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[^{14}C] Linoleic Acid Uptake and Fractionation Assay in *Vibrio cholerae*

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[Abstract] The gram-negative curved bacillus *Vibrio cholerae* (*V. cholerae*) causes the severe diarrheal illness cholera. The work presented here is to assess whether unsaturated fatty acids (UFAs), such as linoleic acid, have the potential to directly affect proteins involved in DNA binding because they are able to enter the cell. In this protocol, we show how to measure linoleic acid entering *V. cholerae* when added exogenously and determine whether it is able to enter the cytoplasm. This protocol will quantify how much linoleic acid is able to enter the cell and then identify the amount of linoleic acid that stays in the membrane or ultimately enters the cytoplasm.

Materials and Reagents

1. Scintillation vials (Thermo Fisher Scientific, catalog number: 03-337-20)
2. Autoclaved 1.7 ml microcentrifuge tubes (BioExpress, catalog number: C-3262-1)
3. Test Tubes (Thermo Fisher Scientific, catalog number: 14-955E)
4. 15 ml conical screw cap tubes (BioExpress, catalog number: C-3394-2)
5. *Vibrio cholerae* classical biotype strain O395
6. ^{14}C linoleic acid (PerkinElmer, catalog number: NEC501050UC)
7. Scintillation cocktail (Thermo Fisher Scientific, catalog number: SX18-4)
8. Tryptone (Thermo Fisher Scientific, catalog number: B211705)
9. Yeast Extract (Thermo Fisher Scientific, catalog number: B288620)
10. Sodium chloride (NaCl) (Thermo Fisher Scientific, catalog number: BP358-212)
11. Potassium chloride (KCl) (Thermo Fisher Scientific, catalog number: BP366-500)
12. Sodium phosphate (Na_2HPO_4) (Thermo Fisher Scientific, catalog number: BP332-500)
13. Potassium phosphate (KH_2PO_4) (Thermo Fisher Scientific, catalog number: BP362-500)
14. Tris-Base (Thermo Fisher Scientific, catalog number: BP152-500)
15. 95% ethanol (Decon Labs, catalog number: 2805HC)
16. Dry ice
17. LB-Lennox (pH 6.5) (see Recipes)
18. 10x PBS (see Recipes)
19. 20 mM Tris-Base (pH 8.5) (see Recipes)

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20. 500 mM NaCl (see Recipes)

Equipment

1. Shaker-capable of shaking at 200 rpm at 37 °C (VWR International, New Brunswick Scientific, model: Excella E25)
2. Water bath shaker-capable of shaking at 30 °C at 200 rpm (VWR International, New Brunswick Scientific, model: Classic C76)
3. LS6000IC liquid scintillation counting system (Beckman Coulter)
4. Timer
5. Autoclave
6. Biomate 3S Spectrophotometer-capable of reading at OD_{600} (Thermo Fisher Scientific)
7. Semimicro Cuvettes (Thermo Fisher Scientific, catalog number: 14-955-127)
8. Table top Centrifuge-capable of spinning at 15,000 rpm at 4 °C (Eppendorf, catalog number: 5424)
9. Micropipettes (1,000 µl, 200 µl, 20 µl)
10. 250 ml Erlenmeyer flask

Procedure

A. [^{14}C] linoleic acid uptake

1. Use one colony of *V. cholerae* classical biotype strain O395 to start an overnight culture in a test tube containing 5 ml standard LB at 37 °C and 200 rpm shaking.
2. After overnight growth, subculture the bacteria 250 µl in 10 ml LB pH 6.5 (1:40 ratio) in an Erlenmeyer flask, and grow for 2 h in a water bath shaker at 30 °C.
3. At 2 h, record the OD_{600} of the culture. Expect an OD_{600} of between 0.2 and 0.3.
4. Transfer the culture to a 15 ml falcon tube and add 0.1 µCi (1 µl) of ^{14}C-radiolabeled linoleic acid for each milliliter of the subculture (final concentration of about 3.2 mM linoleic acid). This tube can stay on the bench top for the duration of the experiment or, if desired, put at 37 °C without agitation between aliquots.
5. Upon addition of the radiolabeled linoleic acid, extract 1 ml of the culture and immediately centrifuge in a 1.5 ml microcentrifuge tube at 15,000 rpm for 3 min at room temperature. This is t = 0. In order to compare the counts per minute (CPM) of ^{14}C in the supernatant and the cell pellet, transfer the supernatant in a 1.5 ml tube and wash the cell pellet 3 times with 1 ml of 1x PBS and centrifuge each for 3 min.
6. Resuspend the cell pellet in 100 µl of 1x PBS and add to 5 ml of scintillation cocktail per scintillation vial. In order to compare the amount of ^{14}C-radiolabeled linoleic acid not taken up by *V. cholerae*, add 100 µl of the supernatant fraction to another vial with 5 ml scintillation cocktail.

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7. The same procedure can be repeated for other aliquots of the subculture at times 5, 15, and 30 min or whatever time points are desired. All scintillation vials can be stored at room temperature and read together after the last time point.
8. After uptake, counts per minute are measured for each time point using a scintillation counting system.
9. Data are then analyzed using the cpm determined from the cell pellet fraction and an equation of best fit determined. To determine how much ^{14}C-radiolabeled linoleic acid is not taken up as a check of accuracy, use cpm determined from the supernatant fraction and multiply by 10 as only 100 µl of the 1 ml was used. The amount of ^{14}C-radiolabeled linoleic acid in the cell pellet is graphed below for each time point:

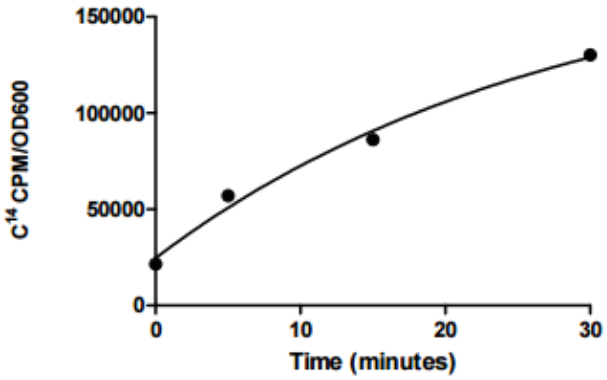

Figure 1. ^{14}C-radiolabeled linoleic acid uptake by *V. cholerae*. The equation of best fit is $y = 24622 + 156786*(1 - e^{-0.036x})$ with an R^2 value of 0.989.

B. Fractionation of *V. cholerae* to determine localization of [^{14}C] linoleic acid

1. After overnight growth at 37 °C as described in step A1, subculture *V. cholerae* O395 classical biotype 250 µl in 10 ml LB (pH 6.5) (1:40 ratio) and grow in a water bath shaker at 30 °C for 2 h.
2. At 2 h, add 0.1 µCi of ^{14}C-radiolabeled linoleic acid to 1 ml of the subculture and incubate at room temperature for 1 h without agitation. This time is chosen to ensure the bacteria are still in log-phase growth.
3. Harvest the bacteria by centrifugation (15,000 rpm) at room temperature for 3 min and wash the pellet three times with 1 ml 1x PBS at 15,000 rpm for 3 min each time.
4. Resuspend the bacteria in a 500 µl solution of 20 mM Tris-Base (pH 8.5) and 500 mM NaCl. Freeze the suspension in an ethanol (at least 95%) and dry ice bath for 2 minutes ensuring the tube is fully submerged, and then put at 37 °C until thawed. Repeat this freeze-thaw process for a total of three freeze-thaw cycles to ensure complete fractionation.
5. Fractionate the bacteria by centrifugation for 10 min at 15,000 *x g* at 4 °C to separate the membrane and cytoplasm. The cytoplasm is taken as the supernatant and the

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pellet (cell envelope fraction) is washed 3 times in 500 µl of 1x PBS by centrifuging for 3 min at 15,000 *x g*. Expect a very small pellet at the bottom of the tube. The amount of ^{14}C-linoleic acid in each fraction is determined by adding the entire fraction to 5 ml scintillation cocktail, followed by measurement of CPM in a scintillation counter.

6. Data are then analyzed as percent ^{14}C-linoleic acid in each fraction. A representative graph is shown below:

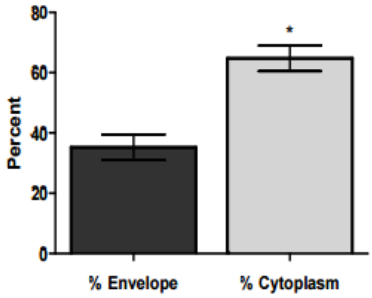

Figure 2. Percentages of ^{14}C-radiolabeled linoleic acid in cytoplasm and envelope fractions

Recipes

1. LB-Lennox (500 ml) (pH 6.5)
   5 g tryptone
   2.5 g NaCl
   2.5 g yeast extract
   Add about 400 ml of water to dissolve and mix the components
   pH to 6.5 with HCl and bring up to 500 ml with water
   Autoclave before use
2. 10x PBS (1 L)
   80 g NaCl
   2 g KCl
   14.4 g Na_2HPO_4
   2.4 g KH_2PO_4
   To use, dilute 1:10 (such as 10 ml brought up to 100 ml with autoclaved water)
3. 20 mM Tris-base (pH 8.5) (100 ml)
   0.24 g Tris-base
   pH to 8.5 with HCl and bring up to 100 ml with water
   Autoclave before use
4. 500 mM NaCl (100 ml)
   2.92 g NaCl

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This work is modified from previous work done in our laboratory (Thomson and Withey, 2014).

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