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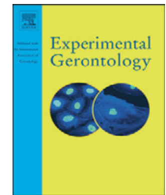

Lifespan regulation under axenic dietary restriction: a close look at the usual suspects

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ABSTRACT

In *Caenorhabditis elegans*, there are several ways to impose dietary restriction (DR) all of which extend lifespan to a different degree. Until recently, the molecular mechanisms underlying the DR-mediated lifespan extension were completely unknown but extensive efforts led to the identification of several key players in this process. Culture in sterile axenic medium is a method of DR (ADR), leading to an impressive doubling of lifespan. Earlier, we established that ADR-mediated longevity is independent of Ins/IGF signaling and *eat-2*. The only gene reported to be indispensable for the ADR lifespan effect is *cbp-1* (Zhang et al., 2009) which was confirmed in this study. In an attempt to identify more genes involved in ADR-mediated longevity, we tested several candidate genes known to regulate lifespan extension in other DR regimens. We found that *cup-4* is equally important as *cbp-1* in ADR-mediated longevity and we identified some genes that may contribute to ADR-induced longevity, but are not required for the full lifespan effect.

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1. Introduction

Dietary restriction (DR), the restriction of nutrients without malnutrition, has been shown to extend lifespan in a wide variety of species (Masoro, 2005). In *Caenorhabditis elegans*, several methods to impose DR were developed and they all extend lifespan to a different degree (reviewed in Greer and Brunet (2011)). The standard food source for *C. elegans* in laboratory cultures is the bacterium *Escherichia coli*, of which the OP50 strain is most often used (Brenner, 1974). The most straightforward DR method is reduction of the amount of bacteria, termed bacterial dilution, which can be conducted either in liquid (BDR) (Klass, 1977) or on solid agar culture (sDR) (Greer et al., 2007). Other plate methods are peptone dilution (PD) which leads to reduction of bacterial growth (Hosono et al., 1989), dietary deprivation (DD), which is the total absence of bacteria (Kaeberlein et al., 2006) and intermittent fasting (IF), in which worms are fed only every other day (Honjoh et al., 2009). Mutation in the gene *eat-2* decreases food intake by reducing pharyngeal pumping rate and is often used as well (Avery, 1993; Lakowski and Hekimi, 1998). DR-like phenotypes can also be induced using chemically defined or undefined liquid media in the absence of bacteria, called axenic dietary restriction (ADR) (Vanfleteren, 1976).

Until recently, it was completely unknown which signaling pathways are possibly involved in the DR-mediated lifespan extension. Using BDR and the *eat-2* mutant, Bishop and Guarente (2007) showed that the transcription factor SKN-1 in the ASI neurons is indispensable and other transcriptional regulators, such as PHA-4, HIF-1, HSF-1 and CBP-1 were identified as important players mediating the DR effect (Chen et al., 2009; Panowski et al., 2007; Steinkraus et al., 2008; Zhang et al., 2009). Furthermore, other genes, including the energy sensor *aak-2* (Greer et al., 2007) and two downstream targets of SKN-1, *cup-4* and *nlp-7* (Park et al., 2010) are involved in the lifespan-extending effect of DR. However, the overview in Greer and Brunet (2011) indicates that none of these genes is equally important in all DR methods, suggesting that different DR methods may activate (partially) separate pathways, instead of a single universal DR pathway, to extend lifespan.

ADR worms share the typical characteristic traits of worms subjected to other DR methods: slowed development, reduced fecundity and prolonged lifespan (Houthoofd et al., 2002; Vanfleteren, 1976). Since axenic medium is rich in peptides and amino acids, carbohydrate, vitamins and minerals, it is unclear why culturing worms in this medium leads to such an extensive lifespan extension. *C. elegans* is a filter-feeder, taking in fluids containing suspended particles and spitting out the fluid while retaining the particles (Avery, 1993). In axenic medium, there are no suspended particles of bacterial size and worms probably encounter difficulties taking up nutrients from this medium, leading to DR. Although unconfirmed, it has been suggested that axenic culture induces a failure of endocytotic uptake of nutrients in the intestinal

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lumen (Vanfleteren, 1974, 1980). It is likely that ADR-cultured worms experience cues of a rich nutritious environment but are unable to take up these nutrients efficiently. Furthermore, the ADR-mediated longevity may be partially mediated by the absence of the harmful effects of proliferating *E. coli*. It has been shown that prevention of bacterial proliferation can extend lifespan (Garigan et al., 2002; Gems and Riddle, 2000). However, this cannot completely account for the effects of culture in axenic medium, since the magnitude of lifespan increase in axenic medium (~100%) is larger than that resulting from bacterial killing (20–40%) (Garigan et al., 2002; Gems and Riddle, 2000).

Previously, it was shown that ADR-induced longevity is still occurring in *daf-16*, *daf-2* and *eat-2* mutants. Culturing *eat-2* mutants in axenic medium extends lifespan even further, suggesting that both DR methods act through separate molecular mechanisms (Houthoofd et al., 2003). Recently, Zhang et al. (2009) established that ADR-mediated lifespan extension is almost completely abolished in the absence of CBP-1 and that this gene is indispensable for lifespan extension by other DR methods as well. This was the first report on a gene indispensable for ADR-mediated lifespan extension. With our study, we attempt to identify more genes that underlie the molecular mechanism of ADR-mediated longevity.

We were able to confirm that CBP-1 is required for ADR-mediated lifespan extension and found that CUP-4 is equally important. In addition, we identified several other genes which may have a partial effect on ADR-mediated longevity.

2. Methods

2.1. C. elegans and RNAi strains

The wild-type (WT) *C. elegans* used was Bristol N2 male stock (*Caenorhabditis* Genetics Center). The mutant alleles were *aak-2(ok524)*, *cup-4(ok837)*, *ucp-4(ok195)*, *crh-1(tz2)*, *hif-1(ia4)*, *hsf-1(sy441)*, *trx-1(ok1449)*, *cep-1(gk138)*, *jnk-1(gk7)*, and *sir-2.1(ok434)* and were provided by the CGC. *skn-1(zu135);nT1[qIS51]* was a kind gift from N. Bishop and *nlp-7(tm2984)* was provided by the National Bioresource project. Most alleles are the result of deletions and are considered null alleles. *hsf-1(sy441)* and *skn-1(zu135)* are the result of a substitution and are considered nonsense alleles.

cbp-1 and *pha-4* dsRNA expressing bacterial strains were from the genomic RNAi library (produced by J. Ahringer at the Wellcome/CRC Institute). As a control, the bacterial strain containing the empty vector L4440 was used.

2.2. Axenic culture conditions

Axenic basal medium consists of 3% soy peptone (Sigma-Aldrich, St. Louis, MO) and 3% yeast extract (Becton-Dickinson, Franklin Lake, NJ), final concentrations (f.c.). Since *C. elegans* is not capable of heme synthesis, after autoclaving, the basal medium was supplemented with 0.05% hemoglobin f.c. (bovine; Serve, Heidelberg, Germany) diluted from a 100× stock in 0.1 M KOH (autoclaved for 10 min). To obtain solid plates containing axenic medium, 2% agar No. 1 (f.c.) (OXOID, Hampshire, United Kingdom) was added to the above described composition.

In the rich axenic medium bacteria can grow rapidly. Hence, it is important to handle all equipment in a sterile manner and therefore all preparations were made in a laminar flow cabinet to ensure sterility. When using axenic solid plates for lifespan determination, the assay was set-up with a sufficient number of worms (double compared to fully fed or axenic liquid conditions), as they tend to crawl off the plates in search of food.

Before starting the lifespan assays, all WT and mutant strains, except for WT worms that are used for RNAi treatment, are cultured in liquid axenic medium from egg until adulthood. This medium needs supplementation with skimmed milk to 20% final concentration to allow faster developmental rates (Houthoofd et al., 2002).

2.3. Lifespan determination

For the lifespan assays, gravid WT and mutant adults were subjected to a microbleaching procedure. Approximately ten worms were brought in a drop of 10 μL sterile distilled water. Ten microliter of a concentrated bleach solution (stock concentration 13.5° hypochlorite and 1 M NaOH) was added and left to incubate until all adults were dissolved or for maximally 10 min. Then 5 mL of axenic medium containing 20% sterile skimmed milk was added. The eggs were allowed to hatch and were incubated at 20 °C until adulthood. At L4 stage, 100 μM of FUdR was added to prevent progeny production. At adulthood, worms were transferred and exposed to the experimental conditions.

For RNAi experiments, N2 worms were first grown to adulthood on standard nutrient agar plates seeded with the *E. coli* OP50 bacteria. RNAi was carried out following standard bacterial feeding protocols (Timmons et al., 2001) in these young adult worms for five days before they were transferred to the experimental conditions.

For each strain, ten worms were placed on small NGM plates seeded with *E. coli* OP50 as fully fed (FF) control. For ADR, ten worms were placed on axenic plates, prepared as described by Lenaerts et al. (2008) (ADR solid; ADRs) or three worms were transferred with sterile Pasteur pipettes to small screw-cap tubes (5 mL tube, 75 × 12 mm, PS, Sarstedt, Nümbrecht, Germany) containing 0.3 mL of liquid axenic medium (ADR liquid; ADRl). To prevent bacterial contamination, 0.1 g/L (final concentration) ampicillin was added to the axenic conditions containing RNAi treated worms. Progeny production was avoided by the addition of 200 μM and 100 μM FUdR to the FF and ADR cultures, respectively. Survival was scored at regular time intervals: daily for the FF conditions, every other day for the ADR conditions. In solid conditions, worms were considered dead if they did not respond to gentle prodding with a platinum wire. In liquid conditions, worms were scored dead if no movement could be detected, even after gently tapping the tubes. Worms that died of ruptured vulva or crawling off the plates were censored. All lifespan assays were conducted at 20 °C.

2.4. Analysis

For statistical analysis of the obtained data, we used the online application for survival analysis (OASIS) as described by Yang et al. (2011). The relative importance of a certain gene to the lifespan-extending effect of ADR was calculated as:

$$\text{Relative importance} = 1 - \frac{\frac{\text{Mut(ADR)}}{\text{Mut (FF)}} - 1}{\frac{\text{N2(ADR)}}{\text{N2(FF)}} - 1}.$$

In which N2(FF) is the mean lifespan of wildtype under fully-fed conditions and Mut(ADR) is the mean lifespan of the mutant under axenic dietary restriction. A gene that does not contribute to the lifespan extension under ADR will have a relative importance of 0, a gene that is fully responsible for this effect will have a relative importance of 1. Because this formula cannot take into account complex genetic interactions, the calculated relative importance values may exceed the [0,1] interval. To assess whether the relative importance differs significantly from 0, the one-sample *t*-test was used. We used a Student's *t*-test to assess whether the relative importance of two genes differs significantly.

For the analysis of differences in ADRl- and ADRs-induced lifespan extension, data of all wild-type survival assays were pooled (16 experiments). The relative lifespan extension was calculated and averaged and a *t*-test was performed to assess whether the difference between ADRl- and ADRs-treated worms was statistically significant.

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