of individual nutrients is challenging due to the complexity of both the animal and its diet. Here, we used an interspecies systems biology approach with *Caenorhabditis elegans* and two of its bacterial diets, *Escherichia coli* and *Comamonas aquatica*, to identify metabolites that affect the animal's gene expression and physiology. We identify vitamin B12 as the major dilutable metabolite provided by *Comamonas aq.* that regulates gene expression, accelerates development, and reduces fertility but does not affect lifespan. We find that vitamin B12 has a dual role in the animal: it affects development and fertility via the methionine/S-Adenosylmethionine (SAM) cycle and breaks down the short-chain fatty acid propionic acid, preventing its toxic buildup. Our interspecies systems biology approach provides a paradigm for understanding complex interactions between diet and physiology.

INTRODUCTION

Our diet provides building blocks for development and reproduction, as well as energy to sustain daily cellular and organismal activities. Complex diets consist of macronutrients such as carbohydrates, fats, and proteins but also provide micronutrients such as vitamins that function as cofactors in metabolic reactions. In mammals, nutrients are provided not only by diet but are also synthesized by the gut microbiota (Hooper et al., 2002). A major challenge is to unravel the contributions of individual metabolites to cellular and organismal physiology and to dissect the metabolic and genetic underpinnings of physiological responses to changing diets.

The nematode *C. elegans* is an emerging model to study the effects of diet on life history traits such as developmental rate, fertility, and aging (Coolon et al., 2009; Gracida and Eckmann, 2013; MacNeil et al., 2013). *C. elegans* is a relatively simple model organism composed of fewer than 1,000 somatic cells. It lives in temperate climates around the globe and subsists on diets of various bacterial species growing on rotting vegetation. These bacteria also inhabit the *C. elegans* intestine to serve as its microbiota (Félix and Duveau, 2012). In the laboratory, *C. elegans* are grown monoxenically on *E. coli* OP50, but many other bacterial strains and species have been fed to worms as well (Avery and Shtonda, 2003; Coolon et al., 2009; MacNeil et al., 2013; Soukas et al., 2009). Bacteria supply *C. elegans* with metabolites that can greatly affect its life history traits. For instance, bacterially derived nitric oxide and folate extend and limit the lifespan of the animal, respectively (Gusarov et al., 2013; Virk et al., 2012). The effects of these metabolites were identified by a hypothesis-driven approach (nitric oxide) or serendipitously by a mutation in the bacteria (folate). Because both *C. elegans* and its bacterial diet are genetically tractable, we reasoned that this predator-prey combination could be used for the unbiased identification of nutrients that drive transcriptional and physiological responses in the animal.

We previously found that, relative to *E. coli* OP50, a diet of *Comamonas* DA1877 accelerates *C. elegans* development and decreases fertility and lifespan (MacNeil et al., 2013). These physiological effects are accompanied by dramatic changes in gene expression (MacNeil et al., 2013). For instance, the acyl-CoA dehydrogenase-encoding gene *acdh-1* is repressed several hundred fold on the *Comamonas* DA1877 diet relative to *E. coli* OP50. We created transgenic animals harboring the *acdh-1* promoter driving expression of GFP to generate a transgenic "dietary sensor" strain with which the transcriptional response to the *Comamonas* DA1877 diet can be readily monitored in living animals (MacNeil et al., 2013). Remarkably, the effects of *Comamonas* DA1877 on *C. elegans* gene expression and development persist even when these bacteria are mixed in small amounts with the *E. coli* OP50 diet, indicating that *Comamonas* DA1877

generates one or more dilutable compounds to which *C. elegans* responds. Using the dietary sensor strain, we identified a *C. elegans* network consisting of metabolic and regulatory genes that, when perturbed, interferes with the transcriptional response to *Comamonas* DA1877 (Watson et al., 2013).

Here, we used an interspecies systems biology approach to identify bacterial metabolites that affect *C. elegans* gene expression and life history traits. We performed genetic screens in *E. coli* and *Comamonas* to identify bacterial genes that, when mutated, result in aberrant repression or activation of *Pacdh-1::GFP* in *C. elegans*. We performed a secondary metabolite screen by supplementing 25 candidate metabolites to *Pacdh-1::GFP* animals. Eight compounds activated the dietary sensor, including branched-chain amino acids, threonine, and propionic acid. Two compounds repressed the sensor: methyl-cobalamin (Me-Cbl) and adenosyl-cobalamin (Ado-Cbl), the two biologically active forms of vitamin B12. We demonstrate that vitamin B12 is generated by *Comamonas* DA1877, but not by *E. coli*, and that it drives many of the gene expression and physiological changes in *C. elegans* induced by the *Comamonas* diet. Interestingly, all eight activating metabolites are closely connected in the *C. elegans* metabolic network to the two enzymes that require vitamin B12 as a cofactor. We find that vitamin B12 fulfills two important physiological roles in *C. elegans*: it regulates development through the synthesis of the major methyl donor SAM and alleviates toxic buildup of the short-chain fatty acid propionic acid. Our interspecies systems biology approach provides a powerful paradigm for gaining insight into the complex interactions between diet, metabolic regulation, and physiology.

RESULTS

Genetic Screens in Bacterial Diets

We previously determined that *Comamonas* DA1877 bacteria must produce a dilutable compound to which *C. elegans* responds with altered gene expression programs, accelerated development, and reduced fertility (MacNeil et al., 2013). We reasoned that we could discover bacterial metabolites that affect *C. elegans* by identifying bacterial genes involved in the generation, processing, or transport of these small molecules. We used the *C. elegans Pacdh-1*::GFP dietary sensor as a reporter for diet-induced gene expression changes—GFP expression is high when the animals are fed *E. coli* OP50 but is barely detectable when the animals are fed *Comamonas* DA1877. We performed genetic screens in each of these bacteria to identify mutant strains that, when fed to *C. elegans*, alter GFP expression.

First, we fed dietary sensor animals the Keio *E. coli* BW25113 collection, which contains deletion mutants for 3,985 of the 4,290 protein-coding genes (Baba et al., 2006). We visually examined whether GFP expression was decreased or increased relative to animals fed the wild-type strain (Figure 1A). In total, 70 mutant *E. coli* strains decreased GFP expression, and seven caused an increase compared to the parent strain (Table S1 available online). Second, we performed a transposon-based mutagenesis screen of *Comamonas* DA1877 bacteria (Figure 1B). Using a nonreplicating transposon, we generated 5,760 *Comamonas* DA1877 mutants, each with a single transposon insertion.

Five mutant *Comamonas* DA1877 strains failed to repress GFP expression when fed to the sensor strain (Table S2).

To facilitate the mapping of transposon insertions and identification of the disrupted genes, we sequenced the *Comamonas* DA1877 genome and annotated protein-coding sequences and RNA genes (Figure S1A). Because there was no systematic report on the taxonomic identity of *Comamonas* DA1877 before this study, we first used the single 16S rRNA gene to identify this strain at the species level as *Comamonas aquatica*, and we will henceforth refer to *Comamonas* DA1877 as *Comamonas aq.* DA1877.

We reasoned that bacterial mutants identified affect *C. elegans* gene expression due to either the buildup or reduction in particular metabolites. A total of 77 *E. coli* and 5 *Comamonas aq.* DA1877 genes (Tables S1 and S2) were identified in the bacterial screens. We focused on bacterial genes encoding metabolic enzymes, transporters, and transcription factors known to regulate metabolic operons. Mapping these genes onto bacterial metabolic networks revealed that perturbation of several different pathways affected GFP expression in *C. elegans* (Figure 1C). For instance, mutations in enzymes from *E. coli* purine metabolism, propionic acid metabolism, the tricarboxylic acid (TCA) cycle, and the biosynthesis of siderophores (iron scavengers) decreased dietary sensor activity. Conversely, *E. coli* and *Comamonas* mutations in branched chain amino acid biosynthesis and vitamin B12 biosynthesis/ import resulted in increased *C. elegans* dietary sensor activity.

A Metabolite Screen in *C. elegans* Identifies Vitamin B12 as the Candidate Dilutable *Comamonas* Molecule

We selected a subset of metabolites implicated by the bacterial screens for use in a secondary screen in which their effect on the *C. elegans* dietary sensor was tested by direct supplementation to either diet. We focused on 18 metabolites implicated by bacterial genes encoding metabolic enzymes, transporters, and transcription factors known to regulate metabolic operons. We included seven additional metabolites that were implicated from our earlier *C. elegans* genetic screens (Table S3) (Watson et al., 2013). We supplemented the metabolites at various concentrations to the *Pacdh-1::GFP* sensor strain fed either bacterial diet (Figure 2A). Six metabolites caused mild, dose-dependent increases in GFP expression when supplemented to the *Comamonas aq.* DA1877 diet, including the amino acids valine, isoleucine, threonine, and glycine, as well as homocysteine and vitamin B6. Two metabolites, α-ketobutyrate and propionic acid, more dramatically increased GFP expression when supplemented to *Comamonas aq.* DA1877 and also further increased GFP expression on the *E. coli* OP50 diet (Figure 2B).

Only two metabolites mimicked the strong repressive effect of the *Comamonas aq.* diet on the *Pacdh-1::GFP* sensor when supplemented to *E. coli* OP50: the two biologically active forms of vitamin B12, Ado-Cbl and Me-Cbl (Figure 2C). Vitamin B12 is an attractive candidate to be the dilutable molecule produced by *Comamonas aq.* DA1877 (MacNeil et al., 2013) for several reasons. First, two of five *Comamonas aq.* DA1877 genes identified in the transposon screen encode vitamin B12 biosynthetic enzymes (*cbiA* and *cbiB*, Table S2 and Figure S1B). Second, vitamin B12 was also implicated by the *E. coli* deletion collection screen,
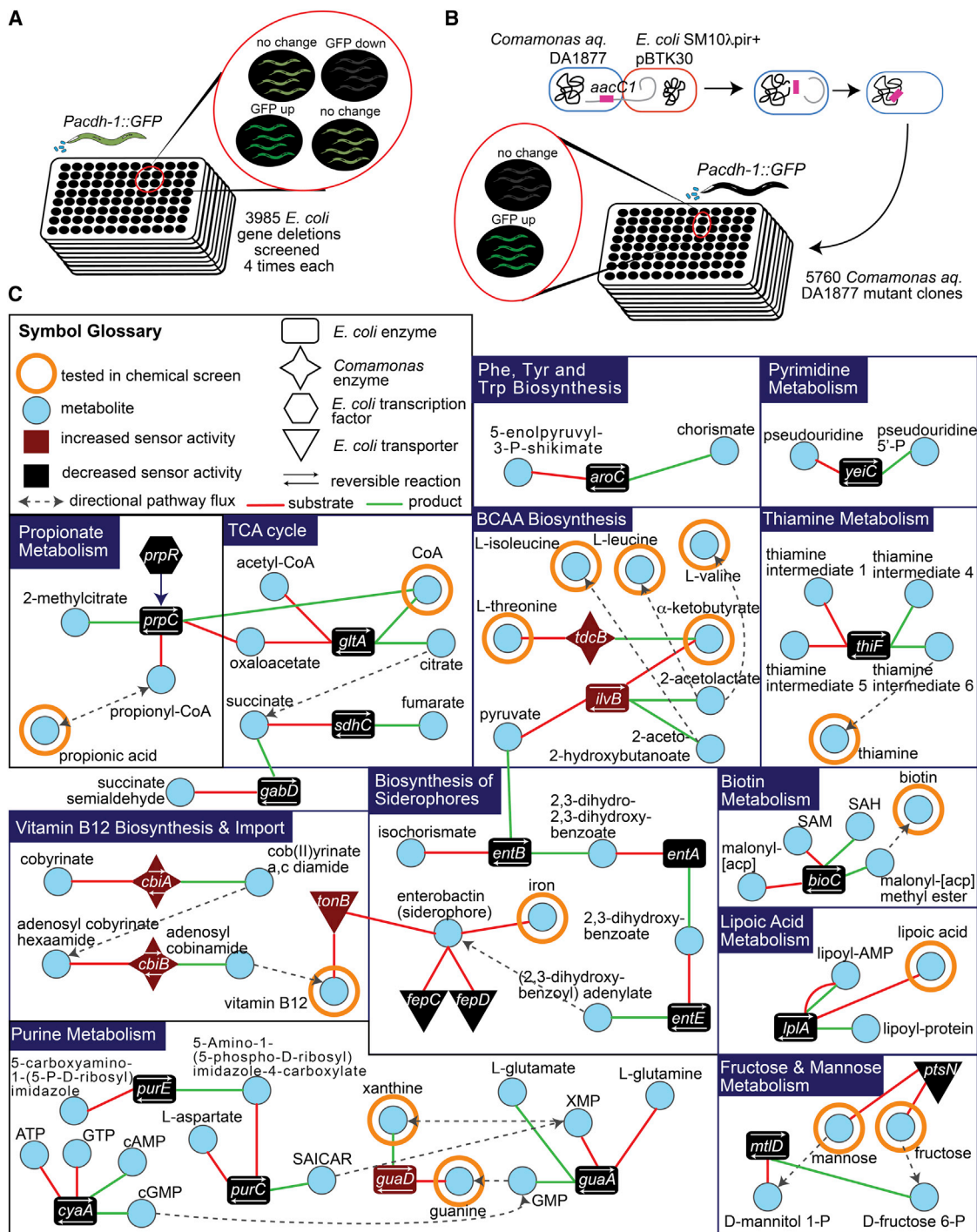
Figure 1. Bacterial Screens

(A) Diagram of the Keio *E. coli* deletion collection screen.

(B) Diagram of *Comamonas* DA1877 transposon mutagenesis screen.

(C) Network of metabolites implicated by bacterial screens. SAICAR, 5-amino-4-imidazole-N-succinocarboxamide ribonucleotide; [acp], acyl carrier protein. See also Figure S1 and Tables S1 and S2.

even though *E. coli* do not synthesize this cofactor (see below). An *E. coli* strain with a deletion in *tonB*, which encodes a protein required for vitamin B12 import (Bassford et al., 1976; Kadner,

1990), resulted in even greater levels of GFP expression when fed to the sensor strain (Figure S1C). Third, Ado- and Me-Cbl were the only repressors identified in the chemical screen, and
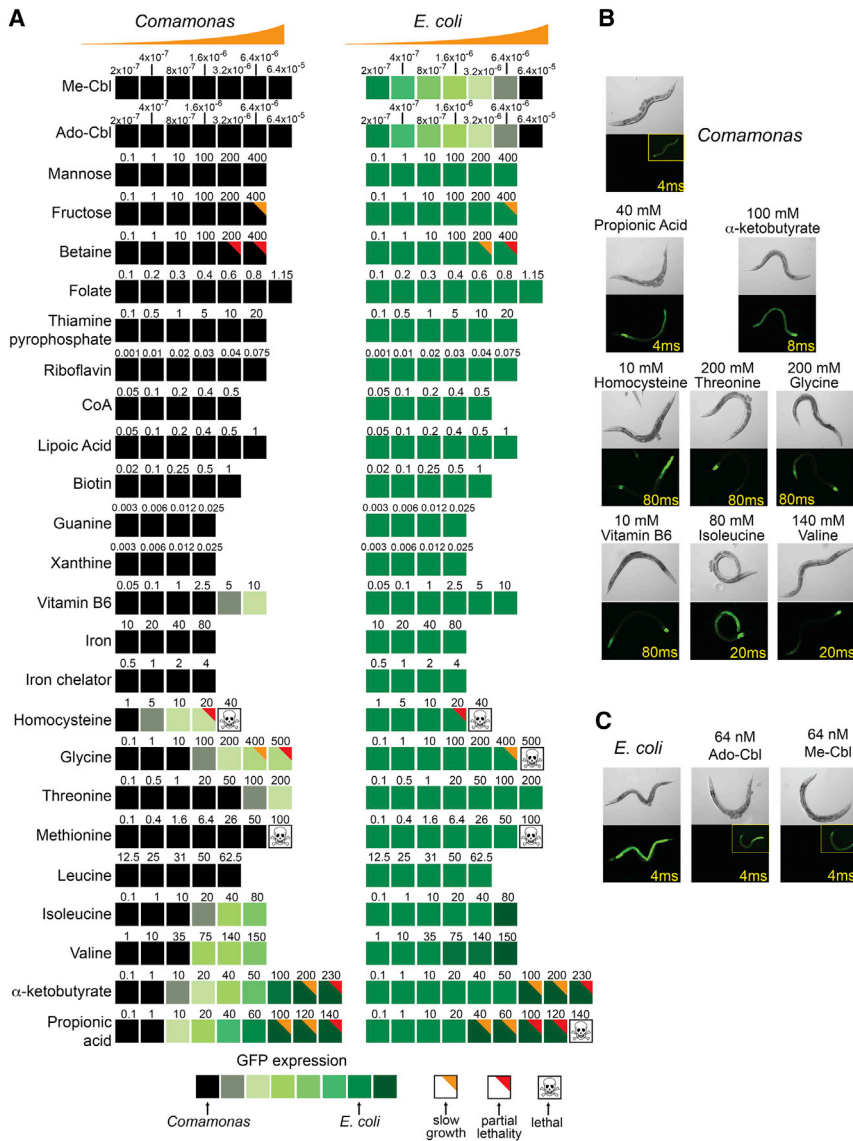
A

Comamonas | E. coli

Me-Cbl	2x10⁻⁷ 4x10⁻⁷ 8x10⁻⁷ 1.6x10⁻⁶ 3.2x10⁻⁶ 6.4x10⁻⁶ 6.4x10⁻⁵
Ado-Cbl	2x10⁻⁷ 4x10⁻⁷ 8x10⁻⁷ 1.6x10⁻⁶ 3.2x10⁻⁶ 6.4x10⁻⁶ 6.4x10⁻⁵
Mannose	0.1 1 10 100 200 400
Fructose	0.1 1 10 100 200 400
Betaine	0.1 1 10 100 200 400
Folate	0.1 0.2 0.3 0.4 0.6 0.8 1.15
Thiamine pyrophosphate	0.1 0.5 1 5 10 20
Riboflavin	0.001 0.01 0.02 0.03 0.04 0.075
CoA	0.05 0.1 0.2 0.4 0.5
Lipoic Acid	0.05 0.1 0.2 0.4 0.5 1
Biotin	0.02 0.1 0.25 0.5 1
Guanine	0.003 0.006 0.012 0.025
Xanthine	0.003 0.006 0.012 0.025
Vitamin B6	0.05 0.1 1 2.5 5 10
Iron	10 20 40 80
Iron chelator	0.5 1 2 4
Homocysteine	1 5 10 20 40
Glycine	0.1 1 10 100 200 400 500
Threonine	0.1 0.5 1 20 50 100 200
Methionine	0.1 0.4 1.6 6.4 26 50 100
Leucine	12.5 25 31 50 62.5
Isoleucine	0.1 1 10 20 40 80
Valine	1 10 35 75 140 150
α-ketobutyrate	0.1 1 10 20 40 50 100 200 230
Propionic acid	0.1 1 10 20 40 60 100 120 140

GFP expression

Comamonas ← → E. coli; slow growth, partial lethality, lethal

B

Comamonas
40 mM Propionic Acid — 4ms
100 mM α-ketobutyrate — 8ms
10 mM Homocysteine — 80ms
200 mM Threonine — 80ms
200 mM Glycine — 80ms
10 mM Vitamin B6 — 80ms
80 mM Isoleucine — 20ms
140 mM Valine — 20ms

C

E. coli — 4ms
64 nM Ado-Cbl — 4ms
64 nM Me-Cbl — 4ms

Figure 2. Metabolite Screen

(A) Summary of the observed effects of supplementing metabolites to *Pacdh-1::GFP* animals fed either *Comamonas aq.* DA1877 (left column) or *E. coli* OP50 (right column). Shades of green represent relative GFP expression. Numbers indicate metabolite concentrations (in mM).

(B) *Pacdh-1::GFP* animals fed *Comamonas aq.* DA1877 bacteria alone or supplemented with the indicated compounds that activate GFP expression. Exposure time is indicated in yellow. Exposure time for inset images is 400 ms.

(C) *Pacdh-1::GFP* animals fed *E. coli* OP50 bacteria supplemented with the indicated compounds that repress GFP expression. Exposure time is indicated in yellow. Exposure time for inset images is 400 ms.

See also Table S3.

their respective pathways, are required for *Comamonas aq.* DA1877-induced gene expression changes (Watson et al., 2013). Taken together, these results suggest that vitamin B12 may be the dilutable molecule provided by the *Comamonas aq.* DA1877 diet that represses *Pacdh-1::GFP*.

Vitamin-B12-Producing Bacteria Repress the Dietary Sensor

Our results predict that vitamin B12 levels are higher in the *Comamonas aq.* DA1877 than in the *E. coli* OP50 diet. To directly compare the amounts of vitamin B12, we measured Ado-Cbl by mass spectrometry. Ado-Cbl levels are much higher in *Comamonas aq.* DA1877 than in *E. coli* OP50 (Figure 3A) but are reduced to background in the *Comamonas aq. cbiA* mutant, indicating that this mutant indeed fails to synthesize vitamin B12.

both robustly repress *Pacdh-1::GFP* in low (nM) doses. This fits with the observation that mixing small amounts of the *Comamonas aq.* DA1877 diet into the *E. coli* OP50 diet is sufficient to exert gene expression and physiological changes (MacNeil et al., 2013). Finally, mutations in the two *C. elegans* enzymes that use vitamin B12 interfere with the transcriptional response to the *Comamonas aq.* DA1877 diet (Watson et al., 2013). Vitamin B12 is an essential nutrient for most animals but is only synthesized by some species of bacteria (Bender, 2003). It is used as a cofactor by the same two enzymes in all vitamin B12-dependent animals—methylmalonyl-CoA mutase (MUT; MMCM-1 in *C. elegans*), which is involved in propionyl-CoA breakdown, and methionine synthase (MS; METR-1 in *C. elegans*), which is involved in the methionine/SAM cycle. Methylmalonyl-CoA mutase uses Ado-Cbl, whereas methionine synthase uses Me-Cbl (Figure S1D). We previously found that both of these vitamin-B12-dependent enzymes, as well as others in
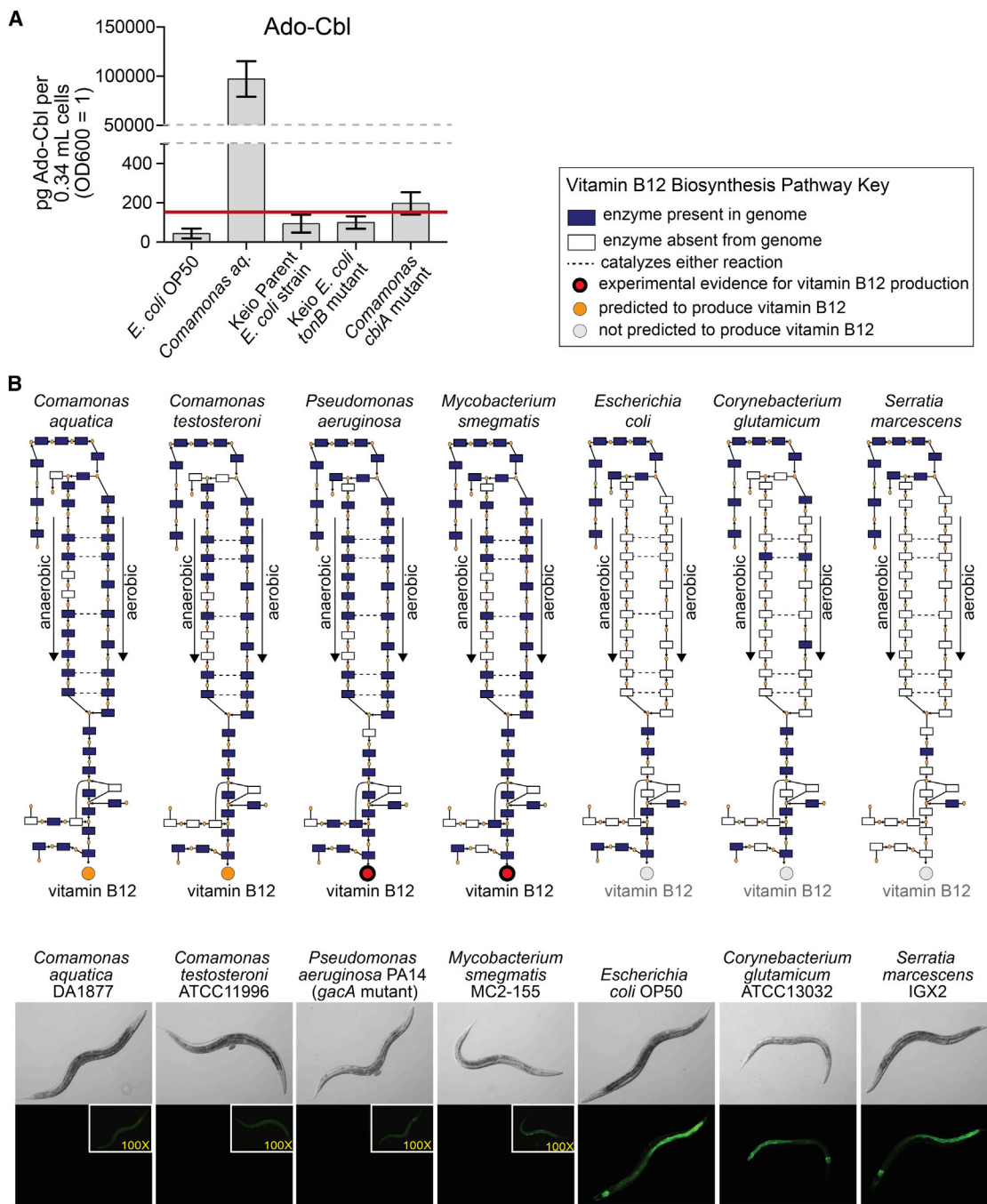
We fed additional bacterial species to *Pacdh-1::GFP* dietary sensor animals and correlated their effect on GFP expression with the presence or absence of a vitamin B12 biosynthesis pathway. Four of these seven bacterial species have the capacity to synthesize vitamin B12 (Karasseva et al., 1977; Lee et al., 2012), and three do not (Figure 3B). We found that the presence or absence of a vitamin B12 biosynthetic pathway in the genome of the bacterial diet correlates perfectly with the repression or activation of the *acdh-1* promoter in *C. elegans*, respectively (Figure 3B).

Requirement of *C. elegans* Vitamin B12 Processing and Utilization

Vitamin B12 repressed GFP expression equally well whether supplemented to live or UV-killed *E. coli* OP50 diets, indicating that its effects do not depend on *E. coli* modification or metabolism (Figure S2A). We wondered why Ado-Cbl and Me-Cbl repress the dietary sensor equally well (Figure 2). From studies of human

Figure 3. Correlation between Vitamin B12 Biosynthesis and Repression of the Dietary Sensor

(A) Ado-Cbl content measured by mass spectrometry in indicated bacterial strains. The red line indicates background levels of Ado-Cbl in the bacteria-free control. Mean ± SEM is plotted from three replicate experiments.

(B) Correlation between vitamin B12 biosynthesis pathway presence and dietary sensor repression. Images of *Pacdh-1::GFP* animals fed various bacterial strains are located below a cartoon that indicates pathway status. Differences in exposure times for the inset images are indicated in yellow.

See also Figure S2.

cobalamin deficiency disorders, it is known that, after vitamin B12 is imported into the cell, it is stripped of its upper axial ligand, undergoes several processing steps, is modified into Ado-Cbl and Me-Cbl, and is distributed to the two enzymes that use it (Figure S2B). Our supplementation experiments likely cannot discriminate between individual effects of these two forms of vitamin B12 because they can be interconverted. We wondered whether vitamin B12 processing and/or distribution in *C. elegans* are
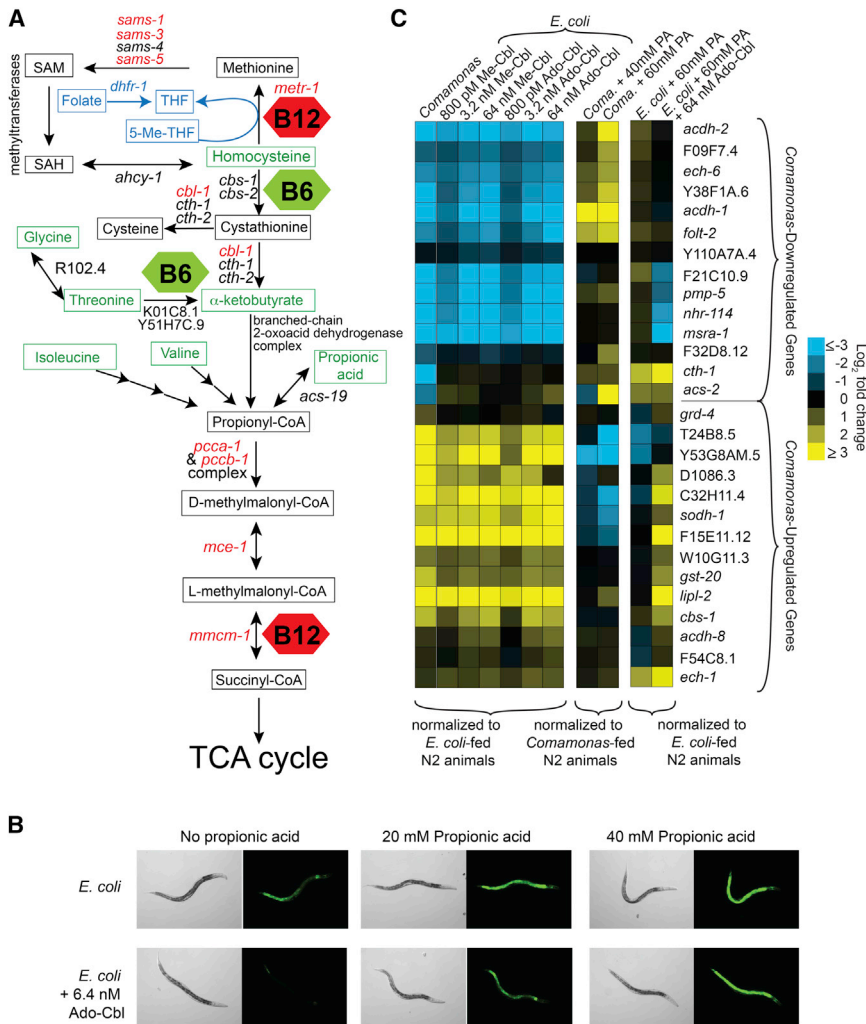
Figure 4. Chemical Epistasis with Vitamin B12 and Propionic Acid on *C. elegans* Gene Expression

(A) Network of the two vitamin-B12-dependent pathways. Metabolites are indicated in rectangles. Green metabolites activate the dietary sensor when supplemented to the bacterial diet. Genes encoding the metabolic enzymes involved are indicated with arrows between metabolites. Genes that, when mutated, activate the dietary sensor are indicated in red. Red hexagons, vitamin B12; green hexagons, vitamin B6; blue, folate metabolism. 5-Me-THF, 5-methyltetrahydrofolate; THF, tetrahydrofolate, the biologically active form of folate.

(B) Chemical epistasis of combined supplementation of Ado-Cbl and propionic acid on *Pacdh-1::GFP* dietary sensor animals fed the *E. coli* OP50 diet.

(C) qRT-PCR of 14 *Comamonas*-upregulated and 14 *Comamonas*-downregulated genes in wild-type animals fed indicated diets and supplemented metabolites. PA, propionic acid; N2, wild-type animals. Changes in expression for each gene are plotted as log₂ fold change compared to respective mRNA levels in wild-type animals fed *E. coli* OP50 (first and third columns) or *Comamonas aq.* DA1877 (middle column).

See also Table S4.

Taken together, vitamin B12 processing/ distributing genes and intact vitamin-B12-dependent metabolic pathways are required for the effect of this cofactor on *C. elegans*.

Propionic Acid Can Override Vitamin B12 to Activate the Sensor

Vitamin B12 represses the dietary sensor, whereas vitamin B6, homocysteine, glycine, threonine, isoleucine, valine, α-ketobutyrate, and propionic acid activate the *acdh-1* promoter (Figure 2A). Interestingly, the breakdown of each of these amino acids, as well as α-ketobutyrate and propionic acid, involves conversion to propionyl-CoA (Figure 4A). Vitamin B6 functions as a cofactor in two reactions that lead to the production of propionyl-CoA (Figure 4A). Because vitamin B12 increases flux through the propionyl-CoA breakdown pathway, we hypothesized that the balance between vitamin B12 and propionyl-CoA levels may be the driving force controlling *acdh-1* promoter activity. To test this hypothesis, we performed a chemical epistasis experiment and found that excess propionic acid can override the repressive effects of vitamin B12 on the dietary sensor in a dose-dependent manner (Figure 4B). This suggests that the effects of vitamin B12 on the *acdh-1* promoter may depend on its capacity to repress the buildup of an activator, propionic acid.

Vitamin B12 Mimics Broad *Comamonas aq.* DA1877-Mediated Gene Expression Changes

The *Comamonas aq.* DA1877 diet affects the expression of many *C. elegans* genes, including *acdh-1* (MacNeil et al., 2013). To

required for its repressive effect on *Pacdh-1::GFP*. In our previous *C. elegans* genetic screens, we did not retrieve any vitamin B12 import and processing genes (Watson et al., 2013), potentially due to inherently high false-negative rates in RNAi screens (Kamath et al., 2003). We predicted *C. elegans* vitamin B12 processing genes based on homology with known human genes (Figure S2B). RNAi of each of two genes tested, *mtrr-1* and *Y76A2B.5*, resulted in failure of both Me-Cbl and Ado-Cbl to repress the dietary sensor (Figure S2C). This indicates that supplemented vitamin B12 must be properly processed and regenerated into its active forms within *C. elegans* to repress *Pacdh-1::GFP*.

We previously found that enzymes in propionyl-CoA breakdown and the methionine/SAM cycle are required for the repressive effect of the *Comamonas aq.* DA1877 diet on the dietary sensor (Watson et al., 2013). Thus, one would predict that these enzymes are also required for the repressive effects of vitamin B12 supplementation. We used two deletion mutants in each of these pathways to test whether they are also involved in mediating the response to vitamin B12. Indeed, supplementation of vitamin B12 to either propionyl-CoA breakdown or methionine/SAM cycle mutants failed to repress *acdh-1* promoter activity (Figure S2D).
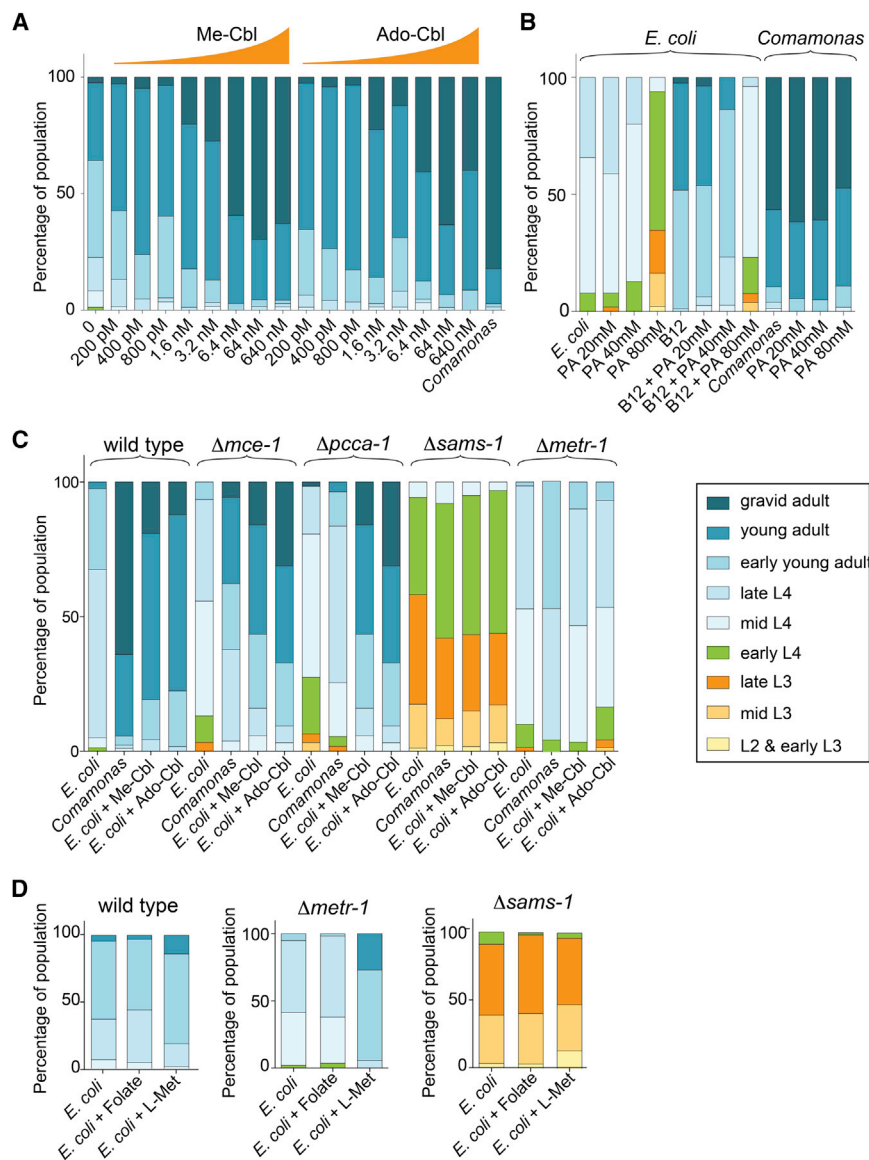
Figure 5. Effects of Metabolite Supplementation on *C. elegans* Development

(A) Developmental progression of synchronized wild-type populations of animals fed indicated diets and supplemented metabolites. The parental generation was cultivated on *E. coli* OP50.

(B) Developmental progression of synchronized wild-type populations of animals fed indicated diets and supplemented metabolites. PA, propionic acid; vitamin B12, 64 nM Ado-Cbl. The parental generation was cultivated on *E. coli* OP50.

(C) Developmental progression of synchronized wild-type and mutant populations of animals fed indicated diets and supplemented metabolites. 64 nM of each variant of vitamin B12 was used.

(D) Developmental progression of synchronized wild-type and mutant populations of animals fed indicated diets and supplemented metabolites. L-Met, 4 mM methionine. 56 μM folate was used. See also Figure S3.

propionic acid could override the repressive effect of vitamin B12 on these genes. Interestingly, a subset of the vitamin-B12-downregulated genes did not respond to propionic acid. A similar trend was observed among the vitamin-B12-activated genes; whereas some responded to propionic acid, others did not. Therefore, we determined that there are at least two classes of vitamin-B12-responsive genes: those that respond to propionic acid (hereafter referred to as type 1) and those that do not (type 2).

Vitamin B12 Accelerates *C. elegans* Developmental Rate and Egg Laying Timing and Requires the Methionine/SAM Cycle

Because vitamin B12 mimics the effects of the *Comamonas aq.* DA1877 diet on *C. elegans* gene expression, we next tested whether it also mimics the accelerated development, reduced fertility, and accelerated aging induced by this bacterial diet (MacNeil et al., 2013). Vitamin B12 supplementation affected neither the mean nor the maximum lifespan of *C. elegans* (Figure S3A). This indicates that another factor must be responsible for the *Comamonas aq.* DA1877 effect on aging, which is in agreement with the observation that a diet consisting of *E. coli* OP50 supplemented with a small amount of *Comamonas aq.* DA1877 does not shorten lifespan (MacNeil et al., 2013).

Addition of vitamin B12 to the *E. coli* OP50 diet did accelerate development, although it did not fully recapitulate the extent of developmental rate increase elicited by the *Comamonas* diet (Figure 5A). This indicates that additional growth-enhancing bacterial factors may be provided by *Comamonas*. Propionic acid supplementation to the *E. coli* OP50 diet slowed *C. elegans* development, and supplementation of both propionic acid and

assess whether vitamin B12 can elicit similar broad effects on gene expression, we used quantitative RT-PCR (qRT-PCR) to determine relative expression levels of 14 representative *Comamonas*-downregulated genes and 14 representative *Comamonas*-upregulated genes in wild-type animals fed *E. coli* OP50 with or without three doses of supplemented Me-Cbl or Ado-Cbl. All but one of the genes tested changed in expression on the *Comamonas aq.* DA1877 diet, as described previously (MacNeil et al., 2013). Most of the *Comamonas*-downregulated genes were also downregulated when animals were fed *E. coli* OP50 supplemented with either form of vitamin B12 (Figure 4C). Likewise, most *Comamonas*-upregulated genes were also activated by supplementation of vitamin B12 (Figure 4C). Thus, vitamin B12 supplementation to the *E. coli* OP50 diet induces similar gene expression changes as those elicited by the *Comamonas aq.* DA1877 diet.

Six vitamin B12-downregulated genes, including *acdh-1*, were upregulated in response to propionic acid (Figure 4C). Further,

vitamin B12 resulted in intermediate growth rates (Figure 5B). Based on this observation, we hypothesized that propionic acid levels may dictate developmental rate: low concentrations caused by high vitamin B12 would accelerate development, whereas high propionic acid levels that occur when vitamin B12 is limiting would slow development. To test this hypothesis, we measured developmental timing in four metabolic gene mutants, two from each vitamin-B12-dependent pathway: Δmce-1 and Δpcca-1 from propionyl-CoA metabolism and Δmetr-1 and Δsams-1 from the methionine/SAM cycle. When fed E. coli OP50, all four mutants exhibited slow growth compared to wild-type animals (Figure 5C). Surprisingly, supplementation of vitamin B12 accelerated development in Δmce-1 and Δpcca-1 mutant animals but failed to increase developmental rate in Δmetr-1 and Δsams-1 mutants (Figure 5C). Thus, vitamin-B12-induced developmental acceleration requires a functional methionine/SAM cycle, but not the propionyl-CoA breakdown pathway. Δmce-1 and Δpcca-1 mutants fail to catabolize propionyl-CoA (Chandler et al., 2006), likely resulting in a buildup of propionic acid. Neither Δmce-1 nor Δpcca-1 mutants can break down propionic acid even when vitamin B12 is present, yet supplementation of this cofactor does accelerate development in these mutants. These data suggest that the levels of propionic acid are not the driving force behind developmental rate but rather that development is accelerated via the methionine/SAM cycle as a result of buildup or lack of specific metabolites therein.

To further test this model, we first focused on metr-1 (methionine synthase), which converts homocysteine to methionine by using vitamin B12 as a cofactor. In this reaction, 5-methyltetrahydrofolate is also converted to the biologically active form of folate, tetrahydrofolate (Figure 4A). Loss of METR-1 function can be caused either by a mutation in the corresponding gene or as a result of vitamin B12 deficiency. In humans, loss of the corresponding enzyme leads to depletion of tetrahydrofolate and methionine and buildup of homocysteine. Folate supplementation (which is converted to tetrahydrofolate in vivo) did not rescue the slow growth of Δmetr-1 mutants (Figure 5D). Thus, tetrahydrofolate depletion is not a primary cause of developmental rate reduction. Methionine supplementation, on the other hand, largely rescued the developmental rate defect of Δmetr-1 mutants (Figure 5D). Interestingly, methionine supplementation did not accelerate development of Δsams-1 mutant animals, which are impaired in converting methionine to SAM (Figure 5D) (Walker et al., 2011). This suggests that the developmental acceleration by methionine depends on its conversion to SAM. Taken together, these data suggest that vitamin B12 accelerates C. elegans development primarily through its role as a cofactor in the methionine/SAM cycle, rather than in propionyl-CoA breakdown. However, excess propionic acid slows development, which is likely due to toxic effects.

Supplementing E. coli with vitamin B12 reduced total brood size and therefore mimics the Comamonas aq. DA1877 diet with regard to this phenotype as well (Figure S3B). Both the Comamonas aq. DA1877 diet and vitamin B12 supplemented E. coli OP50 altered the dynamics of egg laying; animals laid almost all of their eggs (93% and 96%, respectively) within the first 2 days.

In contrast, animals fed E. coli OP50 alone laid only 70% in the first 2 days (Figure S3C). This vitamin-B12-induced shift to early egg laying was still observed in Δpcca-1 mutants, but the effect was lost in Δmetr-1 mutant animals (Figure S3B). Together, these results indicate that vitamin B12 both accelerates development and shifts egg-laying dynamics via its role in the methionine/ SAM cycle.

Vitamin B12 Protects against Propionic Acid Toxicity and Requires the Propionyl-CoA Metabolism Pathway

The developmental acceleration induced by vitamin B12 did not rely on its role in propionyl-CoA breakdown; however, vitamin B12 did prevent some of the developmental deceleration induced by high doses of propionic acid (Figure 5B). At such high doses, propionic acid is toxic to the animal (Figures 2A and 6A). We asked whether vitamin B12 supplementation could alleviate the toxic effects of excess dietary propionic acid in wild-type and Δpcca-1 mutant animals. Vitamin B12 increased tolerance to propionic acid in wild-type animals, but not in Δpcca-1 mutant animals, which are much more sensitive to propionic acid supplementation in general (Figure 6A). This is in agreement with the notion that animals lacking pcca-1 accumulate propionic acid and cannot utilize vitamin B12 to metabolize it. Interestingly, wild-type animals fed Comamonas aq. DA1877 are much more resistant to propionic acid than those fed E. coli OP50 supplemented with 64 nM vitamin B12 (Figure 6A). This indicates that Comamonas aq. DA1877 may provide additional compounds that alleviate propionic acid toxicity or that these bacteria metabolize more propionic acid, thus lowering the effective concentration delivered to the animal.

We wondered whether loss of metr-1 would also increase sensitivity to propionic acid toxicity. Surprisingly, Δmetr-1 mutants tolerated excess propionic acid slightly better than wild-type animals, although this difference was lost when vitamin B12 was in ample supply (Figure 6B). When vitamin B12 is limiting, it is conceivable that MMCM-1 and METR-1 compete for this cofactor. As a result, MMCM-1 might be able to utilize more of the vitamin B12 pool for the breakdown of propionic acid when METR-1 is absent.

Transcription of acdh-1 is strongly activated in response to propionic acid supplementation, vitamin-B12-deficient diets, or mutations in any of the four enzymes required for vitamin B12-dependent propionic acid breakdown. We wondered what the physiological role of this activation could be and reasoned that acdh-1 may function in response to propionic acid buildup, perhaps to help dampen its toxic effects. We tested the sensitivity of Δacdh-1 mutants to propionic acid and found that they are much more sensitive than wild-type animals (Figure 6B). Unlike Δpcca-1 mutants, however, Δacdh-1 mutants survived better on propionic acid when vitamin B12 was supplemented to the diet. This is likely because, unlike in Δpcca-1 mutants, the vitamin-B12-dependent propionyl-CoA breakdown pathway is still intact in Δacdh-1 mutant animals. Taken together, type 1 vitamin B12 response genes that are activated by propionic acid may help to rewire flux through the metabolic network, thereby enabling the breakdown, excretion, or conversion of this metabolite or alleviating its toxic effects indirectly.
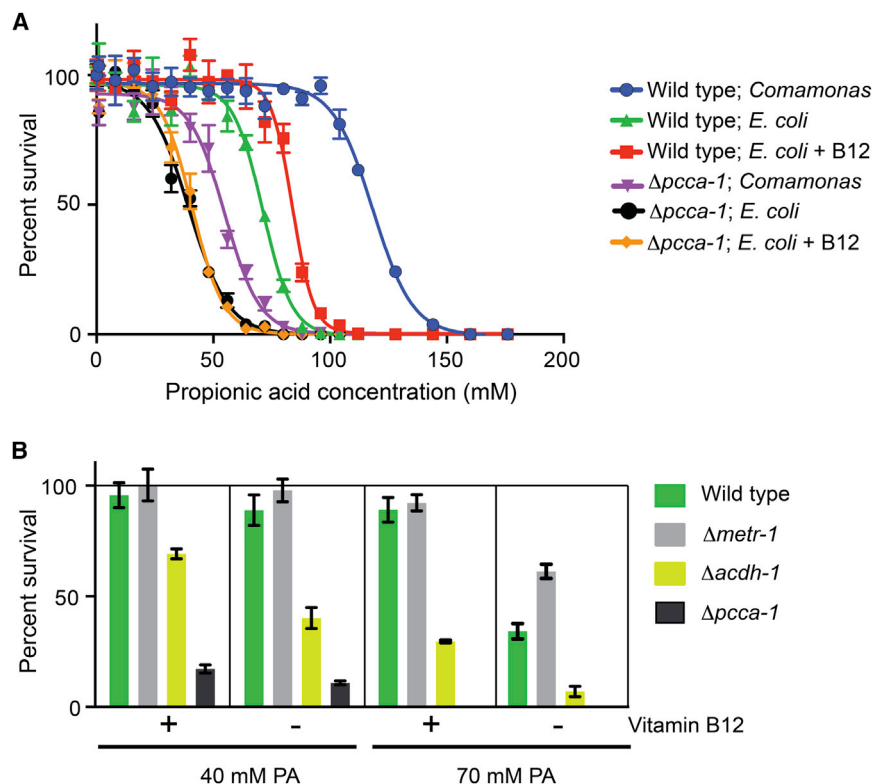
Figure 6. Effects of Vitamin B12 on Propionic Acid Toxicity

(A) Toxicity analysis of incremental doses of propionic acid on wild-type and Δpcca-1 mutant *C. elegans* in the absence and presence of 64 nM Ado-Cbl (B12). Mean ± SEM is plotted from three combined experiments.

(B) Percent survival of different *C. elegans* strains fed *E. coli* OP50 at two concentrations of propionic acid (PA) in the absence or presence of 64 nM Ado-Cbl. Mean ± SEM is plotted from four combined experiments.

DISCUSSION

Using an interspecies systems biology approach with *C. elegans* and two of its bacterial diets, we uncovered metabolites that affect gene expression and life history traits. We identified vitamin B12 as the major dilutable compound produced by *Comamonas aq.* DA1877 that regulates gene expression, accelerates development, and affects fertility (MacNeil et al., 2013; this study). Further, we determined that the balance between vitamin B12 and propionyl-CoA is likely the main driving force in the regulation of type 1 vitamin B12 response genes. This finding may reconcile a puzzling observation, namely that both vitamin B12 supplementation and starvation repress the dietary sensor (MacNeil et al., 2013). How can supplementation of a compound and the absence of a compound have the same effect? In rats, starvation lowers levels of short-chain fatty acids, including propionic acid, as animals catabolize their fat stores (Illman et al., 1986). In worms, starvation and vitamin B12 supplementation may have the same repressive effect on the dietary sensor by reducing the levels of propionyl-CoA.

We identified different physiological functions for the two vitamin-B12-dependent pathways in *C. elegans*: the methionine/SAM cycle affects development and fertility, whereas the propionyl-CoA breakdown pathway mitigates propionic acid-induced toxicity (Figure 7). The observation that the methionine/SAM cycle affects development is compatible with the notion that this is an anabolic pathway that generates SAM, the major methyl donor used to modify proteins such as histones (Liu et al., 2013), and to synthesize phosphatidylcholine (Walker et al.,
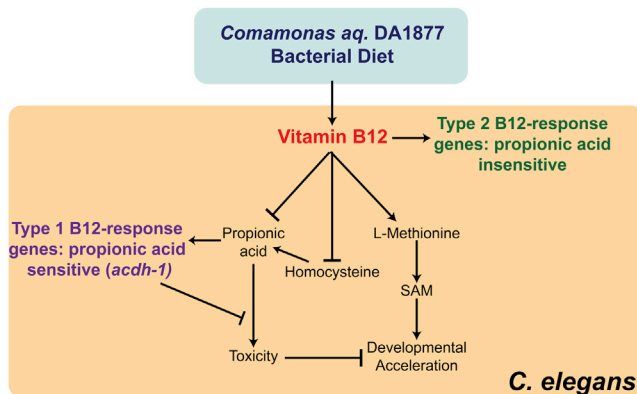
2011), which is vital for the production of cell membranes during development.

Vitamin B12 represses *acdh-1* expression at the level of transcription, though it is unclear whether it acts directly or indirectly. We previously identified several nuclear hormone receptors that control the response of the *acdh-1* promoter to nutritional and metabolic conditions (MacNeil et al., 2013; Watson et al., 2013). Fat-soluble vitamins A and D are direct ligands for nuclear hormone receptors (Chawla et al., 2001). The *C. elegans* genome encodes 274 nuclear hormone receptors (Reece-Hoyes et al., 2005). It is tempting to speculate that vitamin B12 directly interacts with one or more of these nuclear hormone receptors. However, our data indicate that vitamin B12 can only repress *Pacdh-1* when it can efficiently be used to break down propionyl-CoA because metabolic perturbations that block propionyl-CoA breakdown or supplementation of excess propionic acid to wild-type animals can override the repressive effect of vitamin B12 on *acdh-1* expression. We speculate that propionic acid may directly interact with one or more nuclear hormone receptors to regulate type 1 vitamin B12 response genes.

C. elegans is likely to encounter many different bacterial food sources in the wild (Félix and Duveau, 2012). Not all bacterial species synthesize vitamin B12, and *C. elegans* may have evolved an optimal gene expression program to help the animal cope with diets low in vitamin-B12-producing bacteria. One consequence of this gene expression program may be metabolic rewiring; the expression levels of several metabolic genes predicted to function in vitamin-B12-dependent pathways are sensitive to dietary vitamin B12 content (MacNeil et al., 2013; Watson et al., 2013). Turning various enzymes on or off at the level of gene expression may help to optimize metabolic flux. *acdh-1* expression is induced dramatically when vitamin B12 levels are low and propionic acid levels are high. Although the exact metabolic functions of the ACDH-1 enzyme are unknown, we suspect that it may serve to reroute flux around propionyl-CoA within the metabolic network when vitamin B12 is limited to prevent toxic buildup of propionic acid in the animal. This notion is supported by our observation that Δacdh-1 mutants are sensitive to propionic acid toxicity.

Figure 7. Model
Our findings demonstrate that the *Comamonas aq.* DA1877 diet provides high levels of vitamin B12, whereas the *E. coli* OP50 diet is low in this cofactor. Vitamin B12 acts through two different pathways—it lowers levels of propionyl-CoA, thereby preventing propionic acid toxicity, and enables the synthesis of methionine and SAM, thereby accelerating development and reducing fertility. Methionine synthesis reduces levels of homocysteine and likely propionyl-CoA levels. Our data suggest that vitamin B12 may affect these processes by regulating the expression of different sets of genes (type 1 and type 2), some that respond oppositely to propionyl-CoA and others that are insensitive to this metabolite.

Our findings suggest that *C. elegans* development, as it has been studied in the laboratory for decades using *E. coli* OP50, which is low in vitamin B12, only reflects one natural state. It will be interesting to study other characteristics of the animal such as behavior, mating, and movement under ample vitamin B12 conditions.

Vitamin B12 deficiencies in humans can have severe impacts on health, causing anemias and neuropathies, as well as low birth weight, developmental retardation, and defects in cognitive abilities in children of vitamin-B12-deficient mothers (Pepper and Black, 2011). Such deficiencies can lead to propionic and methylmalonic acidemias, homocystinuria, methionine/SAM deficiency, or a combination thereof, and it is not yet fully understood which of these metabolic imbalances lead to physiological and pathological consequences in humans. Each of these different diseases can also be caused by inborn errors in metabolic genes; for instance, propionic acidemia is caused by mutations in the human ortholog of *pcca-1* (Al-Lahham et al., 2010). Altogether, the interspecies paradigm we present provides a model to further characterize vitamin-B12-dependent processes and may provide a platform for the development of dietary or drug treatments for vitamin-B12-related deficiencies.

Vitamin B12 is an essential cofactor in mammals and *C. elegans* (Bito et al., 2013) but is not used at all by plants, yeast, and many insect species. The uneven distribution of vitamin B12 use among eukaryotes is interesting, as is the uneven distribution of vitamin B12 biosynthetic capability among bacterial species. Considering that many species of enteric bacteria can synthesize this essential vitamin suggests that the chemical outsourcing of vitamin B12 production may be an important keystone of a mutually beneficial relationship between gut microbiota and host cells (Albert et al., 1980; Goodman et al., 2009; LeBlanc et al., 2013).

The microbiota greatly affects its host's metabolism (Tremaroli and Bäckhed, 2012). For instance, bacteria in the gut break down dietary fiber, which results in the production of short-chain fatty acids such as propionic acid that are absorbed by intestinal epithelia and are used to generate energy (Al-Lahham et al., 2010). Altogether, the microbiota facilitate digestion, affect xenobiotic response to drugs and other chemicals (Haiser et al., 2013; Maurice et al., 2013), and are critically important in the immunological defense (Kamada et al., 2013). The fact that both *C. elegans* and its bacterial diets are genetically tractable provides a powerful system to further unravel the complex interactions between diet, microbiota, metabolism, physiology and pathology, and the molecular mechanisms involved.

EXPERIMENTAL PROCEDURES

***C. elegans* Strains**
N2 (Bristol) was used as the wild-type strain, and animals were maintained as described at 20°C (Brenner, 1974). *sams-1(ok2946)*, *mce-1(ok243)*, *pcca-1(ok2282)*, *metr-1(ok521)*, and *acdh-1(ok1489)* strains were provided by the *C. elegans* Gene Knockout Consortium and were backcrossed as described (Watson et al., 2013). We previously described the *Pacdh-1::GFP* strain (VL749) (MacNeil et al., 2013).

Bacterial Strains
E. coli OP50, *Serratia marcescens* IGX2, and *Comamonas aq.* DA1877 were obtained from the CGC. *Pseudomonas aeruginosa* PA14 (*gacA* mutant) was a gift from Victor Ambros (UMMS), and *Corynebacterium glutamicum* ATCC13032 and *Comamonas testosteroni* ATCC11996 were obtained from ATCC. *Mycobacterium smegmatis* MC2-155 was a gift from Chris Sassetti (UMMS). For the killed bacteria experiment, *E. coli* OP50 and *Comamonas aq.* DA1877 cultures were grown to log phase in LB, harvested and washed, irradiated with UV (254nm, 5 J/cm^2), concentrated, and added to NGM plates.

***E. coli* Deletion Collection Screen**
Keio *E. coli* deletion collection clones (Thermo Scientific) were grown overnight in Luria Broth (LB) containing 50 μg/ml kanamycin in 96-well deep well dishes. 15 μl of this overnight culture was seeded onto 96-well nematode growth media (NGM) agar plates containing 50 μg/ml kanamycin. *Pacdh-1::GFP* gravid adults fed the Keio parent strain were bleached to harvest embryos, which were subsequently washed in M9 media and allowed to hatch overnight to synchronize the population by L1 arrest. Approximately 25 synchronized L1 animals were added to each well of the seeded plates. After incubating 48 hr at 20°C, animals were visually screened for GFP expression. Each plate from the Keio collection was screened twice; thus, each *E. coli* gene was screened four times. Deletion mutants that affected GFP expression in at least two out of four experiments were considered hits.

***Comamonas aq.* DA1877 Transposon-Based Mutagenesis Screen**
See Supplemental Information.

***Comamonas aq.* DA1877 Genome Sequencing, Assembly, and Annotation**
See Supplemental Information.

Vitamin B12 Biosynthesis Pathway Analysis
To determine the presence or absence of vitamin B12 biosynthesis pathway in bacterial species used in this study, we first gathered EC numbers from KEGG (Kanehisa et al., 2012) and MetaCyc (Caspi et al., 2010) for all reactions comprising this pathway. For *Comamonas aq.* DA1877, we cross-referenced these EC numbers against all EC numbers annotated in the draft metabolic network reconstruction from SEED and determined which vitamin B12 biosynthesis enzymes were encoded in the genome. For other bacterial species, we determined the presence or absence of vitamin B12 biosynthesis genes based

on the same EC numbers but used KEGG and Metacyc databases to guide annotation. If an EC number in the vitamin B12 biosynthesis pathway was annotated as present for a particular species in either or both of these databases, we called it present.

Metabolite Screen

Metabolites were purchased from Sigma-Aldrich and dissolved in water to the maximum soluble concentration. Dilutions were made in water as indicated in Figure 2. For each stock solution, pH was adjusted to 6.0 with sodium hydroxide or hydrochloric acid. Stock solutions were added to NGM in various doses just prior to plate pouring.

Mass Spectrometry

See Supplemental Information.

RNAi Experiments

See Supplemental Information.

qRT-PCR

Embryos from N2 wild-type gravid adults grown on *E. coli* OP50 were harvested, washed, and hatched overnight in M9 to synchronize the population. Synchronized L1s were added to plates containing various concentrations of Me-Cbl or Ado-Cbl (both from Sigma) and/or propionic acid, seeded with *E. coli* OP50 or *Comamonas* DA1877. Animals were harvested for each condition when the majority of the population reached young adult stage. Total RNA was isolated using Trizol (Invitrogen) and then purified of contaminating DNA by DNaseI treatment followed by cleanup using QIAGEN RNeasy columns. cDNA was reverse transcribed from RNA using oligo(dT)12-18 primer and Mu-MLV reverse transcriptase (NEB). Primer sequences for qRT-PCR were generated using the GETprime database (Gubelmann et al., 2011) and are listed in Table S4. qRT-PCR reactions were performed in triplicate using the Applied Biosystems StepOnePlus Real-Time PCR system and Fast Sybr Green Master Mix (Invitrogen). Relative transcript abundance was determined using the ΔΔCt method and was normalized to averaged *ama-1* and *act-1* mRNA levels (Livak and Schmittgen, 2001).

Developmental Rate, Fertility, and Lifespan Assays

For all experiments, animals were grown at 20°C. We measured the developmental state of a population by synchronizing animals by L1 arrest in M9 for 20 hr and then allowing animals to develop for 45 hr at 20°C under various dietary conditions. For L-methionine and folate supplementations, solutions (5 mg/ml L-Met in water or 0.1 mg/ml folate in 20% ethanol) were added on top of plain NGM plates and dried prior to bacterial seeding. After 45 hr, ~35–80 animals were mounted on agarose pads and visually categorized on a compound microscope into age groups based on the development of the vulva and germline as described (MacNeil et al., 2013).

Brood sizes were determined by transferring 30 L4 animals onto individual plates containing *E. coli* OP50, *Comamonas aq.* DA1877, or *E. coli* OP50 supplemented with 64 nM Ado-Cbl. Animals were transferred to new individual plates daily, and the number of viable offspring on the plates was counted after hatching.

For the lifespan assays, ~125 L4 animals were transferred onto NGM plates containing 0.1 mg/ml FUDR, with or without vitamin B12, and seeded with either *E. coli* OP50 or *Comamonas aq.* DA187. Animals were checked for body movement or pharyngeal pumping every 2 days. If no pumping was observed, animals were lightly tapped with a sterile platinum wire, and if animals failed to respond to the touch stimulus, they were considered dead.

Propionic Acid Toxicity Assay

Approximately 100 starved L1 animals were placed on NGM media with increasing concentrations of propionic acid (pH 6.0). After 48 hr, live animals were counted. Each dose of propionic acid was analyzed in triplicate.

ACCESSION NUMBERS

The GenBank accession number for the *Comamonas aq.* DA1877 genome sequence is JBOK00000000.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, three figures, and four tables and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2014.01.047.

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