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Effect of supplementation of Iron and Enterobactin on *C. elegans* behaviour on Keio *E. coli* mutants (6-well plates)



Forked from [Testing the effect of paraquat on *C. elegans* behaviour when on Keio *E. coli* mutants \(6-well plates\)](#).

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Behavioural Genomics



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We use this protocol and it's working

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Abstract

Protocol for screening candidate behaviour-modifying *E. coli* BW25113 single-gene deletion mutants from the 'Keio Collection', to investigate their differential effects on *Caenorhabditis elegans* behaviour when supplemented with iron and enterobactin.



Materials

For bacterial culture:

- 500mL LB
- 50mL Erlenmeyer flasks

For worm maintenance and imaging plates:

- 1L NGM agar (for ingredients, see protocol for making NGM agar)
- 60mm Petri plates ('maintenance plates')
- 90mm Petri plates ('nursery plates')
- 6-well flat bottom plates ('imaging plates')

Supplements:

- Iron(III)chloride (FeCl_3) reagent grade, 97% (157740-100G Sigma-Aldrich, CAS: 7705-08-0)
- Iron(III)sulphate ($\text{Fe}_2(\text{SO}_4)_3 \cdot x\text{H}_2\text{O}$) hydrate (307718-100G Sigma-Aldrich, CAS: 15244-10-7)
- Enterobactin ($\text{C}_{30}\text{H}_{27}\text{N}_3\text{O}_{15}$) from *Escherichia coli*, $\geq 98\%$ (HPLC) (E3910-1MG Sigma-Aldrich, CAS: 28384-96-5)
- KIMTECH Science lint-free precision wipes

Troubleshooting

Safety warnings

- ! Iron and enterobactin are toxic substances, so ensure that you wear gloves and a lab coat when working with them.



Preparing NGM agar + pouring plates

- 1 Prior to screening, prepare the materials needed for screening *C. elegans* on selected Keio *E. coli* mutants:

- 6-well plates (aka. 'imaging plates')
- 15 mL Falcon tubes
- 50 mL Erlenmeyer flasks
- 90 mm Petri plates (aka. 'maintenance plates')
- 150 mm Petri plates (aka. 'nursery plates')

- 2 Make 1L normal Nematode Growth Media (NGM) agar, following the protocol:

Protocol

NAME

Making normal NGM for imaging plates (Cabreiro Lab)

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Preview

- 3 Pour 15 mL NGM agar into each 60 mm maintenance plate, and 35 mL NGM agar into each 90 mm nursery plate, following the protocol for Plate pouring (dx.doi.org/10.17504/protocols.io.6bhhaj6).
Keep the remaining agar warm in a water bath set to 65°C, for pouring into 6-well imaging plates afterwards
- 4 Using the Integra ViaFill, dispense 4 mL NGM agar into each well of the 6-well plates, following the protocol:



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Dispensing agar into multiwell plates

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- 5 Leave the plates on the lab bench (with lids on) until the agar has cooled and solidified (approximately 1 hour, timing depends on humidity)
- 6 Measure the weight of 3 imaging plates (with lids on) and record average plate weight on day of pouring
- 7 Dry the imaging plates under a hood (or drying cabinet) until the plates lose between 3-5% of their original plate weight (with lids on)
- 8 Store the imaging plates upside-down at 4°C until used for experiments

Preparing worms

- 9 Inoculate 10ml LB broth media with *E. coli* BW25113 (Keio background wild-type strain, used as negative control and for raising worms, no Kanamycin) in an Erlenmeyer flask for overnight culture following the protocol:

Protocol

NAME


Inoculating a Liquid Bacterial Culture

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- 10 Place the inoculation in a shaking incubator at 37°C at 200 rpm and leave to grow overnight
- 11 Remove the BW culture from the shaking incubator and place in 4°C fridge until seeding
- 12 Remove the plates from storage and the BW culture from the fridge, and leave on the bench for approximately 30 minutes to acclimate to room temperature
- 13 Using aseptic technique, seed the 60 mm maintenance plates each with approximately 250 µL of BW25113 culture
- 14 Leave under hood until dry (with lids on, timing depends on humidity)
- 15 Using a platinum pick, gently pick 30 adult N2 Bristol *C. elegans* onto each maintenance plate, and store in an incubator at 20°C
- 16 After 24 hours, remove the adult worms, leaving the eggs behind to hatch into L1 larvae
- 17 Inoculate a further 10 mL LB broth with BW25113 bacteria for overnight culture (no Kanamycin), following the protocol in  and place in a shaking incubator at 37°C, 200 rpm
- 18 After 24 hours, remove the culture from the incubator, and the 90 mm nursery plates from storage, and leave to acclimate on bench top for 30 minutes
- 19 Seed the nursery plates each with approximately 1 mL of fresh BW25113 culture. Leave under hood until dry
- 20 Wash the worms off the BW-seeded maintenance plates, into two 15ml Falcon tubes
- 21 Perform an egg prep on worms in the Falcon tubes, following the protocol:



Protocol

NAME

Egg Prep for Bleach Synchronization (Cabreiro Lab)

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- 22 At around noon the next day, wash L1-arrested larvae off the empty plate and re-feed onto the BW-seeded nursery plates using a glass Pasteur pipette. Aim to dispense around 500 worms per plate.
- 23 Incubate at 20°C for 68 hours until the worms are Day 1 adults for the experiment

Preparing bacteria

- 24 Fill 2 separate Erlenmeyer flasks with 25 mL LB. Add 50µg/ml Kanamycin to one flask, and leave the other flask without Kanamycin for the BW25113 control.
- 25 Remove the required Keio frozen stock plates from -80°C containing the strains for antioxidant testing. Gently remove the aluminium film and leave to partially thaw for a minute or so

Safety information

To avoid damaging the bacterial stocks through repeated freeze-thawing, do not let the wells completely defrost. Just enough to be able to pick up some cells with the replicator.

- 26 Inoculate the Erlenmeyer flasks with the desired strains for antioxidant testing from Keio frozen stock plates, following the protocol:



Protocol

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Inoculating a Liquid Bacterial Culture

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- 27 Incubate the cultures overnight at 37°C in a shaking incubator at 200 rpm.
- 28 Remove the overnight cultures from the incubator. Inoculate 2 more Erlenmeyer flasks for a second round of overnight cultures from the first, this time without Kanamycin (to avoid exposing the worms to the antibiotics), and incubate overnight at 37°C at 200 rpm.
- 29 After 24 hours, remove the cultures from the incubator and store at 4°C until used for experiments

Seeding imaging plates (6-well)

- 30 Remove the imaging plates from 4°C storage
- 31 Ensure that imaging plates have lost approximately 3-5% of their original weight (so that they are not too wet for imaging when seeded). Place under a hood or drying cabinet until they have.
- 32 Remove overnight cultures of Keio strains from 4°C storage. Using a pipette, seed 30 µL of bacterial culture into the wells of each 6-well imaging plate.
- 33 Place the seeded plates under a laminar flow hood to dry for 20 minutes, then place in an incubator at 25°C (no shaking) for 7 hours 40 minutes (total lawn growth time: 8 hours)
- 34 After 8 hours total growth time, remove the plates from the incubator and store at 4°C

Adding iron and enterobactin (6-well)

- 35 On the day of tracking, remove the seeded imaging plates from 4°C, and dry for 30 minutes under a laminar flow hood
- 36 Remove the iron(III)chloride, iron(III)sulphate and enterobactin from 4°C.
 - Prepare 100 and 400 mM of iron(III)chloride and iron(III)sulphate (in H₂O)
 - Prepare 1 mg/mL enterobactin solution (in DMSO)
- 37 Using a pipette, dispense 40 µL of iron(III)chloride and iron(III)sulphate into the desired wells (on top of the lawns) of the 6-well imaging plates (for a final concentration of 1 and 4 mM iron in 4 mL agar)
- 38 Using a pipette, dispense 5 µL of enterobactin solution on top of the lawns of the desired wells in the 6-well imaging plates (for a final concentration of 1.25 µg/mL enterobactin in 4 mL agar)
- 39 Leave the plates to dry in a biosafety hood for 30 minutes.
- 40 Record the weight of the plates after drying (as weight at imaging).
- 41 Then leave the plates on the bench (with lids on) for a further 1 hour 30 minutes (total 2 hours with the supplements added) before adding worms.

Picking worms + Hydra tracking (6-well)

- 42 Prior to tracking, ensure that the imaging cave air conditioning is turned on (and there has not been a power-cut) and also empty the dehumidifier waste water tray (see pre-imaging checklist)
- 43 Remove the nursery plates of worms from the 20°C incubator.
- 44 Using a platinum worm pick, carefully pick 10 Day1 worms onto the edge of the bacterial lawns in each well of the 6-well imaging plates, then place in incubator at 20°C until



tracking (after 4 hours on food + supplements).

- 45 30 minutes prior to tracking with the Hydra rig (each run is performed every 20-30 minutes), remove 5 imaging plates at a time from the 20°C incubator and leave to acclimate in the imaging cave.
- 46 Wipe the underside of the lids using KIMTECH Science lint-free precision wipes (to remove any condensation that has formed)
- 47 Place the plates under the Hydra rig and record worm behaviour on the bacterial food for 15 minutes (at the 4-hour timepoint, 25 fps, exposure: 25000 msec, blue-light stimulation)
- 48 After tracking, discard the plates in a biological waste bin
- 49 Check tracking checklist to ensure that all videos have been saved correctly:
'/Volumes/behavgenom\$/Documentation/Protocols/analysis/tracking-checklist-20210210.docx'