66 Measurement of Hydrocarbon Transport in Bacteria

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Abstract: Hydrocarbon uptake by bacteria has not been extensively studied, and strong evidence for active transport of hydrocarbons is lacking. The volatile nature of hydrocarbons, their hydrophobicity, and their relatively low aqueous solubilities can complicate the execution of transport assays and the interpretation of the resulting data. Here we present a detailed protocol for measuring hydrocarbon uptake in bacteria, and suggest key control experiments necessary to obtain conclusive results.

1 Introduction

The mechanism for intracellular accumulation of hydrocarbons by bacteria that utilize these potentially toxic compounds as carbon and energy sources is not well-established. Because hydrocarbons are naturally hydrophobic, they can freely diffuse through the cytoplasmic membrane of cells, and if unregulated can cause intracellular toxicity (reviewed in Sikkema et al., 1995) (Chapter 52, Vol. 2, Part 9). Therefore, bacteria that use toxic molecules as sources of carbon and energy are presented with an interesting problem; cells must acquire sufficient amounts of hydrocarbons to allow growth but also manage that amount such that the concentration does not reach toxic levels. Whether cells that utilize hydrocarbons expend energy to accumulate these types of molecules is a question that remains unanswered (Chapter 50, Vol. 2, Part 8).

Currently, there is little scientific evidence to suggest that active transport mechanisms are involved in hydrocarbon uptake. Although genes and proteins that allow passage of hydrocarbons across the outer membrane have been identified (Kahng et al., 2000; Kasai et al., 2001; Mooney et al., 2006; Wang et al., 1995), there is little compelling evidence for active transport of hydrocarbons across the cytoplasmic membrane. Based upon the hydrophobic nature of these types of molecules, the general argument is that hydrocarbon transporters are not necessary because the molecules can freely permeate the cytoplasmic membrane. In addition, genes encoding cytoplasmic transport proteins have not been identified in known gene clusters for hydrocarbon catabolism, and specific hydrocarbon transport mutants have not been isolated. However, transporter proteins and active transport mechanisms have been identified for aromatic acids, which were also previously thought to accumulate in a passive manner (D'Argenio et al., 1999; Leveau et al., 1998; Nichols and Harwood, 1997). Further scientific investigations are needed to definitively determine whether active transport mechanisms exist for aromatic or aliphatic hydrocarbons.

One possible reason that active transport mechanisms have not yet been identified may be because transport studies using hydrocarbons as substrates are technically difficult to carry out due to the volatile nature of hydrocarbons and the tendency of these molecules to adsorb to plastic and glassware. To definitively determine if active transport is utilized for uptake of hydrocarbons, careful measurement of hydrocarbon accumulation is required. Experiments that examine the possibility of active transport of hydrocarbons should include assays with both wild-type strains and appropriate catabolic mutants to distinguish between hydrocarbon uptake and accumulation of metabolites derived from the hydrocarbon substrate. In particular, for substrates that can diffuse across cellular membranes, it is necessary to rule out the occurrence of diffusion "pulled" by metabolism, which maintains a downhill concentration gradient of substrate. If the role of a putative transport protein is being assessed, results of

assays with the wild-type strain and a mutant lacking the transport protein-encoding gene can be compared. In addition, an experiment in which the transport protein-encoding gene is expressed in a heterologous host that does not metabolize the hydrocarbon of interest is a good way to separate uptake from metabolism. Additional assays in the presence of metabolic inhibitors are also essential to determine whether active (i.e., energized) transport is required for substrate accumulation (see below).

The protocol described in this chapter is a combination of various methods present in the literature that have been designed to assess aromatic acid and aromatic hydrocarbon transport in various microorganisms, using radiolabeled substrates (Bateman et al., 1986; Bugg et al., 2000; Nichols and Harwood, 1997). The general experimental approach described below is designed to address all of the issues discussed above in respiratory bacteria such as pseudomonads. However, appropriate modifications will be necessary for studying hydrocarbon transport in specific bacterial strains. In particular, changes in the growth conditions and cell preparation protocols should be introduced to suit the particular microorganism under study.

2 Experimental Approach

2.1 Procedures

2.1.1 Preparation of Transport Assay Mixture

The most important requirement for the transport cocktail is that it contains a saturating amount of substrate for the transport system under investigation. Saturating levels of substrate need to be experimentally determined for each individual transporter/substrate pair before transport assays can be designed. This can be particularly difficult when assessing a volatile compound. In addition to the substrate being transported, an additional energy source may be supplied to ensure that the cells are energized for active transport. All of the substrates can be diluted in the same buffer system used to harvest and maintain the cell suspension before the assay. For the assay mixture described below, succinate and glucose are provided as energy sources; alternative energy sources may be used depending on the organism under study. Similarly, the choice of buffer (phosphate buffer pH 7 is used here) may be changed for the particular system under study.

Steps in the Procedure

Due to the volatile nature of many hydrocarbons, all of the steps in the assays should be carried out in a fume hood. In addition, established precautions should be taken for containment and proper disposal of radioactive materials.

A1. For one transport assay, combine the following:
1.2 μl 1.0 M glucose
1.2 μl 1.0 M sodium succinate
6.0 μl 5 mM unlabeled (cold) hydrocarbon substrate (see note 1)
3.0 μl 3 mM ^{14}C-labeled hydrocarbon substrate
288.6 μl 25 mM phosphate buffer, pH 7

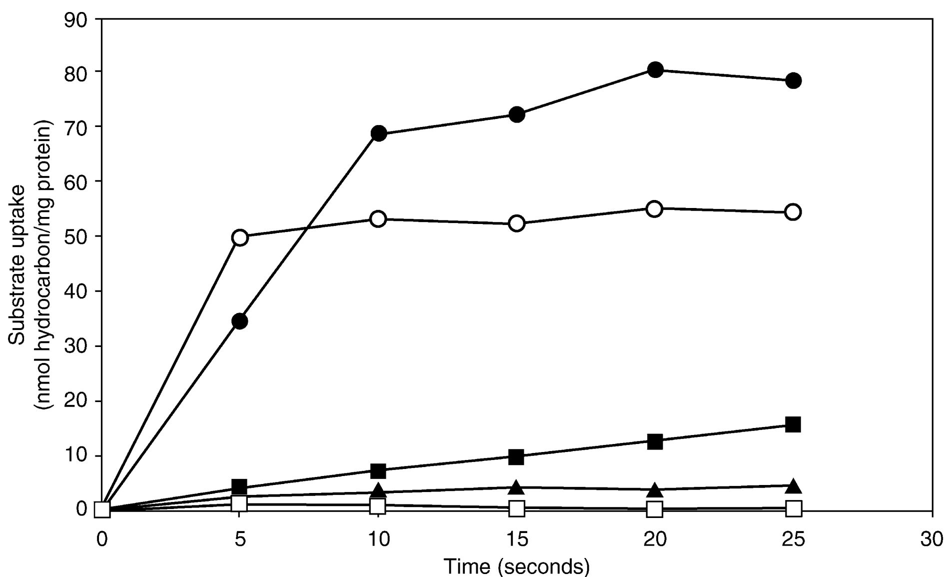

Figure 1

Example of hypothetical hydrocarbon transport data. Transport of radiolabeled hydrocarbon in the native wild-type bacterial strain (closed circles) saturates quickly and metabolism of the hydrocarbon facilitates further uptake of the molecule. Accumulation of hydrocarbons still occurs in a hydrocarbon transport protein mutant strain (closed squares); however, accumulation is non-saturable and based solely on passive diffusion through the cell membrane and subsequent metabolism of the hydrocarbon. In a hydrocarbon transport and metabolism double mutant (closed triangles) accumulation of hydrocarbon is based solely on the external concentration and passive diffusion. When a hydrocarbon transport protein is expressed in a heterologous host, transport of the hydrocarbon saturates quickly (open circles) relative to a vector control (open squares). The uptake rate can be determined from the slope of the linear portion of the graph.

- The above values should be multiplied by the total number of planned assays to make one batch of transport cocktail. Typically, each assay (i.e., strain, growth condition, substrate, etc.) should be carried out in duplicate or triplicate. After mixing, 300 μl aliquots should be distributed into 13 mm or similar treated glass test tubes for each transport assay to be conducted (see note 2).

Notes

1. After addition of cell suspension (see below), this will generate a final hydrocarbon substrate concentration of approximately 65 μM (50 μM unlabeled and 15 μM radio-labeled). The final substrate concentration must be experimentally determined to be saturating for the transport system of interest. The amount of radioactive substrate added to the transport cocktail should have approximately 100,000 disintegrations per minute (DPM) per 100 μl of reaction volume. The cold (unlabeled) hydrocarbon substrate is added to supplement the radiolabeled substrate such that the total hydrocarbon concentration is at saturating levels; this allows use of the radiolabeled substrate as a tracer

while minimizing the amount of radioactivity used. With many hydrocarbon substrates, solubility may be an issue. In these cases, the hydrocarbon may need to be suspended in the presence of a solvent or surfactant to increase solubility. If this is the case, this solubilizing compound should be included in all control solutions.

2. Hydrocarbons can adsorb to glassware. To avoid adsorption of hydrocarbons to the glass, all glassware should be treated with chromic acid overnight, rinsed thoroughly with water, treated with nitric acid overnight, and again rinsed with water (Bugg et al., 2000).

2.1.2 Growth and Preparation of Cells

The appropriate conditions for growth of the cells must be considered for effective transport assays. It is important to determine whether transport of the substrate is inducible. If this is not known, separate cultures should be grown with and without the transport substrate, and results of the assays should be compared relative to the two sets of growth conditions. It is strongly recommended that in addition to wild-type cells, a catabolic mutant not capable of growth on, or metabolism of the transport substrate, be tested if at all possible. Results of these assays should allow clear differentiation between accumulation of radiolabeled substrate and accumulation of radioactive intermediates resulting from metabolism of the transported substrate (*Fig. 1*).

Another important factor for successful transport assays is to use cells that are metabolically active. Therefore, for best results cells should be harvested during mid-exponential phase and used immediately. If putative transport mutants are available, assays can be done with these strains to establish the role of specific genes and proteins in hydrocarbon transport. Expression of transport proteins in a heterologous strain that is incapable of substrate metabolism also allows measurement of uptake in the absence of metabolism (*Fig. 1*).

Steps in the Procedure

B1. Grow cells in 75 ml of a suitable growth medium under appropriate conditions (temperature, aeration, etc.). If transport is inducible, the medium should also contain the inducing substrate. Cells should be harvested at mid-exponential phase.
B2. Collect cells by centrifugation for 10 min at 5,000 x *g* at room temperature.
B3. Wash the cells by resuspending the cell pellet in 50 ml of 25 mM phosphate buffer, pH 7.
B4. Collect cells by centrifugation. Resuspend the cell pellet in approximately 2.0 ml of 25 mM phosphate buffer, pH 7. Final cell density should be $\sim 10^9$–10^{10} cells ml^{-1}.
B5. Aerate the cells by gently bubbling air through a Pasteur pipette until the start of the assay (see note 3).
B6. Remove 100 µl of the cell suspension for determination of protein concentration. (Transport activity will be expressed in terms of substrate accumulated per mg cellular protein). Precipitate the protein with 25% trichloroacetic acid (TCA) (Ausubel et al., 1993). Resuspend the protein pellet in 100 µl 0.1 M NaOH, heat at 95°C for 10 min, and determine the protein content using an appropriate method (Bradford, 1976; Lowry et al., 1951).

Notes

3. Typically, one air bubble per second keeps the cells aerated without damaging or concentrating the cells over the course of the transport assays. In some cases, it may be beneficial

to allow the cell suspensions to be aerated for 15–30 min before starting the transport assays. This short "starvation period" may deplete any remaining intracellular transport substrate, thereby enhancing the detection of intracellular radiolabeled substrate.

2.1.3 Transport Assay

In preparing for the transport assay, it is important to have the cells harvested and aerated and the transport cocktail dispensed and ready for each assay. Once cells have been added to the transport cocktail, immediate timed samples must be taken to assess uptake of the ^{14}C-labeled hydrocarbon. The timing of samples may be an important issue when assessing transport of hydrocarbons, but typically transport should initiate immediately and be measured on the order of seconds to minutes. The transport assay is carried out by mixing cells with labeled substrate, then stopping uptake at timed intervals by filtration and washing. Accumulation of intracellular substrate is measured by scintillation counting of the filters.

Steps in the Procedure

C1. Place 0.2 micron pore size Nucleopore polycarbonate membrane filters (Costar Corp., Cambridge, MA) on the openings of a vacuum manifold (see note 4). One filter should be used for each time point of each assay. Place sealing stoppers on any openings not being used in the assay.
C2. Assemble the vacuum manifold and turn on the vacuum. Leave the vacuum on for the duration of the assay.
C3. Wash the filters by adding 2.0 ml of 25 mM phosphate buffer (pH 7) to each manifold opening. Wait until all of the liquid is pulled through the filters.
C4. Start the transport assay reaction by adding 300 μl of the cell suspension to 300 μl of transport cocktail in the glass test tubes. Start a timer when the cells are added to the cocktail.
C5. At timed intervals, transfer 100 μl of the transport assay mixture to a membrane filter on the vacuum manifold (see note 5).
C6. Immediately after the liquid has been pulled though the filter, wash the cells by adding 2.0 ml of 25 mM phosphate buffer, pH 7 (see notes 6 and 7).
C7. After all samples have been collected and washed, release the vacuum and place the filters in scintillation vials with an appropriate amount of scintillation fluid and determine DPM according to the instructions for your scintillation counter.
C8. From the remaining unused (unfiltered) transport assay mix, remove two 20 μl samples and place each in a scintillation vial to obtain total counts of the ^{14}C-labeled hydrocarbon reaction mixture. In addition, determine background DPM levels for two vials of scintillation fluid, without filters or reaction mix.
C9. Before carrying out additional assays, check the level of liquid in the manifold reservoirs, and empty if reservoirs are full. Discard radioactive waste appropriately.

Notes

4. Before the first assay, wet the openings of the vacuum manifold with ~0.5 ml of 25 mM phosphate buffer, pH 7, before placing the polycarbonate filters on the openings. This will keep the filters in place while you assemble the vacuum manifold for the first time.

5. The appropriate time intervals between samples will need to be experimentally determined for each transport system. A typical set of time intervals may be 5, 10, 15, 20, and 25 s or 15, 30, 60, 90, and 120 s. In some cases, initial time points will need to be taken faster than 5 s.
6. Due to the speed at which this assay occurs, it is often helpful to have two people conducting the assay. One person initiates the assay and removes samples at the timed intervals. The second person can monitor the vacuum manifold openings and wash the cells when the liquid has been completely pulled through the filters.
7. As stated previously, active transport of nonpolar substrates may be difficult to detect because the substrate could rapidly diffuse out of cells, especially in cases (e.g., in assays using a catabolic mutant) where the cells are not actively metabolizing the substrate and "trapping" it. To minimize this possibility, collection of cell pellets by centrifugation through silicone oil may be preferable to filtration and aqueous washing of cells. Silicone oil terminates the reaction by stripping cells of the surrounding medium as the bacterial cells pass through the oil (Klingenberg and Pfaff, 1977; Nichols and Harwood, 1997).

2.1.4 Calculations

If cells accumulate hydrocarbons with the use of a transport protein, the specific activity of the transporter can be calculated.

Steps in the procedure

D1. Subtract background counts from the DPM counts for each time point to obtain corrected DPM values.

D2. Using the two 20 µl unfiltered samples, determine the amount of hydrocarbon substrate per DPM in the reaction. For example: (65 µM hydrocarbon substrate) (0.02 ml) = 1.3 nmol. The average of two 20 µl samples in this example gives approximately 20,000 DPM. Therefore, 1.3 nmol of hydrocarbon substrate/20,000 DPM = 6.5×10^{-5} nmol/DPM.

D3. Determine the amount of substrate retained on each filter. Convert the DPM for each filtered assay sample to nmoles of hydrocarbon substrate accumulated by multiplying the DPM for each filter by the value calculated in step D2. Plot the results versus time to determine the rate of uptake. If the uptake is linear, the slope of the line can be used to determine the rate (*Fig. 1*). Convert the specific rate of uptake into the nmoles accumulated per minute (see note 8).

D4. Correct the values for the amount of protein present in each sample by determining the amount of protein present on each reaction filter.

For example: (mg ml^{-1} protein calculated in step B6) × (0.3 ml cells/0.6 ml total reaction volume) x (0.1 ml volume sample per filter) = total mg protein on each filter.

D5. Determine the specific activity of uptake by dividing the nmoles of hydrocarbon uptake per minute (step D3) by the amount of protein present in the assay (step D4).

Notes

8. If the transport of hydrocarbon saturates quickly, the initial uptake rates should be calculated using time points that have not yet saturated. In some cases, uptake may

be saturated by the first time point. In such cases, redesign the experiment to take earlier time points. If it is not possible to take earlier time points, the initial rate may need to be determined by extrapolating the values to zero.

2.1.5 Determination of Energy Requirement

Because active substrate transport requires energy (ATP or proton motive force), the energy requirement for transport can be assessed by measuring uptake in starved cells. Similarly, metabolic inhibitors that either deplete ATP pools within cells or dissipate the electrochemical gradient across the cell membrane can be used to verify the requirement of cellular energy for transport. Here we discuss the use of sodium azide, which inhibits cytochrome oxidase, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) and dinitrophenol (DNP), which dissipate the proton gradient, and KCN, which eliminates electron transport. Many additional inhibitors are available (Ahmed and Booth, 1982; Bugg et al., 2000; Heytler, 1979; Joshi et al., 1989; Kashket, 1985; Linnett and Beechey, 1979; Nichols and Harwood, 1997). Additional experiments to identify the specific nature of the cellular energy source have been discussed elsewhere (Joshi et al., 1989; Kashket, 1985).

Steps in the Procedure

E1. Grow and wash cells as described in B1–B3.
E2. Deplete cellular energy stores by resuspending cells in an equal volume of growth medium or buffer without added energy source. Shake for 18 h under the same conditions used for growth (see note 9).
E3. Prepare two reaction mixtures as described in A1, except energy sources such as glucose or succinate are omitted from one mixture.
E4. Wash and resuspend cells as described in B2–B6. Carry out transport assays (Section C) in duplicate, using reaction cocktails with and without added energy source. Compare the specific activities for the two conditions (calculate as described in Section D) to determine if uptake occurred in the absence of energy source.
E5. To assess the effect of metabolic inhibitors, treat starved cells as described in note 10.
E6. After inhibitor treatment, incubate cells with an energy source (10 mM glucose or succinate) for an additional 15 min. Perform transport assay to determine if cells exposed to inhibitor can accumulate substrate.
E7. Calculate specific activity of substrate uptake (Section D) and compare to results in the absence of inhibitors.

Notes

9. To verify that energy stores have been depleted, measure intracellular levels of ATP in cells concomitantly with transport assays (Bagnara and Finch, 1972; Joshi et al., 1989).
10. Approximate concentrations and exposure times for metabolic inhibitors: 30 mM sodium azide for 5–10 min, 10 mM potassium cyanide (KCN) for 5 min (Bugg et al., 2000), 50 μM CCCP for 1 min, 5 mM DNP for 1 min (Nichols and Harwood, 1997).

2.1.6 Specialized Materials Required

Sampling vacuum manifold (Millipore 1225, Billerica, MA, or similar apparatus)
Radiolabeled substrate
Scintillation counter

3 Research Outlook

Based solely on the hydrophobic nature of both the cytoplasmic membrane and hydrocarbon substrates, it would seem to be energetically wasteful for cells to utilize active transport mechanisms to accumulate molecules that freely pass through membranes. However, the absence of conclusive evidence for transport across the cytoplasmic membrane does not mean that such active transport mechanisms do not exist. Careful analysis of hydrocarbon uptake with appropriate controls, catabolic mutant strains, and metabolic inhibitors may identify specific transport systems for hydrocarbons. If such systems do indeed exist, isolation of transport mutants should be possible and will allow the identification and characterization of the transport proteins responsible.

References

Ahmed S, Booth IR (1982) The use of valinomycin, nigericin and trichlorocarbanilide in control of the protonmotive force in *Escherichia coli* cells. Biochem J 212: 105–112.

Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K (1993) Current Protocols in Molecular Biology. New York: Wiley.

Bagnara AS, Finch LR (1972) Quantitative extraction and estimation of intracellular nucleoside triphosphates of *Escherichia coli*. Anal Biochem 45: 24–34.

Bateman JN, Speer B, Feduik L, Hartline RA (1986) Naphthalene association and uptake in *Pseudomonas putida*. J Bacteriol 166: 155–161.

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248–254.

Bugg T, Foght JM, Pickard MA, Gray MR (2000) Uptake and active efflux of polycyclic aromatic hydrocarbons by *Pseudomonas fluorescens* LP6a. Appl Environ Microbiol 66: 5387–5392.

D'Argenio DA, Segura A, Coco WM, Bunz PV, Ornston LN (1999) The physiological contribution of *Acinetobacter* PcaK, a transport system that acts upon protocatechuate, can be masked by overlapping specificity of VanK. J Bacteriol 181: 3505–3515.

Heytler PG (1979) Uncouplers of oxidative phosphorylation. Methods Enzymol 55: 462–472.

Joshi AK, Ahmed S, Ferro-Luzzi Ames G (1989) Energy coupling in bacterial periplasmic transport systems: Studies in intact *Escherichia coli* cells. J Biol Chem 264: 2126–2133.

Kahng H-Y, Byrne AM, Olsen RH, Kukor JJ (2000) Characterization and role of *tbuX* in utilization of toluene by *Ralstonia pickettii* PKO1. J Bacteriol 182: 1232–1242.

Kasai Y, Inoue J, Harayama S (2001) The TOL plasmid pWWO *xylN* gene product from *Pseudomonas putida* is involved in *m*-xylene uptake. J Bacteriol 183: 6662–6666.

Kashket ER (1985) The proton motive force in bacteria: a critical assessment of methods. Annu Rev Microbiol 39: 219–242.

Klingenberg M, Pfaff E (1977) Means of terminating reactions. Methods Enzymol 10: 680–684.

Leveau JH, Zehnder AJ, van der Meer JR (1998) The *tfdK* gene product facilitates uptake of 2,4-dichlorophenoxyacetate by *Ralstonia eutropha* JMP134(pJP4). J Bacteriol 180: 2237–2243.

Linnett PE, Beechey RB (1979) Inhibitors of the ATP synthethase system. Methods Enzymol 55: 472–518.

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. J Biol Chem 193: 265–275.

Mooney A, O'Leary ND, Dobson AD (2006) Cloning and functional characterization of the *styE* gene,

involved in styrene transport in *Pseudomonas putida* CA-3. Appl Environ Microbiol 72: 1302–1309.

Nichols NN, Harwood CS (1997) PcaK, a high-affinity permease for the aromatic compounds 4-hydroxybenzoate and protocatechuate from *Pseudomonas putida.* J Bacteriol 179: 5056–5061.

Sikkema J, De Bont JAM, Poolman B (1995) Mechanisms of membrane toxicity of hydrocarbons. Microbiol Rev 59: 201–222.

Wang Y, Rawlings M, Gibson DT, Labbé D, Bergeron H, Brousseau R, Lau PCK (1995) Identification of a membrane protein and a truncated LysR-type regulator associated with the toluene degradation pathway in *Pseudomonas putida* F1. Mol Gen Genet 246: 570–579.