MethodsX 4 (2017) 250–255

Contents lists available at ScienceDirect

MethodsX

journal homepage: www.elsevier.com/locate/mex

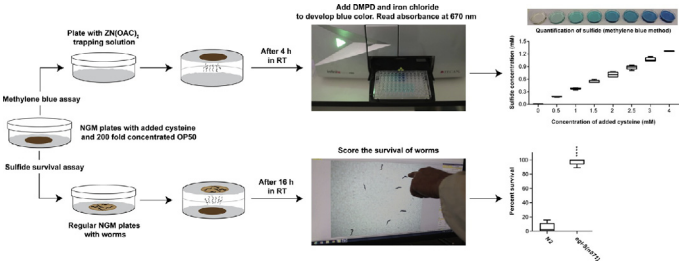
Method Article

A method for measuring sulfide toxicity in the nematode *Caenorhabditis elegans*

Leonid Livshits, Einav Gross*

The Hebrew University of Jerusalem, Faculty of Medicine, IMRIC Dept. of Biochemistry & Molecular Biology, P. O. Box 12271, Jerusalem, Israel

GRAPHICAL ABSTRACT



ABSTRACT

Cysteine catabolism by gut microbiota produces high levels of sulfide. Excessive sulfide can interfere with colon function, and therefore may be involved in the etiology and risk of relapse of ulcerative colitis, an inflammatory bowel disease affecting millions of people worldwide. Therefore, it is crucial to understand how cells/animals regulate the detoxification of sulfide generated by bacterial cysteine catabolism in the gut. Here we describe a simple and cost-effective way to explore the mechanism of sulfide toxicity in the nematode *Caenorhabditis elegans* (*C. elegans*).

- A rapid cost-effective method to quantify and study sulfide tolerance in *C. elegans* and other free-living nematodes.
- A cost effective method to measure the concentration of sulfide in the inverted plate assay.

ARTICLE INFO

Keywords: Sulfide, *C. elegans*, *egl-9*, Methylene blue, Cysteine catabolism

Article history: Received 7 April 2017; Accepted 11 July 2017; Available online 19 July 2017

* Corresponding author.
E-mail address: einavg@ekmd.huji.ac.il (E. Gross).

http://dx.doi.org/10.1016/j.mex.2017.07.005

Method details

The major goal of the method presented here is to provide a robust and economical way to explore sulfide toxicity in *C. elegans*. As such, the method does not require any expensive infrastructure, equipment, or costly sulfide releasing compounds, but rather uses the natural *C. elegans* food source (bacteria) to catabolize cysteine to sulfide. Moreover, the inverted plate setup (described below) provides a simple way to treat the worms with chemicals and RNAi expressing bacteria without the exposure of these agents to the cysteine plates. Notably, the assay can be used to explore the sulfide tolerance of other nematode species [1], and in principle can be adjusted to study the sulfide resistance of other animals/cells. In addition, we demonstrate the use of the methylene blue method [2,3] to measure the production of sulfide in the inverted assay plate. This method is accurate, as well as simple and inexpensive. We anticipate that these methods will enable researchers worldwide to explore the mechanism by which animals tolerate and adapt to high physiological levels of sulfide.

Materials

1000 ml Erlenmeyer flask (IsoLab, Germany)
15 ml sterile polypropylene tube (Miniplast, Ein-Shemer, Israel, Cat. No. 835-015-40-111)
5 ml syringe (BD Plastipak 302187)
50 ml sterile polypropylene tube (Miniplast, Ein-Shemer, Israel, Cat. No. 835-050-21-111).
Analytical balance (Mettler-Toledo)
Bacto agar (BD-Difco, Cat. No. 214010)
Bacto peptone (BD-Difco, Cat. No. 211677)
Bacto tryptone (BD-Difco, Cat. No. 211705)
Bacto yeast extract (BD-Difco, Cat. No. 212750)
Calcium chloride (Sigma, Cat. No. C1016)
Cholesterol (Sigma, Cat. No. C8667)
Corning Costar 96 well cell culture plate
Corning® 500 ml vacuum filter/storage bottle system, 0.22 μm
Costar® 5 ml serological pipettes, (Corning Inc, Product #4487)
Dipotassium hydrogen phosphate trihydrate (Sigma, Cat. No. P3786)
Double distilled water (DDW)
ELMI Intelli RM-2L mixer
Heating bath (Lab Companion BW-10H)
Heraeus™ Megafuge™ 16 centrifuge (ThermoFisher Scientific)
Hydrochloric acid (Sigma, Cat. No. 30721)
Incubating shaker (Lab Companion SIF-5000R, Jeio Tech, Korea)
Iron chloride (Sigma, Cat. No. 157740)
L-Cysteine (Sigma-Aldrich Cat. No. W326305).
Magnesium sulfate (Sigma, Cat. No. M2670)
Microlance hypodermic needle 21G x 1.5" (BD 304432)
Minisart 0.2 μm syringe filter (Sartorius Stedim Biotech, Cat. No. 16534)
N,*N*-Dimethyl-*p*-phenylenediamine (Sigma, Cat. No. 186384)
Nunc 200 ml centrifuge tube (Thermo Fisher Scientific, Cat. No. 376813)
Olympus SZ61 stereo microscope (Olympus)
Parafilm roll (Bemis, USA, Cat. No. PM-996)
Petri dish, 35 × 10 mm (Runlab Labware Manufacturing Co., Taizhou, China, Cat. No. 55801).
pH meter (Mettler-Toledo)
Platinum/iridium 0.25 mm wire (World Precision Instruments, Inc., Cat. No. PT1002)
Potassium dihydrogen phosphate (Merck, Cat. No. 1.04873.1000)
Sodium chloride (DAEJUNG Cat. No. 7548-4400)
Sodium hydroxide (Gadot, Cat. No. 830224310)

Sodium hypochlorite solution (Sigma, Cat. No. 239305)
Sodium sulfide (Sigma, Cat. No. 208043)
Tecan® Infinite® 200 PRO multimode microplate reader (Tecan Trading AG, Switzerland)
Tuttnauer cGMP pharmaceutical autoclave- stream sterilizer, model 5596 Compact
Vortex (Lab Companion AAH360115U)
Zinc acetate (Sigma, Cat. No. 383317)

Sulfide toxicity assay protocol

Generating synchronized young C. elegans *hermaphrodites*

To generate a synchronized population of L1 arrested larvae:

1. Prepare a 12 ml hypochlorite solution by mixing 2.4 ml of 2.5 N sodium hydroxide and 3 ml of 5% solution of sodium hypochlorite with 6.6 ml DDW. The final concentrations of sodium hydroxide and sodium hypochlorite are 0.5 N and 1.25%, respectively.
2. Collect the worms (gravid hermaphrodites) into a 15 ml tube by washing the nematode growth medium (NGM) plates three times with M9 buffer (22 mM KH_2PO_4, 42 mM Na_2HPO_4, 86 mM NaCl, and 1 mM $MgSO_4$).
3. Allow the worms to sediment for ~3 min and remove supernatant until 2 ml of volume remains.
4. Add 2 ml of hypochlorite solution and mix by inverting the tube five times. To assist the release of embryos, use a syringe with a 21 gauge needle to aspirate the worm suspension back and forth several times.
5. After 3 min observe the state of the worms using a dissecting stereoscope. At this stage approximately 50% of worms should appear broken and many of the embryos should float in the solution.
6. Immediately sediment the embryos using centrifugation (1690 *g* for 2 min).
7. Carefully remove the supernatant and add 10 ml of M9 buffer. Repeat the M9 washes three additional times. Remove the supernatant until 2 ml remains.
8. Rotate the tube 16 h at room temperature (RT, 21 °C).
9. Collect the hatched L1 larvae by centrifugation (1690*g* for 3 min) and put ~50 larvae per NGM plate (seeded with 100 μl OP50 bacteria).
10. Grow the L1 larvae until the young adult stage.

Preparing the cysteine plates

1. Make 100 mM cysteine solution by dissolving 0.242 g cysteine in 18 ml of DDW. Adjust the pH to 7.2 with sodium hydroxide (stock solution of 1.25 N), and then add DDW to 20 ml. Sterilize the solution by passing it through a 0.2 μm syringe filter.
2. Prepare 1 M potassium phosphate buffer pH 6 by adding approximately 150 ml of 1 M K_2HPO_4 to 500 ml of 1 M KH_2PO_4 solution of DDW. Adjust the pH to 7.2 with sodium hydroxide (stock solution of 1.25 N), and then add DDW to 20 ml. Sterilize the buffer solution by passing it through a 0.22 μm filter bottle.
3. Make 100 mM solutions of calcium chloride ($CaCl_2$) and magnesium sulfate ($MgSO_4$). Sterilize this solution via a 0.2 μm syringe filter.
4. To make NGM-agar, dissolve 3 g sodium chloride (NaCl) in 900 ml DDW. Add 20 g Bacto agar and 2.5 g of Bacto peptone. Add DDW to make a 1 l suspension. Autoclave, cool to 55 °C in a heating bath and then add using sterile technique the following solutions (mix well after each addition): 1 ml of $CaCl_2$, 1 ml $MgSO_4$, 25 ml of potassium phosphate buffer pH 6, and 1 ml of 5 mg/ml cholesterol (the cholesterol is dissolved in ethanol).
5. To make 3 mM cysteine plates, transfer 42.5 ml of NGM-agar to a 50 ml tube. Work quickly in order to prevent the solidification of the agar. Add 6 ml DDW and 1.5 ml of 100 mM cysteine solution pH 7.2. Vortex and transfer 4 ml to each 35 mm petri dish.

6. Cover the plates with aluminum foil and incubate overnight at RT.

Preparing the concentrated bacterial suspension

1. Make Luria-Bertani (LB) agar plates by dissolving 10 g NaCl, 10 g Bacto tryptone, 5 g Bacto yeast extract, and 15 g Bacto agar in 950 ml DDW. Adjust the pH to 7.0. Add DDW to make a final volume of 1L, autoclave. Cool the solution to 55 °C, and pour 25 ml per 90 mm petri dish. Let the plate to dry for 2 days at RT.
2. Prepare 2X yeast tryptone (YT) medium by dissolving 5 g NaCl, 16 g bacto tryptone, and 10 g bacto yeast extract in 950 ml DDW. Adjust the pH to 7.0. Add DDW to make a final volume of 1L and autoclave.
3. Two days before the experiment, streak an LB plate with OP50 bacteria (from a glycerol stock). Incubate at 37 °C overnight.
4. A day before the experiment, inoculate an OP50 bacteria colony from a freshly streaked LB plate into 3 ml of 2XYT medium in a 15 ml tube. Shake overnight at 37 °C, 250 rpm.
5. On the day of the experiment, transfer 1 ml from the overnight culture into 200 ml 2XYT (in a 1 L Erlenmeyer flask). Shake at 37 °C, 250 rpm, until an OD_{600} of ~0.7.
6. To make 200-fold concentrated OP50, transfer the bacteria to centrifuge bottles and spin the bacteria at 3000 *g* for 5 min. Pour the supernatant and invert the bottles on a Kimwipe to dry the pellet.
7. Resuspended the pellet with 650 μl DDW, add 30 μl 100 mM cysteine, pH 7.2, and add DDW to a final volume of 1 ml. In this way, the OP50 suspension also contains 3 mM cysteine. For control experiments, add DDW instead of the cysteine solution.

Assay procedure

1. Using a cut 1–200 μl pipet tip, put 25 μl from the OP50 suspension in each cysteine plate. For control experiments, use the same amount of OP50, but without added cysteine and use a regular NGM plate.
2. Dry the plates for 1 h at room temperature.
3. Transfer 20 young hermaphrodites to a regular NGM plate. In the experiment shown in moviefile 1, we compared the survival of N2 worms and *egl-9(n571)* mutants.
4. Invert the regular NGM plate onto a cysteine plate (or a regular plate in control experiments).
5. Seal the gap between the plates with parafilm.
6. After 16 h remove the parafilm and expose the plates to air (21% O_2) for 30 min. Use a dissecting stereomicroscope to determine the viability of the worms. Use a platinum wire to gently poke the head and tail regions. Worms that fail to respond are considered dead.
7. We use the Prism 6 software to analyze the data.

Below we present the quantification of survival of N2 wild-type worms and *egl-9(n571)* mutants (Fig. 1).

Sulfide measurement by methylene blue method

1. Make a zinc acetate, $Zn(OAc)_2$, trapping solution by mixing 4 ml sodium hydroxide (NaOH) with 4 ml DDW and 28 ml of 1% solution of $Zn(OAc)_2$. Vortex and put 4 ml of the sulfide trapping suspension in each 35 mm plate.
2. Prepare NGM agar plates containing 0, 0.5, 1, 1.5, 2, 2.5, 3, 3.5 and 4 mM cysteine and seed with 200-fold concentrated OP50 bacteria as described below.
3. Invert each cysteine plate on top of a trapping solution plate.
4. Seal the gap between the plates with parafilm.
5. Shake for 4 h at 250 rpm at RT

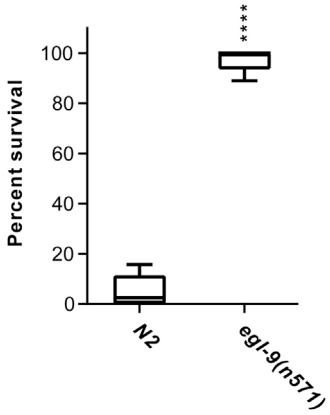


Fig. 1. Box and whisker plot comparing the survival of N2 worms and *egl-9(n571)* mutants. The 25% and 75% quartiles and median are displayed as lower and upper box edges and centreline, respectively. The whiskers specify the total range of values. Asterisks indicate significance for comparisons with N2 worms. Unpaired *T*-test. $^{****}p < 0.0001$. The graph represents the average of six independent experiments.

6. After 4 h remove the parafilm and add 500 μl of 20 mM *N*,*N*-Dimethyl-*p*-phenylenediamine (dissolved in 7.2 M hydrochloric acid) to the $Zn(OAc)_2$ trapping solution.
7. Cover the trapping solution plates with lids and shake for additional 10 min.
8. Add 500 μl of 20 mM iron chloride (dissolved in 1.2 M hydrochloric acid) to each plate.
9. Cover with lids and shake for additional 10 min.
10. To quantify the accumulated sulfide, prepare plates with $Zn(OAc)_2$ trapping solution supplemented with known concentrations of sodium sulfide (Na_2S), *i.e.* 0, 0.1, 0.2, 0.4, 0.8, 1.6, and 3.2 mM sulfide.
11. Follow the procedure described in step 6–9, but omit the 4 h incubation in step 6.
12. Transfer 200 μl of each standard solution and experimental plates to a well in a 96-well plate, make triplicates for each sample.
13. Read the absorbance at 670 nm. We use a Tecan® Infinite® 200 PRO multimode microplate reader.
14. Analyze the data using appropriate software; we use GraphPad Prism7.

Below we present the quantification of sulfide emission from the cysteine plates (Fig. 2).

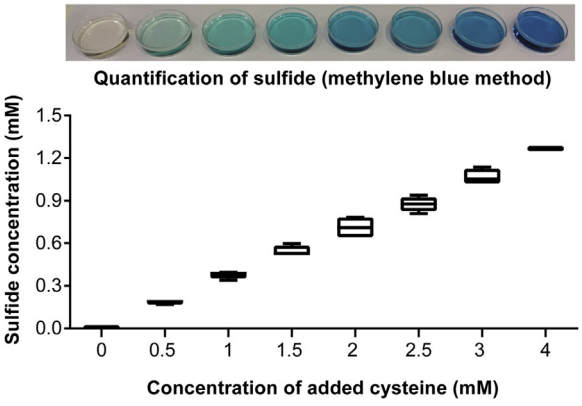


Fig. 2. Measurements of sulfide production by OP50 at divergent cysteine levels. The concentration of sulfide was measured by the methylene blue method. A representative methylene blue development is shown in the picture above the graph. The graph represents the average of four independent experiments.

Notes

1. The sulfide toxicity assay and methylene blue protocols are shown in detail in Moviefile 1.
2. We characterized the kinetics of sulfide release from OP50 bacteria in the presence of 3 mM cysteine (using the lead acetate method). Firstly, we measured the production of sulfide at various time points from the addition of OP50 to the tubes (containing 3 mM cysteine) until 16 h later. The aim of this experiment was to explore whether the production of sulfide inside the tube is constant throughout the course of the survival experiments. The production of sulfide after 16 h was similar to the production at the beginning of the experiment (Supplementary Fig. 1A), indicating that the OP50 bacteria maintain the ability to generate sulfide throughout the course of the assay. Secondly, we measured the accumulation of sulfide throughout the course of a 16 h experiment. The level of sulfide was significantly higher after 16 h incubation compared to 2 h incubation (Supplementary Fig. 1B, $p = 0.0022$, Mann Whitney test), further supporting the conclusion that sulfide is released throughout the assay.
3. The course of work described in this paper accompanies the results described in Livshits et al. [1]. In addition, Images 1 and 2 were adapted from this article.

Acknowledgements

We thank members of the Gross laboratory for comments and advice. Some strains were provided by the CGC, which is funded by National Institutes of Health Office of Research Infrastructure Programs (P40 OD010440), the *C. elegans* Knockout Consortium, and the National BioResource Project (Japan). The research leading to these results received funding from the European Research Council under the European Union's Seventh Framework Programme (FP/2007-2013)/ERC Grant Agreement no. 281844.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.mex.2017.07.005.

References

[1] L. Livshits, A.K. Chatterjee, N. Karbian, R. Abergel, Z. Abergel, E. Gross, Mechanisms of defense against products of cysteine catabolism in the nematode Caenorhabditis elegans, Free Radic. Biol. Med. 104 (2017) 346–359.
[2] A. Ang, A. Konigstorfer, G. Giles, M. Bhatia, Measuring free tissue sulfide, Adv. Biol. Chem. 2 (2012) 360–365.
[3] J.K. Fogo, M. Popowsky, Spectrophotometric determination of hydrogen sulfide, Anal. Chem. 21 (6) (1949) 732–734.