Chemically defined medium and *Caenorhabditis elegans*: A powerful approach

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C. elegans has been established as a powerful genetic system. Growth in a chemically defined medium (*C. elegans* Maintenance Medium (CeMM)) now allows standardization and systematic manipulation of the nutrients that animals receive. Liquid cultivation allows automated culturing and experimentation and should be of use in large-scale growth and screening of animals. Here we present our initial results from developing culture systems with CeMM. We find that CeMM is versatile and culturing is simple. CeMM can be used in a solid or liquid state, it can be stored unused for at least a year, unattended actively growing cultures may be maintained longer than with standard techniques, and standard *C. elegans* protocols work well with animals grown in defined medium. We also find that there are caveats of using defined medium. Animals in defined medium grow more slowly than on standard medium, appear to display adaptation to the defined medium, and display altered growth rates as they change defined medium composition. As was suggested with the introduction of *C. elegans* as a potential genetic system, use of defined medium with *C. elegans* should prove a powerful tool.

Cultivation in chemically defined media was promoted at the initial suggestion of the utility of *C. elegans* as a genetic model system[1]. However, the development of a defined medium (*C. elegans* Maintenance Medium (CeMM)[2]) lagged far behind the development of *C. elegans* as a genetic system[3]. As a result monoxenic cultivation of *C. elegans* on Nematode Growth Medium agar plates with *E. coli* (NGM) is standard[4]. The ability to alter systematically the composition of CeMM should make it a staple in metabolism and aging studies. Additionally, cultivation in CeMM now allows removal of the variables introduced by monoxenic cultivation. Some of these variables impact nematode biology[5], with reduction of lifespan being the impact most recently demonstrated[6]. Monoxenic cultivation also introduces variables into pharmaceutical studies on *C. elegans*[4, 7], an example being lack of drug effect being due to *E. coli* metabolizing the drug. Large scale screening of pharmaceutical and nematicidal compounds on *C. elegans* can now be achieved when CeMM is used with equipment for automated culturing[8] and experimentation[9] (updated version available from Union Biometrica). CeMM may also be useful in growing large numbers of animals for genomic or proteomic work and in development of *C. elegans* biosensors. We made arrangements with Fisher to have a 2x stock of CeMM produced by MediaTech. All studies presented here utilized the same batch, though growth rates did not vary between two batches.

Actively growing cultures may be maintained longer using CeMM than NGM. Growth arrested L1s and dauers are observed after approximately one week on NGM, whereas healthy, reproducing populations were observed for at least one week, at room temperature, in unchanged CeMM, in all tested materials (Table 1). Healthy, reproducing populations were observed for at least one month in the flasks, Opticell™, and FEP bag. With small initial populations healthy, reproducing animals were observed after two or three months on 1.7% agar CeMM plates or in unchanged liquid medium. Significant evaporation of CeMM was noted in 75 cm^2 flasks and 10 mm tubing. The Opticell™ or

FEP bag allowed adequate oxygenation of stationary cultures and minimized evaporation. We presume animal movement was sufficient to mix CeMM, if indeed mixing CeMM is necessary to dilute wastes and evenly distribute nutrients. Use of either device also facilitated transport and shipment of animals. More sedimented animals were observed in FEP bags than in Opticells™, although sedimentation did not have obvious effects on population growth or health. In all devices, we often noted clumps of eggs in the medium, perhaps due to self-association. As many as 80% of these eggs failed to develop when clumps were placed in fresh CeMM. These results demonstrate CeMM can easily be used with a variety of laboratory devices and that CeMM grown animals require less maintenance than NGM grown animals. These results also suggest there are differences animals grown in CeMM versus on NGM.

Animals in CeMM grow more slowly than on NGM and appear to adapt to the medium. As shown in Figure 1, wildtype animals (Strain N2) grown on NGM reached a length of 1 mm in times consistent with those published[10]. Wildtype N2 animals that had been maintained in CeMM for three years prior to this study took two to three times as long to reach 1 mm in length when grown in CeMM (Fig. 1). Animals grown on 1.7% agar CeMM plates, reached a length of 1 mm in times not significantly different from those in liquid CeMM, implying growth rate differences are the effect of CeMM composition but not the physical state (not shown). When animals were switched from NGM into CeMM or from CeMM to NGM, animals grew as predicted for the medium to which they were switched (not shown). Animals developed from eggs transferred into CeMM from NGM had no gross phenotypic differences from animals exclusively grown in CeMM. Animals developed from eggs transferred onto NGM from CeMM displayed a moderate egg-laying defect (36%) and massive internal hatching (8%). These data suggest adaptation to growth in CeMM occurs and we have renamed the wildtype N2 grown in CeMM CC1. Using a wildtype strain maintained in axenic medium for several years in another laboratory (Strain LSJ1) we observed social feeding behavior when grown on NGM. In CeMM LSJ1 took two days longer to reach 1 mm than either of the other wildtype strains (N2, CC1) but started laying eggs at approximately the same time. These results suggest different adaptations to growth in axenic media are possible and that the life-cycle of animals in CeMM is altered compared to that of animals on NGM.

CeMM composition changes as the result of animal growth in the medium and growth rates are consequently altered as the medium changes. When culturing at 22.5°C, CeMM remains at pH 6 for the first week, increases to pH 7 after one week, increases to pH 8.5 after three weeks, and remains at pH 8.5 after six weeks, suggesting that animals condition CeMM. To evaluate growth in used CeMM, we used 75cm^2 flasks to grow animals at 25°C for 4.5 weeks, at which point CeMM had decreased in volume from 10 to 3.5 ml. Animals were killed by heating to 50°C for one hour. New animals added to this medium reached 1 mm one day sooner than animals grown in fresh CeMM subjected to 50°C for one hour. Particulate material, in the form of shed cuticles and dead animals, accumulates in used CeMM. Particulate material has been reported to increase the growth rate of the related species *C. briggsae* by providing physical stimulation[11]. To test if particulate material accounted for the increased growth rate we grew animals in used, heat killed CeMM subject to filtration through a 0.2 micron filter (Millipore). Animals reached a length of 1 mm three days later than animals grown in fresh CeMM, suggesting both that used CeMM is nutritionally depleted or otherwise altered in a toxic fashion, and

that the presence of accumulated particulate material improves the growth rate. It is unlikely that physical stimulation leads to increased growth, since animals grown on CeMM plates do not display similarly improved growth rates. We believe increased growth rate is the result of cannibalism of dead animals in CeMM. Accordingly, we find addition of *E. coli* strain OP50 to fresh CeMM results in growth rates not significantly different from animals grown in used, unfiltered CeMM. Together these results also suggest that animals grown in fresh CeMM are receiving suboptimal nutrients. It is important to note this is defined by growth rate which may or may not be the appropriate end point to measure[12]. These results suggest careful consideration should be given to using animals in fresh versus used media for experimentation and that composition changes must be taken into account when comparing data from separate experiments.

CeMM can be stored, but repeated handling will decrease shelf life, presumably due to degradation of components because of photosensitivity or oxidation. Animals grown at 25°C displayed an unaltered growth rate in CeMM that had been stored unused in the dark at 4°C for one year as compared to fresh CeMM. In contrast, animals displayed a variably decreased growth rate reaching 1 mm two to five days later than animals in fresh CeMM when grown at 25°C in CeMM stored in the dark at 4°C but subject to periodic removal for culturing animals over the course of a year. We find storing and handling media in small aliquots minimizes concerns with media degradation.

Protocols developed for NGM grown animals work with CeMM grown animals. Standard protocols for settling worms or bleaching eggs to create age synchronous populations were easily performed with CeMM grown animals, as were protocols for preparation of dauers, dauer recovery, and freezing of strains[4, 7]. Individual animals were manipulated using a pipettor and microscopic observation and optical measurement of animals were conducted with ease. However, due to CeMM fluorescence at low magnification, but not high, GFP was difficult to observe in transgenic animals (Strains MU1085 (*bwIs2 (flp-1::gfp; rol-6 (su1006))*) and PJ707 (*jIs01 (myo-3::GFP; rol-6 (su1006))*). The difference in GFP visualization may reflect differences in the filter sets used or may be because it is more difficult to visualize GFP at low magnification using a dissecting scope. Consistent with the *a priori* prediction that males would not mate well in liquid, we observed successful matings on CeMM agar plates but not in liquid CeMM, both in 24 well tissue culture dishes (Strains CB1489 (*him-8 (e1489)* IV) and PJ1046 (*cha-1 (p1182^{ts})* IV; *ccIs55 (unc-54::lacZ)* V)). These results demonstrate that although there may be differences between NGM and CeMM grown animals, knowledge of NGM grown animals can be applied to CeMM grown animals.

We have presented technical aspects of our initial work with animals grown in CeMM. These animals are clearly different from those grown on NGM. Those wishing to use CeMM should be aware that, as with all media, there are caveats associated with use of CeMM and potential pitfalls of applying what is known of animals on NGM to animals in CeMM. To provide an additional resource to all wishing to use CeMM we will present baseline data for the life-cycle of *C. elegans* in CeMM elsewhere.

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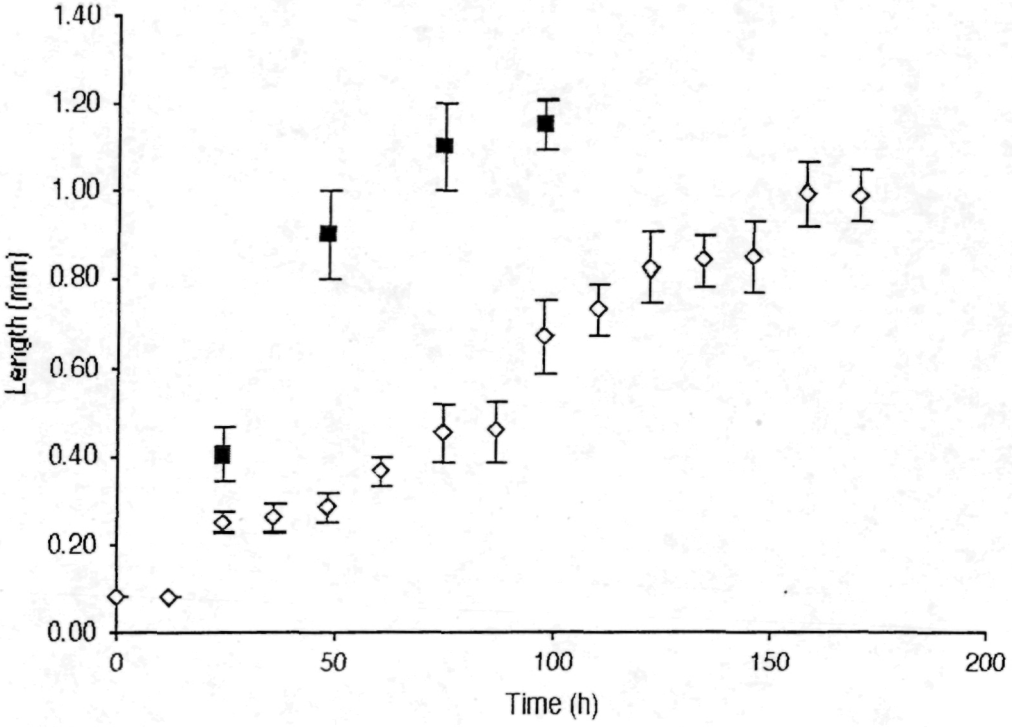
Table 1: Equipment compatibile with *C. elegans* in CeMM

Material:	Manufacturer:
Tubes:	
1 ml	Eppendorf
15 ml conical	Falcon
50 ml conical	Falcon
Dishes:	
60 mm petri	Falcon
100 mm petri	Falcon
60 mm tissue culture	Falcon
100 mm tissue culture	Falcon
24 well tissue culture	Corning
96 well tissue culture	Corning
Flasks:	
25 cm^2 cell culture	Corning
75 cm^2 cell culture	Corning
Membranous devices:	
10 mm membrane tubing	Spectrapor
Opticell™	BioCrystal
FEP bag	American Fluoroseal

Figure 1: Growth curve at 20°C for animals grown on NGM (filled squares) and in CeMM (open diamonds). Eggs were obtained by bleach treatment of animals grown for at least three generations on plates or in liquid CeMM. Eggs were placed back on plates or in CeMM at t=0. Length of ten animals was optically measured every twenty-four (NGM) or twelve (CeMM) hours, until adults were observed in consecutive timepoints.


1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
Length (mm)
0
50
100
150
200
Time (h)