Original Article

Validated Liquid Culture Monitoring System for Lifespan Extension of *Caenorhabditis elegans* through Genetic and Dietary Manipulations

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ABSTRACT: Nutritional and genetic factors influence aging and life expectancy. The reduction of food intake without malnutrition, referred to caloric restriction (CR), has been shown to increase lifespan in a wide variety of species. The nematode *Caenorhabditis elegans* (*C. elegans*) is one of the principle models with which to study the biology of aging and search for anti-aging compounds. In this study, we validated and optimized a high-throughput liquid culture system to monitor *C. elegans* lifespan with minimized mechanical stress. We used alive and ultraviolet (UV)-killed *Escherichia coli* (*E. coli*) OP50 at 10^8 or 10^9 colony-forming units (cfu)/ml to feed Bristol N2 wild-type (WT) and mutant worms of a well-characterized insulin/insulin-like growth factor signaling (ILS) pathway: the insulin receptor homolog *daf-2* (e1370), phosphatidylinositol 3-kinase *age-1* (hx546), and transcription factor FOXO homolog *daf-16* (mu86 and mgDf50). Compared with alive *E. coli* at 10^9 cfu/ml, supplementations of alive *E. coli* at 10^8 cfu/ml or UV-killed *E. coli* at 10^9 cfu/ml dramatically prolonged lifespan in WT and *age-1* mutants, and to a lesser extent, in *daf-2* and *daf-16* mutants, suggesting that signaling pathways in CR and ILS do not overlap fully. Feeding 10^8 cfu/ml UV-killed *E. coli*, which led to maximally saturated longevity in WT and *daf-2* mutant, can prolonged lifespan in *age-1*, but not *daf-16*, mutants. This approach will be useful for investigating the biology of aging, physiological responses and gene functions under CR conditions and also for screening pharmacologic compounds to extend lifespan or affect other biologic processes.

Key words: C. elegans, aging, lifespan, caloric restriction

Aging eventually affects every living thing with an intrinsic and progressive decline in function. For decades, researchers have been exploring aging mechanisms and trying to discover methods to prolong lifespan and reduce age-related diseases [1]. Lifespan is influenced by many factors including nutrients and genetic determinants. Among them, caloric restriction (CR) is one of the most robust and reproducible interventions known to extend lifespan and delay the onset of age-related phenotypes in a wide range of species [2-5]. The efficacy of CR in primates has not yet been fully resolved, but a conclusive settlement is eagerly awaited [6,7].

The nematode *Caenorhabditis elegans* (*C. elegans*) has proved an excellent and convenient model of 2- to 3-week lifespan for the study of dietary and genetic determinants of longevity and aging [1,8]. A good example of the genetic factors in play is the

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insulin/insulin-like growth factor signaling (ILS) pathway [9]. The lifespan of *C. elegans* reportedly doubles owing to mutations in the *age-1* or *daf-2* genes, orthologs of the mammalian phosphatidylinositol 3-kinase and insulin/ insulin-like growth factor-1 receptor, respectively [10,11]. CR can further lengthen lifespan of long-lived *age-1* and *daf-2* mutants [12-14]. In addition, the FOXO transcription factor *daf-16* has been discovered to be the primary transcription factor required for the profound lifespan extension observed in *daf-2* mutants [15]. Thus, *daf-16* null mutants have shortened lifespans.

To accomplish a fixed amount of feeding for CR assays, ultraviolet (UV)-killed *Escherichia coli* (*E. coli*) instead of proliferating alive *E. coli* is provided to *C. elegans*. The feeding of UV-killed *E. coli* reportedly increases *C. elegans* lifespan 20% relative to that of animals maintained on alive *E. coli*. Toxins produced by the alive bacteria and bacterial invasion explain the vulnerability [16].

Despite accumulating results, experimental systems for monitoring lifespan of *C. elegans* are not fixed; in some cases, assays have been carried out in liquid media, whereas other studies have been used nematode growth agar medium (NGM) plates. Whereas NGM agar plates are the standard and common method for the cultivation of *C. elegans*, they have several disadvantages, including losing or killing worms unintentionally during their transfer and through desiccation after they crawl up the wall of the plate. On the contrary, a liquid culture system overcomes these disadvantages and may be more useful and convenient for testing a variety of compounds, fixed concentrations of feeding bacteria, or combinations thereof through addition to the culture media using high-throughput experiments.

In this study, we implemented a *C. elegans* liquid culture system to optimize lifespan extension using Bristol N2 wild-type (WT) and genetic mutant worms fed alive or UV-killed *E.coli* in 96-well plates. This approach will be an excellent model for high-throughput drug and chemical screenings to identify positive candidates for lifespan prolongation. This system will also be useful for studying physiological responses and gene functions in aging processes and under CR conditions.

MATERIALS AND METHODS

C. elegans *strain*

N_2 Bristol (WT), long lived mutants of *daf-2* (e1370) and *age-1* (hx546) and short lived mutants of *daf-16* (mu86) and *daf 16* (mgDf50) were used [3,17,18].
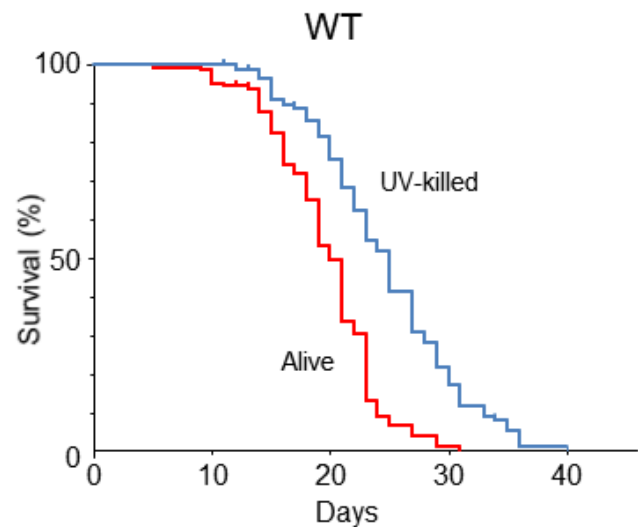

Figure 1. *C. elegans* **survival in an agar-based system**. Kaplan-Meier survival curves of N2 Bristol wild-type (WT) *C. elegans* fed alive *E. coli* (10^9 cfu/ml) and UV-killed *E. coli* (10^9 cfu/ml) on solid nematode growth medium. n = 68 (alive *E. coli*), n = 70 (UV-killed *E. coli*), Log-rank test, *p* < 0.0001. Values represent the mean ± SE of the sum of 2 independent experiments.

Culture conditions

C. elegans strains were maintained at 20ºC on NGM with alive *E. coli* OP50 as the food source as described elsewhere [19]. Liquid culture conditions of *C. elegans* with 96-well plates were used [20] with a minor modification. Briefly, worms were maintained in S complete media containing ampicillin (50 µg/ml) (Wako Pure Chemical Industries, Japan) and amphotericin B (0.1 µg/ml) (Sigma Aldrich, USA). To prevent hatching, we included 50 µg/ml 5-fluoro-2'-deoxyuridine (5-FUdR) in the media. All experiments were performed at 20ºC.

Preparation of E. coli

To assess the lifespan of worms on NGM plates, we used M9 medium before synchronization to prepare *E. coli* OP50. For the liquid culture system, ampicillin-resistant *E. coli* OP50 was used to avoid bacterial contamination. Colony-forming units (cfu) per milliliter of the *E. coli* were determined after overnight cultivation at 37 ºC on agar plates. After being counted, alive *E. coli* were irradiated with 254-nm UV light using a UV Stratalinker model 2400 (Stratagene, USA) at 9,999 J/cm^2 for 30 minutes. The complete killing of *E. coli* was confirmed by spreading UV-exposed *E. coli* on agar plates and incubating overnight at 37ºC.
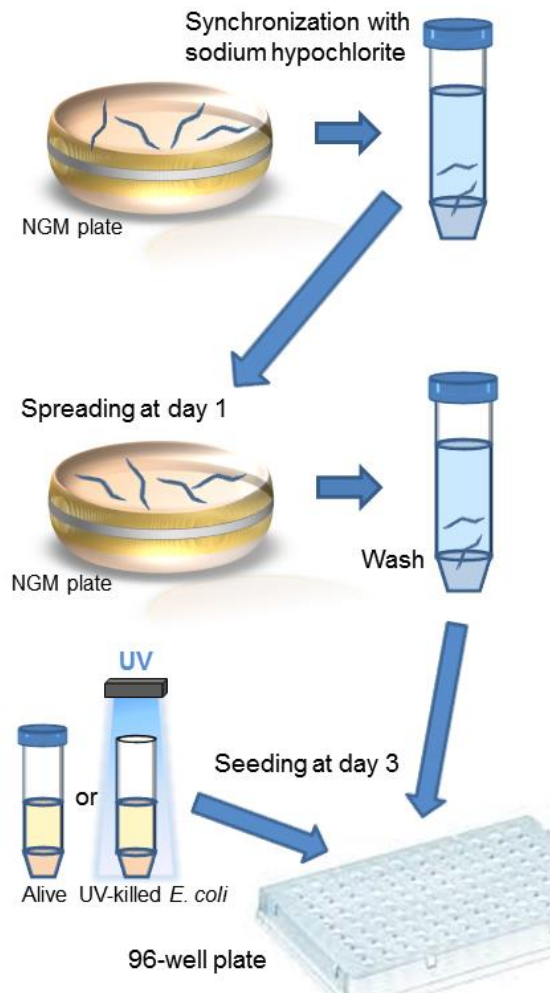
Figure 2. Schematic representation of our liquid culture protocol for monitoring *C. elegans* survival. NGM, nematode growth medium.

Lifespan determination

Worms were synchronized via hypochlorite bleaching, hatched overnight, and subsequently cultured on NGM plates. The day of synchronization was regarded as day 0. Age-synchronized worms were monitored until all were dead, which was declared when the worms did not move after repeated stimuli. For measuring lifespan on NGM plates, WT *C. elegans* were transferred to the NGM plates containing 100 µg/ml 5-FUdR and fed alive or UV-killed *E. coli* after 7 hours of synchronization. Worms were transferred to new plates using a worm picker, and their viability was simultaneously checked every other day. Animals that crawled away from the plate or showed internal hatching were excluded. Experiments were performed twice independently.

For the liquid culture system, worms hatched overnight were transferred to NGM plates (10-cm diameter) after synchronization. At the young adult stage on day 3, worms were transferred to 96-well plates with indicated concentrations of alive or UV-killed *E. coli*. Each well contained 5-15 worms in 150 µl S complete medium containing 50 µg/ml ampicillin and 0.1 µg/ml amphotericin B. After the wells were shaken for 3 minutes, worm survival was checked every other day under an inverted microscope (Axiovert 35, Zeiss, Germany) at 50x or 100x magnification. To confirm whether worms were alive or dead, we activated their movement via exposure to strong lights in some cases. Culture media containing the indicated concentrations of alive or UV-killed *E. coli* were exchanged 4 times per week.

Statistical analysis

Kaplan-Meier curves were generated using survival duration in days for each worm. We compared curves for the 2 groups with the log-rank test. All values are expressed as means ± standard error (SE). Differences in the mean values between 2 groups were assessed using the 2-tailed Student's *t*-test. Differences in the mean values among more than 2 groups were determined with analysis of variance. A value of $p < 0.05$ was considered statistically significant.

RESULTS

Most of the CR studies reported to date have been performed using agar plates to assess the lifespan of *C. elegans*. We examined first whether feeding with UV-killed *E. coli* (10^9 cfu/ml) could affect the lifespan of Bristol N2 WT worms on NGM agar plates compared with lifespan achieved with alive *E. coli* (10^9 cfu/ml) feeding. The mean, median and maximal lifespans of WT were significantly longer in the UV-killed *E. coli* group (20, 18 and 40 days) compared with those in the alive *E. coli* group (16, 15 and 24 days, respectively) (Fig. 1). This difference is compatible to that of previous reports [21].

In this study, we validated and optimized a liquid culture system for *C. elegans* for the observation of lifespan extension using Bristol N2 WT and genetic mutant worms of the insulin receptor homolog *daf-2* (e1370), phosphatidylinositol 3-kinase *age-1* (hx546), and transcriptional factor FOXO homolog *daf-16* (mu86 and mgDf50) through the feeding of alive or UV-killed *E.coli* in 96-well plates (Fig. 2). The *daf-2* and *age-1* mutants have longer lifespans, whereas *daf-16* mutants are models for shorter lifespans, compared with WT worms under non-CR conditions.
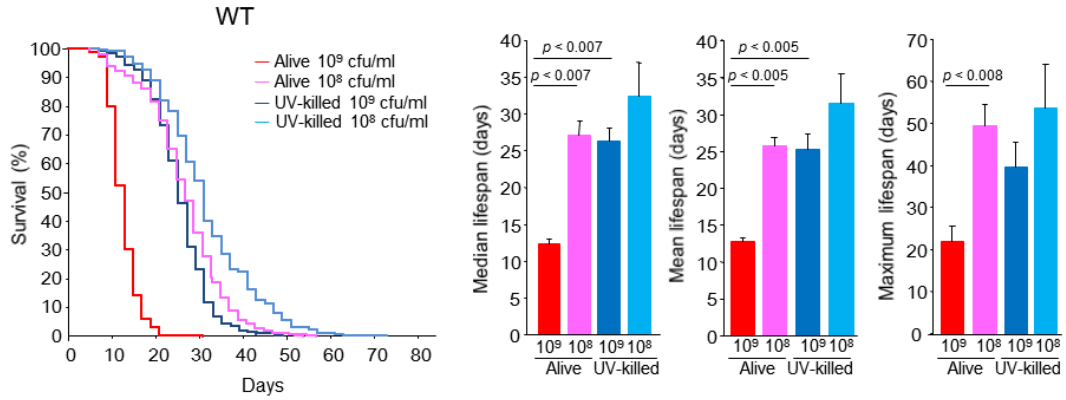
Figure 3. N2 Bristol WT *C. elegans* survival in the liquid culture system. Kaplan-Meier survival curves and median, mean, and maximal lifespans of WT *C. elegans* fed alive *E. coli* (10^9 or 10^8 cfu/ml) and UV-killed *E. coli* (10^9 or 10^8 cfu/ml). n = 396 (10^9 cfu/ml alive *E. coli*), n = 300 (10^9 cfu/ml UV-killed *E. coli*), n = 384 (10^8 cfu/ml alive *E. coli*), n = 300 (10^8 cfu/ml UV-killed *E. coli*), Log-rank test, $p < 0.0001$ between alive *E. coli* at 10^9 and 10^8 cfu/ml, between UV-killed *E. coli* at 10^9 and 10^8 cfu/ml, between 10^9 cfu/ml alive *E. coli* and 10^9 cfu/ml UV-killed *E. coli*, and between 10^8 cfu/ml alive *E. coli* and 10^8 cfu/ml UV-killed *E. coli* 10^8. Values represent the mean ± SE of the sum of 3 independent experiments.

In pilot studies, 3 concentrations of alive or UV-killed *E. coli* (10^9, 10^8 or 10^7 cfu/ml) were fed to the worms in 96-well plates. We found that feeding a 10^7 cfu/ml concentration of alive or UV-killed *E. coli* did not extend lifespan compared with feeding 10^8 cfu/ml *E. coli*, which instead shortened the lifespan of *daf-16* mutants (data not shown), suggesting that feeding 10^7 cfu/ml *E. coli* could be considered a malnutrition condition in our experimental system. Therefore, we used 10^9 and 10^8 cfu/ml of *E. coli* for further studies.

In WT *C. elegans*, feeding 10^8 cfu/ml alive *E. coli* significantly extended the mean, median and maximum lifespans of *C. elegans* 101.6%, 119.5%, and 125%,

respectively, of that achieved feeding 10^9 cfu/ml alive *E. coli* (Fig. 3). The results clearly demonstrated that CR conditions prolonged WT *C. elegans* lifespan and slowed the aging of worms in this liquid culture system. Feeding with non-proliferating and UV-killed *E. coli* is also one of the CR methods used to examine *C. elegans* longevity [22]; fixed and stable concentrations of *E. coli* are provided to the worms. Feeding 10^9 cfu/ml UV-killed *E. coli* significantly prolonged the mean and median lifespans of WT *C. elegans* to the levels seen under the CR condition with 10^8 cfu/ml alive *E. coli* (Fig. 3).
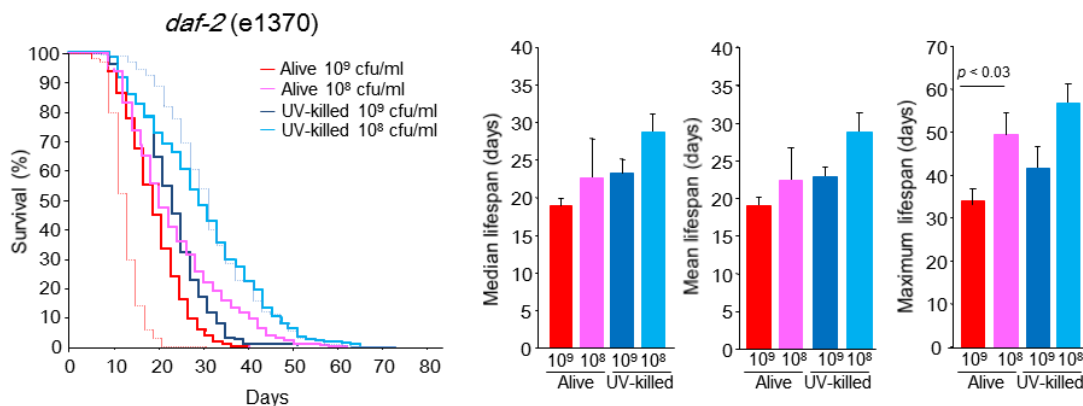

Figure 4. *daf-2* (e1370) *C. elegans* survival in the liquid culture system. Kaplan-Meier survival curves and median, mean, and maximal lifespan of *daf-2* (e1370) *C. elegans* fed alive *E. coli* (10^9 or 10^8 cfu/ml) and UV-killed *E. coli* (10^9 or 10^8 cfu/ml). Dashed red and blue lines indicate Kaplan-Meier survival curves of WT *C. elegans* fed 10^9 cfu/ml alive *E. coli* and 10^8 cfu/ml UV-killed *E. coli*, respectively. n = 350 (10^9 cfu/ml alive *E. coli*), n = 300 (10^9 cfu/ml UV-killed *E. coli*), n = 350 (10^8 cfu/ml alive *E. coli*), n = 300 (10^8 cfu/ml UV-killed *E. coli*), Log-rank test, $p < 0.0001$ between alive *E. coli* at 10^9 and 10^8 cfu/ml, between UV-killed *E. coli* at 10^9 and 10^8 cfu/ml, between 10^9 cfu/ml alive *E. coli* and 10^9 cfu/ml UV-killed *E. coli*, and between 10^8 cfu/ml alive *E. coli* and 10^8 cfu/ml UV-killed *E. coli*. Values represent the mean ± SE of the sum of 3 independent experiments.
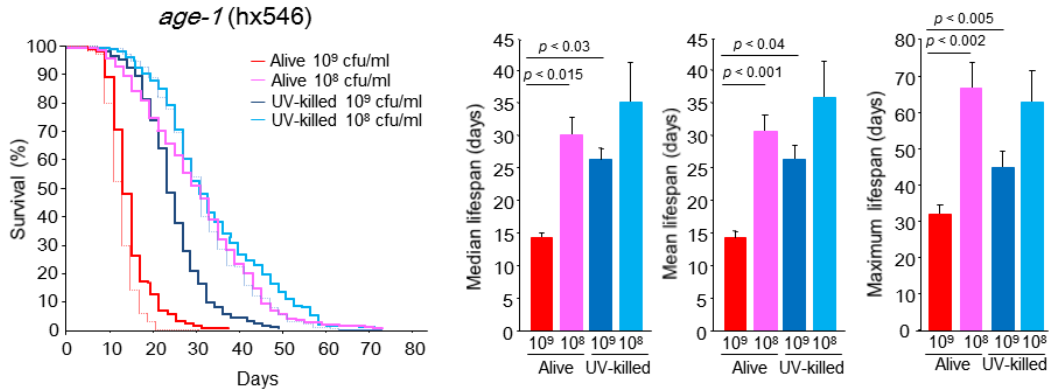
Figure 5. *age-1* **(hx546)** *C. elegans* **survival in the liquid culture system**. Kaplan-Meier survival curves and median, mean, and maximal lifespan of *age-1* (hx546) *C. elegans* fed alive *E. coli* (10^9 or 10^8 cfu/ml) and UV-killed *E. coli* (10^9 or 10^8 cfu/ml). Dashed red and blue lines indicate Kaplan-Meier survival curves of WT *C. elegans* fed 10^9 cfu/ml alive *E. coli* and 10^8 cfu/ml UV-killed *E. coli*, respectively. n = 400 (10^9 cfu/ml alive *E. coli*), n = 300 (10^9 cfu/ml UV-killed *E. coli*), n = 400 (10^8 cfu/ml alive *E. coli*), n = 300 (10^8 cfu/ml UV-killed *E. coli*), Log-rank test, $p < 0.0001$ between alive *E. coli* at 10^9 and 10^8 cfu/ml, between UV-killed *E. coli* at 10^9 and 10^8 cfu/ml, between 10^9 cfu/ml alive *E. coli* and 10^9 cfu/ml UV-killed *E. coli*, and between 10^8 cfu/ml alive *E. coli* and 10^8 cfu/ml UV-killed *E. coli*. Values represent the mean ± SE of the sum of 3 independent experiments.

Next, we undertook experiments with longer survival worm models of *daf-2* (e1370) and *age-1* (hx546) mutants. The *daf-2* mutants fed 10^8 cfu/ml alive or UV-killed *E. coli* showed survival curves that were significantly shifted to the right, but the mean and median lifespan values were not significantly longer than that of worms given 10^9 cfu/ml alive *E. coli* (Fig. 4). The *daf-2* mutants fed 10^9 cfu/ml alive *E. coli* survived longer than WT worms did under this culture condition (Figs. 3 and 4). When *age-1* mutants were subjected to 10^8 cfu/ml alive *E. coli*, a significant increase in survival was noted, and their mean, median, and maximal lifespans were significantly expanded by 107.5%, 121.6% and 106.3%, respectively, compared with those under the feeding condition of 10^9 cfu/ml alive *E. coli* (Fig. 5). In case of feeding UV-killed *E. coli*, we also observed significantly longer survival of *age-1* mutants at the concentration of 10^8 cfu/ml compared with 10^9 cfu/ml (Fig. 5). Even in the longer survival models of *daf-2* and *age-1* mutants, 10^8 cfu/ml UV-killed *E. coli* yielded maximally saturated and prolonged survival curves similar to those seen in WT worms (Figs. 4 and 5). Non-overlapping signaling pathways between *daf-2* and *age-1* mutants may be involved in survival under the feeding condition of 10^9 cfu/ml alive *E. coli*.
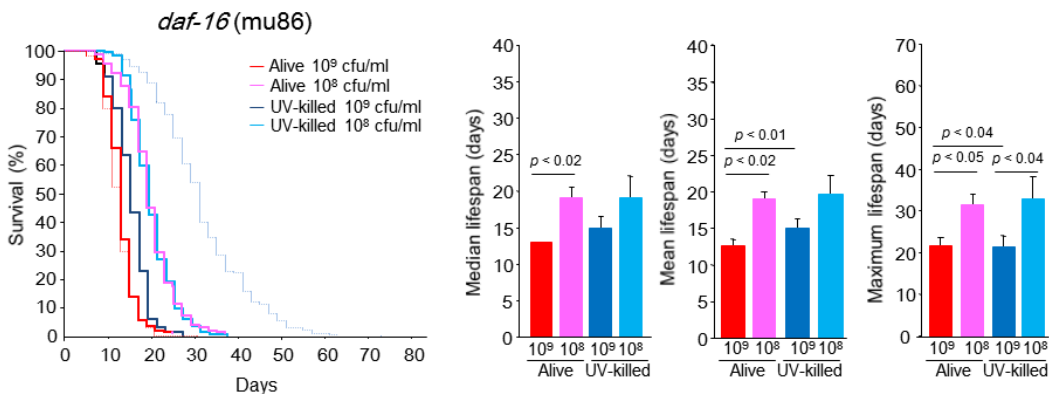

Figure 6. *daf-16* **(mu86)** *C. elegans* **survival in the liquid culture system**. Kaplan-Meier survival curves and median, mean, and maximal lifespan of *daf-16* (mu86) *C. elegans* fed alive *E. coli* (10^9 or 10^8 cfu/ml) and UV-killed *E. coli* (10^9 or 10^8 cfu/ml). Dashed red and blue lines indicate Kaplan-Meier survival curves of WT *C. elegans* fed 10^9 cfu/ml alive *E. coli* and 10^8 cfu/ml UV-killed *E. coli*, respectively. n = 400 (10^9 cfu/ml alive *E. coli*), n = 300 (10^9 cfu/ml UV-killed *E. coli*), n = 400 (10^8 cfu/ml alive *E. coli*), n = 300 (10^8 cfu/ml UV-killed *E. coli*), Log-rank test, $p < 0.0001$ between alive *E. coli* at 10^9 and 10^8 cfu/ml, between UV-killed *E. coli* at 10^9 and 10^8 cfu/ml, and between 10^9 cfu/ml alive *E. coli* at 10^9 and 10^9 cfu/ml UV-killed *E. coli*. Values represent the mean ± SE of the sum of 3 independent experiments.

When the short lifespan models of mutant worms of *daf-16* (mu86) and *daf-16* (mgDf50) were fed 10^9 cfu/ml alive *E. coli*, their survival curves were very similar to those of WT worms fed 10^9 cfu/ml alive *E. coli* (Figs 3, 6 and 7). This result led us to suspect that DAF-16 activity would be inhibited in WT animals fed 10^9 cfu/ml alive *E. coli*. The CR-induced lifespan extension of *daf-16* mutants occurred to lesser degree than that in WT worms (Figs. 6 and 7). However, in both the *daf-16* (mu86) and

daf-16 (mgDf50) mutants fed 10^8 cfu/ml alive *E. coli*, the mean, median and maximal lifespans were significantly 50%, 46.2% and 43.2% longer and 42.7%, 42.0% and 40.9% longer, respectively, than that of the groups fed alive *E. coli* at 10^9 cfu/ml (Figs. 6 and 7). The feeding of UV-killed *E. coli* improved *C. elegans* longevity but the increase did not reach the level seen in WT worms fed 10^8 cfu/ml UV-killed *E. coli* (Figs. 3, 6 and 7).
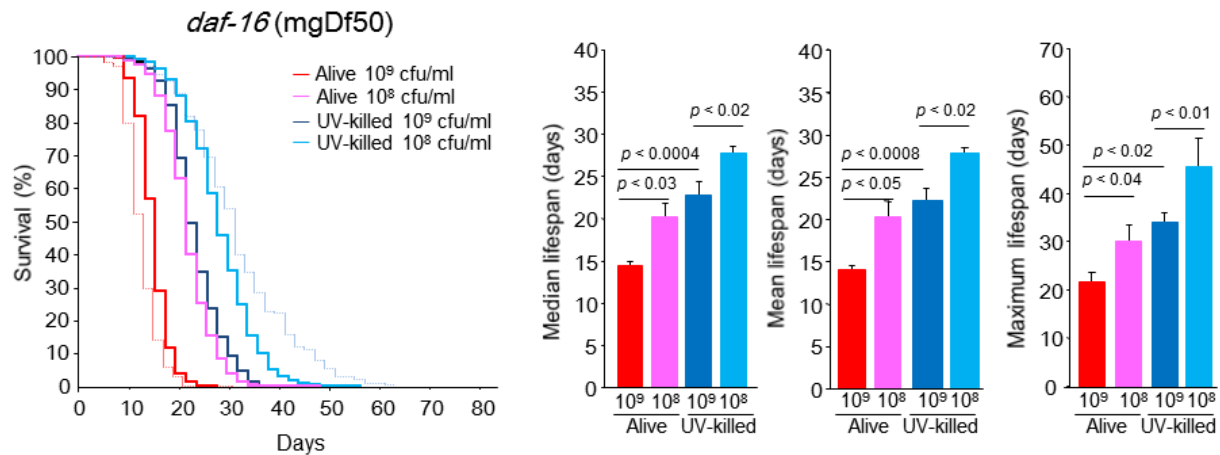

Figure 7. *daf-16* (mgDf50) *C. elegans* survival in the liquid culture system. Kaplan-Meier survival curves and median, mean, and maximal lifespan of *daf-16* (mgDf50) *C. elegans* fed alive *E. coli* (10^9 or 10^8 cfu/ml) and UV-killed *E. coli* (10^9 or 10^8 cfu/ml). Dashed red and blue lines indicate Kaplan-Meier survival curves of WT *C. elegans* fed 10^9 cfu/ml alive *E. coli* and 10^8 cfu/ml UV-killed *E. coli*, respectively. n = 400 (10^9 cfu/ml alive *E. coli*), n = 300 (10^9 cfu/ml UV-killed *E. coli*), n = 400 (10^8 cfu/ml alive *E. coli*), n = 300 (10^8 cfu/ml UV-killed *E. coli*), Log-rank test, $p < 0.0001$ between alive *E. coli* at 10^9 and 10^8 cfu/ml, between UV-killed *E. coli* at 10^9 and 10^8 cfu/ml, between 10^9 cfu/ml alive *E. coli* and 10^9 cfu/ml UV-killed *E. coli*, and between 10^8 cfu/ml alive *E. coli* and 10^8 cfu/ml UV-killed *E. coli*. Values represent the mean ± SE of the sum of 3 independent experiments.

DISCUSSION

CR is one of the well-characterized strategies for retarding senescence, slowing aging, reducing mortality, and prolonging lifespan. In *C. elegans*, the CR condition of 5 x 10^8 bacteria/ml alive *E. coli* on agar plates has been reported to increase mean lifespan by 13.2% compared with that of the non-CR condition of 5 x 10^{10} bacteria/ml alive *E. coli ad libitum* [23]. Both dilution of food bacteria and deprivation of the bacteria are known to extend the lifespan of WT *C. elegans* [24]. In mice, a 40% decrease in food intake reportedly extends lifespan up to 43% [25]. Numerous hypotheses and pathways underlie CR-mediated longevity: reduction of metabolic rate and ILS pathways, activation of AMP-activated protein kinase, glyoxylate shunt, SKN-1, and *daf-16* dependence [26-28].

In this study, we reproduced and observed significant prolongation of lifespan in *C. elegans* of *daf-2* (e1370) and *age-1* (hx546) mutants and shortened lifespan in *daf-16* (mu86) and *daf-16* (mgDf50) mutants using our liquid culture system, compared with that in WT worms at the same concentration of *E. coli* feeding (Figs. 3-7). The liquid culture system has advantages of minimized mechanical stress to *C. elegans* and a convenient method for testing compounds. CR of *E. coli* feeding from 10^9 to 10^8 cfu/ml also significantly extended lifespan in WT and all *C. elegans* mutants examined with the system (Figs. 1 and 3-7). We thus conclude that this liquid culture system will be useful for identifying positive candidates for lifespan prolongation via high-throughput drug and chemical screening. As an example of pharmacological intervention, treatment of metformin, a biguanide drug, significantly extended the

lifespan of WT worms fed with 10^9 cfu/ml alive *E. coli* (data not shown). The beneficial effect of metformin has been reported previously [29]. We are confident that this liquid system will be commonly implemented in the near future. It will be powerful to be able to screen chemicals or compounds that prolong the lifespans of *daf-2* mutants by feeding 10^8 cfu/ml UV-killed *E. coli*, which showed the maximal longest lifespan, and those of *daf-16* mutants by feeding 10^9 cfu/ml alive *E. coli*, which showed the shortest lifespan in this study. We hope that novel pathways or unique genes will be identified in these screenings using our system.

In conclusion, we used for the first time a liquid culture system to optimize lifespan extension in *C. elegans* with Bristol WT and genetic mutant worms by feeding alive or UV-killed *E. coli*. This approach was evaluated as a useful tool in the high-throughput screening of drugs, chemicals and natural compounds for lifespan prolongation and in the study of physiological responses and gene functions under CR conditions and in aging processes.

Acknowledgements

Disclosure Statement

The authors declare no competing interests.

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