Diet-Induced Developmental Acceleration Independent of TOR and Insulin in C. elegans

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SUMMARY

Dietary composition has major effects on physiology. Here, we show that developmental rate, reproduction, and lifespan are altered in C. elegans fed Comamonas DA1877 relative to those fed a standard E. coli OP50 diet. We identify a set of genes that change in expression in response to this diet and use the promoter of one of these (acdh-1) as a dietary sensor. Remarkably, the effects on transcription and development occur even when Comamonas DA1877 is diluted with another diet, suggesting that Comamonas DA1877 generates a signal that is sensed by the nematode. Surprisingly, the developmental effect is independent from TOR and insulin signaling. Rather, Comamonas DA1877 affects cyclic gene expression during molting, likely through the nuclear hormone receptor NHR-23. Altogether, our findings indicate that different bacteria elicit various responses via distinct mechanisms, which has implications for diseases such as obesity and the interactions between the human microbiome and intestinal cells.

INTRODUCTION

The amount and nutritional content of food are important determinants of organismal health and influence life-history traits such as developmental rate and fecundity. Thus, cells and organisms must sense and interpret dietary state and alter their physiology accordingly. Although the influence of caloric intake on organismal health and life-history traits is well appreciated, little is known about the physiological effects of different diets and the mechanisms involved in coordinating diet and physiology.

In response to differences in nutrient availability from different diets, metabolic networks are modulated to meet cellular and organismal needs. Further, as it is not beneficial to turn on catabolic and anabolic fluxes simultaneously, metabolites generated by a specific catabolic pathway often act as inhibitors of the opposing anabolic pathway and vice versa. Metabolic network modulation also occurs at the level of transcription; complex information processing mechanisms relay nutritional input to signal transduction pathways that impinge upon transcription factors to regulate metabolic gene expression.

The nematode C. elegans is a powerful genetic model to study the impact of diet on gene expression and life-history traits such as developmental rate, reproduction, and lifespan. C. elegans is a soil-dwelling bacterivore with a simple anatomy of fewer than 1,000 somatic cells, 20 of which form the intestine, a single organ that functions as both gut and liver with digestive as well as endocrine functions. In the laboratory, C. elegans can be fed a variety of bacterial species and strains (Avery and You, 2012; Coolon et al., 2009). The standard laboratory diet for C. elegans is E. coli OP50. In the wild, however, C. elegans is not likely to encounter E. coli; rather, its diet constitutes a variety of bacteria that grow on rotting vegetation. A number of bacteria have been isolated from soil samples containing C. elegans, including Comamonas (Avery and Shtonda, 2003). Altering the C. elegans diet can affect a number of traits, including roaming time, lifespan, and fecundity and pharyngeal pumping rate (Coolon et al., 2009; Shtonda and Avery, 2006; Soukas et al., 2009).

In response to starvation, animals can enter into larval stage 1 (L1) diapause, which is a short-term developmental delay. If food does not become available, C. elegans also employs a long-term survival strategy by entering into dauer, an alternate L3 phase (Sommer and Ogawa, 2011). Caloric restriction has been shown to decrease fecundity and developmental rate and to increase lifespan (Lakowski and Hekimi, 1998). Starvation and caloric restriction versus ample food availability exemplify extreme conditions in the dietary spectrum. However, challenges are not simply the presence or absence of food, but instead, diverse food sources with different content and quality may be encountered.

Several nutrient-response systems have been studied in numerous model systems. The target of rapamycin (TOR) pathway, for instance, detects a variety of conditions, including amino acid availability, energy levels, and stress and affects
numerous physiological processes, including growth, metabolism, and lifespan (Laplante and Sabatini, 2012). In C. elegans, the TOR ortholog let-363 is essential for development (Long et al., 2002). Other phenotypes associated with perturbation of the TOR pathway include decreased brood size and developmental rate and increased lifespan (Honjo et al., 2009; Korta et al., 2012; Pan et al., 2007; Soukas et al., 2009). The insulin/IGF signaling pathway is another major pathway that regulates lifespan, diapause, and stress response, for instance, in response to a lack of nutrients (Narasimhan et al., 2009). Insulin signaling occurs through the DAF-2 receptor and impinges on the FoxO transcription factor DAF-16. Finally, nuclear hormone receptors (NHRs) sense a variety of signals produced under specific metabolic and environmental conditions. NHRs are ligand-regulated transcription factors that affect numerous physiological processes, including development, growth, and metabolism (Pardee et al., 2011; Sonoda et al., 2008). Remarkably, the C. elegans genome encodes 271 NHRs (Reece-Hoyes et al., 2005), whereas the human genome encodes fewer than 50 (Reece-Hoyes et al., 2011; Sonoda et al., 2008). Most C. elegans NHRs are homologs of HNF4, and only a few have been characterized experimentally.

Here, we use C. elegans to investigate the relationships between diet and life-history traits and the mechanisms involved. We find that, when fed the soil bacteria Comamonas, C. elegans develop faster, lay fewer eggs, and live shorter than when fed E. coli OP50. By expression profiling, we identify a core set of C. elegans genes that differ in expression on the different diets. We establish a transgenic dietary sensor strain that harbors the promoter of one of these genes, acd-7, to drive expression of the green fluorescent protein (GFP). On the standard laboratory diet of E. coli OP50, GFP expression levels are high. In contrast, GFP expression is barely detectable when the animals are fed Comamonas DA1877. This demonstrates that the dietary response occurs at the level of transcription. Remarkably, when Comamonas DA1877 is dramatically diluted with E. coli OP50, GFP expression is low and developmental rate is accelerated. This shows that the Comamonas DA1877 effect is “dominant” over that of E. coli OP50 and that the response does not simply reflect differences in caloric intake, for instance, by mimicking starvation. We show that the developmental acceleration caused by a Comamonas DA1877 diet is independent of TOR and insulin signaling. Instead, we find that Comamonas DA1877 affects cycling gene expression during larval molts, likely through NHR-23.

RESULTS

Dietary Modification of Life-History Traits
We measured developmental rate, fecundity, and lifespan in C. elegans fed three different diets: E. coli OP50; E. coli HT115, a strain used in RNA interference (RNAi) by feeding experiments (Timmons et al., 2001); and Comamonas DA1877. The latter has been proposed to be a healthier diet because it alleviates developmental delays in Eat mutants that have defects in pharyngeal pumping and a compromised ability to eat (Avery and Shtonda, 2003; Shtonda and Avery, 2006). To assess developmental rate, we synchronized animals fed each of the diets in the L1 stage and selected one time point to monitor the developmental age of a population of animals (Figure S1 available online). We found an increased number of older animals in the Comamonas DA1877-fed population than in that fed E. coli OP50 or HT115, suggesting that Comamonas DA1877-fed animals develop faster (Figure 1A). In order to separate the effects of diet on developmental rate from recovery from L1 starvation, we switched animals from an E. coli OP50 to a Comamonas DA1877 diet midway through development. Even following recovery from starvation on E. coli OP50, animals fed a Comamonas DA1877 diet displayed accelerated development (Figure 1B). This demonstrates that effects on development are not the result of differences in recovery from starvation and are not limited to early developmental time points.

To investigate the timing of the changes in developmental rate, we used a transgenic strain harboring a molting-dependent reporter Pmlt-10::GFP-pest. This transgenic strain expresses a destabilized GFP protein under the control of the mlt-10 promoter, a gene whose expression oscillates with molting (Frand et al., 2005). We examined GFP expression during development in animals fed E. coli OP50 or Comamonas DA1877. As reported previously, animals fed E. coli OP50 displayed oscillatory GFP expression (Frand et al., 2005). Surprisingly, the amplitude of GFP oscillations was dramatically reduced in all four molting cycles in animals fed Comamonas DA1877 (Figure 1C). Thus, Comamonas DA1877 may alter developmental rate by modulating the molting program.

We measured two additional life-history traits in C. elegans fed the different diets and found that animals fed Comamonas DA1877 have a decreased average brood size of 158 eggs compared to 234 and 232 for animals fed E. coli OP50 or HT115, respectively (Figure 1D). Finally, animals fed Comamonas DA1877 exhibit a much-reduced lifespan (Figure 1E).

The Dietary Effect on Development and Lifespan Is Not Due to Pathogenic Infection
In addition to serving as food, some bacteria can also be pathogenic to C. elegans (Gravato-Nobre and Hodgkin, 2005; Tan et al., 1999). To test whether the life-history trait changes result from a pathogenic response, we used killed bacteria. Comamonas DA1877 proved difficult to kill using standard protocols (Sutphin and Kaeberlein, 2009) (data not shown). However, a combination of UV irradiation, peptone-free media, and antibiotics effectively killed both E. coli OP50 and Comamonas DA1877. On killed bacteria, both diets result in similar brood sizes (Figure 1F). In contrast, there was still a diet-induced difference in developmental rate and lifespan (Figures 1G and 1H). This demonstrates that the short lifespan and fast development are not the result of a pathogenic infection but rather are more likely to be caused by a dietary effect. Thus, we focused on these phenotypes in the rest of our study.

Diet-Induced Changes in Gene Expression
Next, we examined the gene expression changes elicited by the three different bacterial diets in young adult animals using microarray expression profiling. In animals fed Comamonas DA1877, 389 genes changed significantly (≥2-fold; p < 0.001) when compared to E. coli OP50 (Figures 2A and 2B).
Figure 1. A *Comamonas* DA1877 Diet Affects *C. elegans* Life-History Traits

(A) Developmental progression on three different diets. Synchronized N2 wild-type animals (L1 stage) were grown on three different diets as indicated on the x axis and scored after 43 hr. Larval stage was visually determined based on the stage of vulval development (see Figure S1).

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Gene ontology (GO) analysis (Ashburner et al., 2000) of the genes that change on a Comamonas DA1877 diet revealed an enrichment of terms related to molting (Figure 2C). This is consistent with the ability of Comamonas DA1877 to affect the cycling expression of mit-10 (Figure 1C). Thus, it is likely that Comamonas DA1877 affects the C. elegans molting program and, hence, the expression of associated genes. However, in contrast to what we observed with the mit-10 reporter, the majority of the molting-associated genes that change in response to diet decrease in expression in response to Comamonas DA1877. Because animals fed E. coli OP50 and Comamonas DA1877 grow at different rates, animals were collected for RNA isolation based on visual examination of developmental age in the population. We therefore wondered whether some changes in the diet-induced expression profiles could result from differences in staging of the population. Therefore, we performed an additional microarray expression profiling experiment at a later time point that is well separated from the oscillations that occur during molting. At this stage, even more genes changed in expression in response to Comamonas DA1877 (Figure 2D). This is likely due, at least in part, to the onset of reproduction. A comparison between the two expression profiling experiments revealed a set of 87 “core” genes that are affected by a Comamonas DA1877 diet at both stages (Figure 2E and Table S2). The core includes upregulated and downregulated genes that encode metabolic enzymes, as well as numerous worm-specific genes.

A Dietary Sensor in Living Animals

The expression profiles do not reveal whether these changes occur at the level of transcription or messenger RNA (mRNA) stability. Among the core genes, acdh-1 exhibits the most dramatic change in expression (Figure 2A and Table S1). We previously generated a transgenic strain that expresses GFP in the intestine and hypodermis under the control of the acdh-1 promoter and wondered whether we could use this as a “dietary sensor” in living animals (Arda et al., 2010). We integrated the Pacdh-1::GFP transgene into the C. elegans genome and fed the animals different diets. GFP expression was high on E. coli OP50, intermediate on E. coli HT115, and barely detectable on Comamonas DA1877, which recapitulates the microarray data (Figure 3A). The effect on GFP expression was transmitted to the progeny and maintained prior to the start of feeding (Figure 3B). To test whether the sensor can respond to individual nutrients, we exposed the Pacdh-1::GFP transgenic animals to 5 mM glucose on each of the three diets and found that this results in an increase in GFP expression, most notably on an E. coli HT115 diet (Figure 3C). Animals fed Comamonas DA1877 required higher glucose concentrations to induce acdh-1 promoter activity (Figure 3D). Previously, it has been shown that acdh-1 expression is reduced upon starvation (Van Gilst et al., 2005a). When deprived of food, GFP expression was indeed reduced in Pacdh-1::GFP transgenic animals (Figure 3E). Altogether, these results demonstrate that acdh-1 expression is modulated in response to dietary conditions at least in part through the activity of its promoter and thus at the level of transcription. We obtained similar results with a strain that expresses GFP under the control of acdh-2, which is homologous to acdh-1 and also changes in response to diet (Figure S2).

The Dietary Effect Is Distinct from the Starvation Response

Both a diet of Comamonas DA1877 and starvation repress the dietary sensor. Previous studies have demonstrated that caloric restriction affects life-history traits (Lakowski and Hekimi, 1998), as does a Comamonas DA1877 diet. Although—superficially—this may suggest that caloric intake differs between the different bacterial diets, caloric restriction would be expected to reduce developmental rate and increase lifespan, which is opposite to the effects of Comamonas DA1877. We performed two experiments to test whether Comamonas DA1877 confers a (partial) starvation response. In the first experiment, we used nCounter technology (Geiss et al., 2008) to compare the expression changes of endogenous acdh-1 and acdh-2 to three starvation-induced genes, acs-11, gst-4, and cpt-3, as well pgm-1, a stress-responsive gene (Tawe et al., 1998; Van Gilst et al., 2005a). We did not observe significant changes in the expression of these genes on any diet (Figure 4A). In the second experiment, we reasoned that, if Comamonas DA1877 lacks specific nutrients and thereby induces a starvation response, then combining it with E. coli OP50 would alleviate this and increase acdh-1 expression. Interestingly, however, a mixed diet of E. coli OP50 and Comamonas DA1877 resulted in barely detectable GFP expression, similar to Comamonas DA1877 alone (Figure 4B). Remarkably, even when diluted dramatically with E. coli OP50, Comamonas DA1877 still exerted a repressive effect (Figure 4B). Together, these observations demonstrate that Comamonas DA1877 is not simply nutrient poor. Indeed, we did not observe major differences in bulk protein, carbohydrate, or lipid between the different bacteria (Figure S3). A second implication of these observations is that Comamonas DA1877 generates a signal to which the animal responds.

Diluting Comamonas DA1877 with E. coli OP50 was sufficient to induce an increase in developmental rate (Figure 4C) but did not affect lifespan (Figure 4D). This suggests that developmental rate and lifespan are regulated by different Comamonas-derived effects.
signals. Alternatively, the effects may be caused by the same signal, but lifespan may be more dose dependent. Lifespan experiments require animals to be maintained on bacterial plates for longer periods of time during which the Comamonas DA1877 signal may diminish.

We performed microarray expression profiling on animals fed diluted Comamonas DA1877 (0.1%, Figure 4E). The expression profile of animals grown on diluted bacteria was very similar to that of animals grown on undiluted Comamonas DA1877. In fact, of 87 “core” genes, 67 also changed on the diluted dietary condition, although in general, the magnitude of these changes was lower (Figure 4E). These observations indicate that a small amount Comamonas DA1877 is sufficient to change gene expression as well as accelerate development.

Amphid Sensing Is Not Required for the Dietary Response

C. elegans senses many environmental cues neuronally and relays the information to the rest of the animal through the use of secreted ligands and neuropeptides (Ezcurra et al., 2011). Therefore, we tested whether amphid neurons that are exposed to the environment and that sense external signals are involved in the response to Comamonas DA1877. We crossed the dietary sensor into daf-6(e1377) animals in which socket cells are abnormal, preventing the interaction of amphid neurons with the exterior environment (Albert et al., 1981). We found that daf-6(e1377) mutants still respond to Comamonas DA1877 (Figure 5A). The dietary response of endogenous core gene acdh-1, acdh-2, and ech-6 expression is also unaffected by a mutation in daf-6 (Figure 5B). Together, these observations demonstrate that the Comamonas DA1877 signal is not sensed by amphid neurons but may rather be interpreted as the bacteria traverse the digestive tract upon ingestion.

Effects of TOR and Insulin Pathways on the Dietary Response

Insulin and TOR signaling act as nutrient response pathways and affect life-history traits in many organisms (Laplante and Sabatini, 2012; Narasimhan et al., 2009). To test their possible roles in dietary response, we knocked down known pathway components and examined the effects on GFP expression in the dietary sensor using RNAi by feeding with E. coli HT115 bacteria on which the sensor displays intermediate levels of GFP expression (Figure 3A). Although acdh-1 was previously reported as a target of daf-16 (Murphy et al., 2003), knockdown of neither daf-2 nor daf-16 markedly affected GFP expression (Figure S4). We crossed the dietary sensor into daf-2(e1370) daf-16(e1370) double mutants and observed a slight increase in GFP on an E. coli OP50 diet (Figure 5C). When these animals were fed Comamonas DA1877, however, GFP expression decreased as in wild-type sensor
animals. Similarly, in the absence of daf-16, neither the dietary response nor the developmental acceleration by Comamonas DA1877 was affected (Figures 5D and 5E). Altogether, these findings demonstrate that the insulin signaling pathway is not required for the dietary sensor and developmental timing aspects of the response to Comamonas DA1877.

Knockdown of several components of the TOR pathway reduced GFP expression in the dietary sensor, including rict-1 and ruvb-1 (Figures 5F and S4). There are two TOR complexes, TORC1 and TORC2, that induce different signaling pathways, and inhibition of either pathway decelerates development (Pan et al., 2007; Soukas et al., 2009). Wild-type animals fed E. coli HB101 develop faster than those grown on E. coli OP50, and this acceleration is suppressed by a mutation in rict-1, a TORC2 component (Soukas et al., 2009). To compare the effect of Comamonas DA1877 to that of E. coli HB101, we measured development in wild-type and rict-1(ft7) mutant animals. As previously reported, E. coli HB101 accelerated growth in wild-type animals, but not rict-1(ft7) mutant animals. In contrast, Comamonas DA1877 accelerated growth in both wild-type and rict-1(ft7) mutant animals (Figure 5G). We next tested whether rsks-1, a TORC1 pathway component, was required for accelerated growth on Comamonas DA1877. As previously reported (Pan et al., 2007), rsks-1 mutants develop more slowly than wild-type animals; however, they do develop faster on Comamonas DA1877 than on E. coli OP50 (Figure 5H). Together, these results demonstrate that, although the dietary sensor is affected by TOR pathway inhibition, the accelerated developmental rate of animals fed Comamonas DA1877 is independent of TOR.

**Nuclear Hormone Receptors Affect the Dietary Sensor**

NHRs are ligand-regulated transcription factors that act as sensors for hormones, vitamins, and lipids and play broad roles in development and physiology (Sonoda et al., 2008). Therefore, they are excellent candidates to mediate the response to dietary signals. By RNAi, we found that nhr-49, a known nutrient-response mediator (Van Gilst et al., 2005b), does not affect the dietary sensor (Figure S4).

We previously identified NHR-10 as a direct regulator of acdh-1 (Arda et al., 2010). We crossed the dietary sensor into nhr-10(tm4695) mutant animals that carry a deletion in the nhr-10 gene. As expected, we observed a strong decrease in GFP expression in nhr-10 mutants on E. coli OP50 (Figure 6A). However, GFP expression was still readily detectable, most notably in the posterior intestine. Interestingly, in nhr-10 mutants, GFP expression was further reduced on Comamonas DA1877 (Figure 6A). In addition, although nhr-10 mutants developed more slowly than wild-type animals, their...
development was still accelerated on a *Comamonas* DA1877 diet (Figure 6B). This demonstrates that NHR-10 is involved in *acdh-1* regulation but is not solely responsible for the dietary response.

The effect on the molting program by *Comamonas* DA1877 is either a cause or a consequence of accelerated development. In order to discriminate between these possibilities, we compared the oscillation of the *mlt-10* molting reporter between animals fed *Comamonas* DA1877 and *E. coli* HB101. Remarkably, although animals fed *E. coli* HB101 develop more rapidly, the oscillatory pattern of GFP expression was not affected (Figure 6C). This demonstrates that the effect is specific to *Comamonas* DA1877 and not solely a result of accelerated growth.

NHR-23 is a regulator of molting, and *mlt-10* is a known transcriptional target of NHR-23. Therefore, we asked whether *nhr-23* may be involved in the dietary response. In our microarray experiment of young adult animals, we found that the GO term “molting cycle” is enriched in the genes that change in expression on a *Comamonas* DA1877 diet. Many of these genes also change in expression in a published study where *nhr-23* knockdown was compared to control L1 larvae (Kouns et al., 2011). *nhr-23* is an essential gene, and its knockdown causes larval arrest due to molting defects (Kostrouchova et al., 1998). To circumvent this, we diluted bacteria producing *nhr-23* double-stranded RNA (dsRNA) 20-fold with bacteria containing vector alone. We examined larval development in both animals with decreased and increased *nhr-23* expression (from transgene gaIs269 that expresses an NHR-23::GFP fusion protein). Surprisingly, both knockdown and overexpression of *nhr-23* slowed development (Figure 6D). Knockdown of the exogenous copy of *nhr-23* by GFP knockdown partially suppressed the latter effect, demonstrating that it is a result of an increase in *nhr-23*. Thus, both a reduction and an increase in *nhr-23* expression decelerate development.
oscillations in mlt-10 expression are part of a tightly regulated system, controlled, at least in part, by nhr-23. Changing nhr-23 expression levels may perturb these oscillations, and adjusting to these perturbations may result in a developmental delay.

Diluted nhr-23 RNAi results in a dramatic reduction in GFP expression in adults (Figure 6E). NHR-10 is a direct regulator of acdh-1 (Arda et al., 2010). NHR-23 could therefore act indirectly, for instance, by activating NHR-10. Alternatively, NHR-23 may act in parallel to NHR-10 to affect acdh-1, either directly or indirectly. To test whether NHR-23 mediates its effect on acdh-1 expression through NHR-10, we performed nhr-23 RNAi in the nhr-10(tm4695) mutant animals carrying Pacdh-1::GFP. If NHR-23 affects acdh-1 expression by modulating NHR-10, we would expect GFP levels following RNAi of nhr-23 in an nhr-10 null animal the same as in the nhr-10 mutant alone. However, knocking down nhr-23 further reduced GFP expression (Figure 6F), demonstrating that the effect of nhr-23 on acdh-1 expression is not mediated through nhr-10.

**DISCUSSION**

Diet has major effects on the development and health of complex multicellular organisms. We and others have used the nematode *C. elegans* together with a variety of bacterial diets to unravel the phenotypic adjustments of the animal in response to diet, as well as the mechanisms involved (Coolon et al., 2009; Shtonda and Avery, 2006; Soukas et al., 2009). Different bacteria likely provide different degrees and types of nutrition to the nematode. We focused on the effects of the soil bacterium *Comamonas* that was isolated together with *C. elegans* and therefore likely represents a natural food source (Avery and Shtonda, 2003). Relative to the standard laboratory diet of *E. coli* OP50, a diet of *Comamonas* DA1877 accelerates development, reduces fecundity, and shortens lifespan. In contrast, *unc-119(ed3)* mutant animals produce more offspring on *Comamonas* DA1877 than on *E. coli* OP50 (data not shown). Thus, our findings indicate that bacterial diets are not simply healthy or unhealthy but that specific qualities of these diets may be optimal under different conditions and for different life-history traits. In addition, the response to *Comamonas* DA1877 is likely not a trait that was acquired when *C. elegans* was established as a genetic model system in the laboratory because another nematode species, *C. briggsae*, exhibits similar changes in life-history traits when fed the different diets (data not shown).

A complication in understanding the influence of diet on *C. elegans* physiology is the fact that bacteria are not only a source of food; they can also be pathogenic (Tan and Shapira, 2011). The observation that *Comamonas* DA1877 can exert its effects on development and lifespan when killed demonstrates that these effects are not due to pathogenicity. However, we noted that several genes previously reported to change in response to specific pathogens were also changed in response to the diets we tested (data not shown). In studies of the *C. elegans* pathogen response, pathogenic bacteria also serve as food, and therefore, it will be important to disentangle dietary from pathogenic effects in the future.

In the wild, it is likely that *C. elegans* encounters complex mixtures of bacterial species. Our observation that the effect of *Comamonas* DA1877 on development and gene expression occurs even when mixed with *E. coli* OP50 illustrates that the animal is capable of responding to even low amounts of particular types of bacteria. Further, this indicates that it is not the caloric content that is responsible for the effects on physiology and gene expression but rather that the bacteria generate a signal that is interpreted by the nematode. We demonstrate that this signal is not sensed by amphid neurons. Rather, it is likely that this response occurs after ingestion of the bacteria, potentially directly by cells in the intestine. Relative to *E. coli* OP50, diluted *Comamonas* DA1877 accelerated *C. elegans* development but did not affect lifespan. This may suggest that different effects of *Comamonas* DA1877 may be elicited by different signals. Future biochemical fractionation experiments with *Comamonas* DA1877 extracts may shed light on the nature of these signals and thereby further illuminate the mechanism by which it is interpreted by the nematode.

TOR and insulin represent major nutrient-sensing pathways in a number of organisms (Laplante and Sabatini, 2012; Narasimhan et al., 2009). TOR signaling is regulated by amino acid availability and stress and affects downstream processes, including protein synthesis, energy metabolism, and proliferation. Similarly, insulin signaling regulates the starvation response and lifespan. TOR and insulin signaling pathways regulate developmental rate and likely adjust this rate in accordance with cellular and environmental states. Remarkably, the developmental acceleration of *C. elegans* in response to *Comamonas* DA1877 is independent of both of these pathways. We did, however, observe changes in the expression of GFP in the dietary sensor in response to perturbation of TOR. This is likely related to the response of the dietary sensor to food deprivation, as loss of TOR signaling may mimic starvation.

NHR-10 directly binds and activates the acdh-1 promoter (Arda et al., 2010) (this study). However, NHR-10 is not solely responsible for the dietary response. In the accompanying paper in this issue of *Cell*, we find that NHR-10 is partly responsible for the response to endogenous metabolic network perturbations and identify additional NHRs that affect the dietary sensor (Watson et al., 2013). Here, we identify NHR-23 as a candidate mediator of the response to *Comamonas* DA1877. Together, these results indicate that a variety of NHRs function coordinately to ensure appropriate transcriptional and physiological responses to different exogenous and endogenous cues.

*Comamonas* DA1877 affects the *C. elegans* molting program, which is also regulated by nhr-23. *Comamonas* DA1877 dampens the oscillatory activity of the mlt-10 promoter. Further, in young adult animals, many molting genes are repressed in response to this diet—of 266 genes downregulated by nhr-23 knockdown, 39 are also repressed by *Comamonas* DA1877 (Kouns et al., 2011) (this study). These changes could be either the cause or consequence of the effect that *Comamonas* DA1877 exerts on cyclic gene expression during molting. Our observation of dampened mlt-10 oscillations demonstrates that this diet does in fact affect molting. mlt-10 is a target of NHR-23, suggesting that this NHR may be activated by
Figure 5. Analysis of Known Genes and Pathways Indicates that the Comamonas DA1877 Effect on Development Is Independent of TOR and Insulin Signaling

(A) The dietary sensor is not affected by a loss-of-function mutation in daf-6.

(B) nCounter analysis of diet-responsive genes in N2 wild-type and daf-6(e1377) mutant animals.

(C) daf-2(e1370) mutant animals respond to Comamonas DA1877 like wild-type animals.

(D) daf-16(mgDf50) mutant animals respond to Comamonas DA1877 like wild-type animals.

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**Comamonas DA1877.** In adults fed this diet, however, the expression of other NHR-23 targets is decreased, suggesting that NHR-23 may be repressed by this diet. NHRs can both activate and repress transcription (Pardee et al., 2011).

mRNA expression does not change in response to diet (data not shown), suggesting a modulation of its activity at the protein level. The *C. elegans* genome encodes six NHR-23 variants that differ in their N-terminal domains. One possibility is that diet affects different NHR-23 variants that have distinct sets of target genes at different stages in the animal’s lifetime.

Oscillating gene expression is pivotal in numerous biological processes, including the cell cycle, molting cycles, and circadian rhythms. Our data show that diet can affect oscillatory gene expression, which likely affects developmental rate. *Comamonas* DA1877 both dampens *mlt-10* oscillation and shortens its period. On the other hand, *E. coli* HB101 shortens the period without dampening the amplitude. Thus, both diets likely accelerate development by impinging on the molting program. Whereas *Comamonas* DA1877 affects developmental rate in a TOR-independent manner, *E. coli* HB101 accelerates growth in a TOR-dependent manner. Nutrition also affects developmental rate in *Drosophila* (Layalle et al., 2008). In flies, the nutritional effect can occur via the TOR pathway, which impinges on the molting hormone Ecdysone (Layalle et al., 2008). Ecdysone initiates a transcriptional cascade that activates the

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(E) Developmental progression of animals at 43 hr post-L1 synchronization of *daf-16(mgDf50)* and wild-type (N2) animals grown on *E. coli* OP50 or *Comamonas* DA1877.

(F) RNAi analysis of TOR pathway components. Animals harboring the dietary sensor were fed *E. coli* HT115 bacteria that express dsRNA for the indicated genes. See also Figure S4.

(G) Developmental progression of animals at 48 hr post-L1 synchronization of *rict-1(ft7)* mutant animals fed the indicated diets.

(H) Developmental progression of animals at 50 hr post-L1 synchronization of *rsks-1(ok1255)* mutant animals fed the indicated diets.
that even small amounts of one diet can elicit a physiological response in the presence of another. This indicates that “unhealthy” foods can illicit physiological responses in the presence of an otherwise “healthy” diet or vice versa. This has major implications for treatments for diseases affected by diet such as diabetes, obesity, and cancer. Another implication of our study for human health relates to the observation that bacteria can generate a signal that is interpreted by gene regulatory networks in nematode cells, most likely in the intestine. Numerous bacterial species, known as the microbiome, colonize the human intestine. These commensal bacteria provide numerous benefits to our health—they are important in immunity to ward off harmful bacteria, produce essential nutrients and vitamins, and regulate gut development. However, under adverse conditions, the gut flora can inflict infections or affect disease progression. It is likely that the microbiota generates a cacophony of signals that can affect the metabolic network of the surrounding intestinal cells. We propose that the nematode C. elegans can be used as a model to provide further insights into the communication between microbes and mammalian cells.

EXPERIMENTAL PROCEDURES

Strains
C. elegans strains were cultured and maintained by standard protocols (Brenner, 1974). Construction Pacdh-1::GFP was previously described (Arda et al., 2010). The extrachromosomal array was integrated by UV irradiation using standard methods (Evans, 2006) to generate VL749 wvls24 [Pacdh-1::GFP + unc-119(+)]. Integrated lines were outcrossed three times to N2 wild-type animals. The nhr-10(tm4695) mutant was kindly provided by the National Bioresource Project, Japan. Additional strains were obtained from the C. elegans Genetics Center (CGC). The daf-6(e1377) mutant was crossed into VL749 to generate VL840. Animals were genotyped, and dye filling was performed to verify the identity of the daf-6(e1377) mutant, which is dye-filling defective. Bacterial strains E. coli OP50, E. coli HT115(DE3), and Comamonas DA1877 were obtained from the CGC. GR1395 mgl519 [mt-10::GFP-pest; ttx-1::GFP] was used to monitor molting (Frand et al., 2005). The RW11029 strain [gals269 [nhr-23::TY1::EGFP·3xFLAG + unc-119(+)]] was obtained from the CGC.

Phenotypic Analysis of Life-History Traits
Animals were grown at 20°C. For all diet-specific assays, animals were grown on the appropriate diet for at least one generation prior to the assay. We measured development by first synchronizing animals by L1 arrest. Briefly, animals were grown on the relevant diet, and eggs were collected by bleaching, washed three times in M9 buffer, and allowed to hatch in M9 buffer for 18 hr. Following synchronization, animals were transferred to nematode growth media (NGM) plates and incubated at 20°C. At the indicated times in Figures 1, 4, 5, and 6, animals were washed off the plates, mounted on agarose pads, and examined on a compound microscope. Animals were visually categorized into age groups based on the development of the vulva (Figure S1). At least 40 animals were scored for each diet.

Brood sizes were determined by picking individual L4 animals onto plates containing different diets. Animals were transferred daily, and number of offspring on the plates was counted. For lifespan analysis, L4 animals were transferred onto NGM plates seeded with either of the three diets. The following day, the animals were transferred to NGM plates seeded with the appropriate bacteria. Every two days, animals were checked for pharyngeal pumping. If pumping was not observed, animals were lightly prodded with a platinum wire. If animals did not respond, they were considered dead and were scored and removed. For lifespan analysis using diluted bacteria, animals were cultured on peptone-free NGM. Killed bacterial lawns were prepared using an overnight culture of bacteria (E. coli OP50 or Comamonas DA1877). Bacterial cultures were concentrated 2-fold and
seeded on peptone-free NGM containing tetracycline and spectinomycin. When lawns were dry, plates were UV irradiated using a Stratagene crosslinker as described (Sutphin and Kaebelerin, 2009).

Expression Profiling Analysis
N2 wild-type animals were grown on each diet for one generation prior to egg collection. Eggs were collected and synchronized in L1. All animals were grown on standard NGM plates. Animals fed Comamonas DA1877 developed faster, and thus, all samples could not be collected simultaneously but were instead collected when most animals on the plates reached the young adult stage as judged by the presence of a fully developed vulva or as gravid adults as judged by the presence of eggs in the gonad. Animals were washed twice in M9 buffer, pelletized by centrifugation, and frozen at −80°C in Trizol. RNA was collected using Trizol extraction followed by DNase I treatment and cleanup using RNeasy prep kit (QIAGEN). Three biological replicates were prepared for each condition. Microarray expression profiling was performed by the Genomics Core facility at University of Massachusetts Medical School using C. elegans genome arrays that contain probes for predicted coding sequences (Affymetrix). The RMA method in the Affy package from Bioconductor was used in R to summarize the probe level data and to normalize the data set to remove across-array variation. Log-transformed data were used in subsequent analyses. Moderated T statistics in Limma (Smyth, 2004) was used to calculate significance. Significance was determined using an adjusted p value (Benjamini and Hochberg, 1995). Changes in gene expression that were 2-fold (p < 0.001) or greater were considered significant. Microarray data have been submitted to GEO (GSE43959).

nCounter mRNA Quantification
nCounter assays were performed as per manufacturer’s instructions (NanoString Technologies). Probes were hybridized with 300 ng of total RNA. Resulting counts were normalized to ama-1 mRNA levels. Probe sequences are provided in Table S3.

Mixing Bacterial Diets
Liquid cultures were grown overnight at 37°C in Luria-Bertani (LB) broth. E. coli OP50 and Comamonas DA1877 bacterial cultures were diluted to the same OD600 and mixed in ratios indicated in Figures 3F and 3G. Bacterial suspensions were spread onto peptone-free NGM to minimize bacterial growth. Eggs were immediately added to plates, and animals were allowed to develop to adults. For growth assays, eggs were prepared by hypochlorite bleaching of animals grown on E. coli OP50. Eggs were allowed to hatch in M9 to generate a synchronized L1 population.

Starvation Assay
To assay the response to starvation, animals were first grown on E. coli OP50 bacteria. L4 animals were washed five times in M9 and then transferred to unseeded peptone-free NGM plates. After 24 hr, animals were collected and examined for GFP expression.

Gene Ontology Analysis
Gorilla software (Eden et al., 2009) was used to identify GO term enrichment. Up- or downregulated genes were compared to all genes present on the array. GO terms with a p value < 10−4 are provided in Figure 2C.

RNAi Gene Knockdown
Plates were prepared by adding IPTG to a final concentration of 5 mM to NGM agar. RNAi clones were obtained either from the ORFeome RNAi library (Rual et al., 2004), the Arrhinger RNAi library (Karnath et al., 2003), or cloned into the RNAi Gateway Destination vector from ORFeome clones or using genomic DNA. E. coli HT115 RNAi cultures were grown in LB broth containing 100 µg/ml ampicillin to log phase and were concentrated by centrifugation, resuspended in 1/10 volume of M9, and added to RNAi feeding plates. VL74 or N2 animals were synchronized by hypochlorite bleaching, washed in M9 buffer, and added to the E. coli HT115 seeded plates. For nhr-23 RNAi cultures, were grown as described above. Following growth of the cultures, HT115 bacteria containing the nhr-23 RNAi construct were diluted 1/20 with HT115 bacteria containing the empty RNAi vector alone.

Generation of Molting Curves
In order to generate molting curves, embryos were collected from E. coli OP50 grown adults by hypochlorite bleaching. Animals were allowed to hatch and were synchronized in L1 by incubation in M9 for 18 hr. Animals were then added to the specified foods and incubated at 20°C. For the longer time course (Figure 1), two experiments were started 12 hr apart, and animals were scored every hour for 12 hr. Animals were scored on a fluorescence-equipped dissecting microscope. Animals with appreciable GFP expression were scored positive. At least 50 animals were scored for each time point.

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REFERENCES


EXTENDED EXPERIMENTAL PROCEDURES

Overnight cultures of *E. coli* OP50, *E. coli* HT115 and *Comamonas* DA1877 grown in LB were pelleted by centrifugation and washed five times in PBS. Bacteria were lyophilized for further characterization. Gross lipid content was measured using the sulfo-phospho-vanillin method (Izard and Limberger, 2003). Oleic acid was used as a standard to derive lipid content per dry weight. Carbohydrate content was measured using the phenol-sulfuric acid method (Dubois et al., 1951). Glucose was used as a standard to derive carbohydrate content per dry weight. Gross protein content was measured using the Bio-Rad DC Protein Assay using BSA as a standard (Lowry et al., 1951). Each assay was performed in triplicate.

SUPPLEMENTAL REFERENCES

Figure S1. Stages of Vulval Development, Related to Figure 1
**Figure S2. Effect of Bacterial Diet on Gene Expression, Related to Figure 3**

*Pacdh-1::GFP* and *Pacdh-2::GFP* transgenic animals grown on different bacterial diets. Asterisks indicate exposure times that are 10 times that of controls. Extended analysis of dietary effect as per Figure 3A.
Figure S3. Content of Bacterial Diets, Related to Figure 4
Analysis of total bulk carbohydrate, lipid and protein in *E. coli* OP50, *E. coli* HT115 and *Comamonas* DA1877. Error bars represent standard deviation from three technical replicates.
Figure S4. Effects of Nutrient-Sensing Pathways on Sensor Activity, Related to Figure 5
Analysis of GFP expression in VL749 animals (Pacdh-1::GFP) following knockdown of indicated genes.