Journal of Microbiology (2013) Vol. 51, No. 6, pp. 791–799
DOI 10.1007/s12275-013-3259-6

Trans-Membrane Transport of *n*-Octadecane by *Pseudomonas* sp. DG17

Fei Hua, Hong Qi Wang*, Yi Li, and Yi Cun Zhao

College of Water Sciences, Beijing Normal University, Beijing 100875, P. R. China

(Received October 15, 2012 / Accepted July 29, 2013)

The trans-membrane transport of hydrocarbons is an important and complex aspect of the process of biodegradation of hydrocarbons by microorganisms. The mechanism of transport of ^{14}C *n*-octadecane by *Pseudomonas* sp. DG17, an alkane-degrading bacterium, was studied by the addition of ATP inhibitors and different substrate concentrations. When the concentration of *n*-octadecane was higher than 4.54 μmol/L, the transport of ^{14}C *n*-octadecane was driven by a facilitated passive mechanism following the intra/extra substrate concentration gradient. However, when the cells were grown with a low concentration of the substrate, the cellular accumulation of *n*-octadecane, an energy-dependent process, was dramatically decreased by the presence of ATP inhibitors, and *n*-octadecane accumulation continually increased against its concentration gradient. Furthermore, the presence of non-labeled alkanes blocked ^{14}C *n*-octadecane transport only in the induced cells, and the trans-membrane transport of *n*-octadecane was specific with an apparent dissociation constant K_t of 11.27 μmol/L and V_{max} of 0.96 μmol/min/mg protein. The results indicated that the trans-membrane transport of *n*-octadecane by *Pseudomonas* sp. DG17 was related to the substrate concentration and ATP.

Keywords: Pseudomonas, n-octadecane transport, facilitate, energy-dependent, specific

Introduction

Alkanes, the major components of oil and environmental pollutants, are saturated hydrocarbons that are virtually insoluble in water (Alexander *et al*., 2007). Studies have shown that a large variety of microorganisms, including *Rhodococcus* sp. (Sharma and Pant, 2000), *Pseudomonas* sp. (Murielle and Vandecasteele, 2008), and *Acinetobacter* sp. (Throne-Holst *et al*., 2006), can biodegrade these hydrophobic compounds and remove them from the environment. Microbial resources can physically access soluble, emulsified hydrocarbons and large oil droplets, transport these substrates across cell membranes (Rosenberg, 1993; Bouchez-Naitali *et al*., 1999), and form inclusions before the hydrocarbons are metabolized (de Andres *et al*., 1991; Alvarez *et al*., 1997). The mechanisms of uptake of hydrocarbons has thus been the subject of many studies, the majority of which have focused on biosurfactants. However, the transport mechanisms by which hydrocarbons travel across the cell membrane have received comparatively little attention, especially alkanes.

Previous studies have reported on the trans-membrane transport of benzoate (Harwood and Gibson, 1986), 4-hydroxybenzoate (4-HBa) (Merkel *et al*., 1989), 4-toluene sulfonate (Locher *et al*., 1993), 2-nitrophenol (Folsom, 1997), and toluene (Kahng *et al*., 2000). Solute transport across cell boundaries has been demonstrated to occur by one of three main mechanisms: (1) passive diffusion; (2) passive facilitated uptake; or (3) energy-dependent active uptake. For organic acids, the uptake can be effectively driven by intracellular/extracellular gradients of pH and substrate concentration (Yuroff *et al*., 2003). Meanwhile, how hydrophobic compounds, including alkanes and aromatic hydrocarbons, travel across the cell membrane was also reported. Bateman *et al*. (1986) found that the uptake of naphthalene by the *Pseudomonas putida* strain PpG1064 was not associated with an active transport system, but with diffusion because trans-membrane transport was not susceptible to inhibitors such as cyanide and CCCP (carbonyl cyanide *m*-chlorophenylhydrazone). In contrast, different inhibitors such as sodium azide, CCCP, NDP (2, 4-dinitrophenol), and cyanide could reduce the levels of cellular PAHs (polycyclic aromatic hydrocarbons) (Beal and Betts, 2000; Bugg *et al*., 2000; Miyata *et al*., 2004; Kallimanis *et al*., 2007). For instance, Aristeidis *et al*. (2007) showed that the uptake of phenanthrene in phenanthrene-grown cells was dramatically reduced by treatment with an inhibitor, which showed that phenanthrene travels across the cell membrane via a phenanthrene-inducible active transport mechanism that is dependent on the PMF (proton motive force). However, phenanthrene can travel across glucose-grown cells by passive diffusion. Whitman *et al*. (1998) indicated that naphthalene uptake by *Pseudomonas fluorescens* Uper-1 occurs via an energy-linked active transport system. Similarly, Kim *et al*. (2002) found that *R. erythropolis* S+14He was able to selectively discriminate and preferentially transport *n*-hexadecane from mixtures of structurally similar alkanes into intracellular inclusions by an energy-driven transport system. Miyata *et al*. (2004) demonstrated PAH transport in the *Mycobacterium* sp. strain RJGII-135 and provided the earliest report on the presence of a saturable, energy-dependent system involved in the specific binding of PAH to the bacteria cell. In this case, the substrate transport process not only requires energy, but it also requires a membrane transporter.

Furthermore, studies with microorganisms using diverse carbon sources, such as *Rhodococcus erythropolis* sp. (Kim *et al*., 2002), *Acinetobacter* sp. (Kennedy and Finnerty, 1975), fungus *Ashbya gossypii* (Stahmann *et al*., 1994), and *Rhodo-*

*For correspondence. E-mail: whongqi310@sohu.com; Tel.: +86-010-588 07810; Fax: +86-010-58802739

coccus opacus sp. (Alvarez *et al.*, 1996), have shown that growth in the presence of alkanes resulted in the intracellular accumulation of the substrate in inclusion bodies. These inclusions, which are transparent in transmission-electron micrographs, were shown to contain unmodified storage hydrocarbons (Kennedy and Finnerty, 1975) or fatty acids (de Andres *et al.*, 1991). Thus, in this case, the inclusions could provide indirect evidence for hydrocarbons trans-membrane transport.

The trans-membrane mechanism of the transport of alkanes into bacterial cells has been less well studied, but some results provide a possible explanation. In the present study, the nature of the trans-membrane transport mechanism of *n*-octadecane by *Pseudomonas* sp. DG17, a newly isolated strain from crude oil-polluted soil, was investigated with respect to the role of energy and a substrate concentration gradient. The aim of this study was to investigate the trans-membrane transport of *n*-octadecane with regard to the substrate concentration and the effects of ATP inhibitors in *Pseudomonas* sp. DG17 as well as to explore the specific binding of *n*-octadecane. To our knowledge, the trans-membrane transport dynamics and kinetic equations for *n*-octadecane have not been reported previously.

Materials and Methods

Culture and growth conditions

Pseudomonas sp. DG17, a culture maintained at the China General Microbiological Culture Collection Center (CGMCC ID: 5052; GenBank ID: JN 216878), was isolated from petroleum-contaminated soils (Da Gang oilfield, China) by an enrichment culture technique using crude oil as the sole source of carbon and energy. The culture was maintained at 4°C on glucose medium and transferred monthly as uninduced cells. Meanwhile, growth of DG17 on *n*-octadecane in a domestication experiment was conducted in 250-ml flasks at 30°C with shaking (150 r/min) in mineral salt medium (MSM) containing (per liter at pH 7.0) 0.4 g Na_2HPO_4, 0.15 g KH_2PO_4, 0.1 g NH_4Cl, 0.05 g $MgSO_4{\cdot}7H_2O$, 0.0015 g $CaCl_2$, 0.1 g $NaNO_3$, 1 ml trace medium (per 100 ml solution containing 0.5 mg $CuSO_4{\cdot}5H_2O$, 1.0 mg H_3BO_3, 1.0 mg $MnSO_4{\cdot}5H_2O$, 7.0 mg $ZnSO_4$). *n*-Octadecane (400 mg/L) was added as the carbon source before the MSM was sterilized by autoclaving at 121°C for 20 min. The domestication experiment was carried out three times in two months. Each time, 10 ml of the fermentation broth was transferred into new MSM. The DG17 culture was maintained at 4°C on a solid octadecane medium and transferred monthly as induced cells. Previous studies have shown that *Pseudomonas* sp. DG17 grows well on *n*-octadecane and could produce a rhamnolipid biosurfactant (Hua and Wang, 2011).

Batch-cultured cells were used for transport experiments and were harvested in late log phase. Before the assays, DG17 cells inoculated from a glucose solid medium were transferred into 150 ml MSM containing 100 mg/L glucose and grown for three days as uninduced cells. Meanwhile, cells inoculated from *n*-octadecane medium were added to 150 ml MSM containing 400 mg/L *n*-octadecane and grown for six days. All incubations were conducted on a rotary shaker at 30°C. The cells were collected by centrifugation at 4,000×g for 20 min and washed twice with 0.1 mol/L potassium phosphate buffer (pH 7.0) before being re-suspended in mineral salt medium (pH 7.0). The optical density at 600 nm (A_{600}) was approximately 1.5 as measured by spectrophotometer (Varian, USA).

^{14}C *n*-octadecane transport assay

Radiolabeled compound used in these assays was ^{14}C *n*-octadecane (ARC 1261, 99% pure, 55 mCi/mmol) obtained from American Radiolabeled Chemicals, Inc. (USA). The trans-membrane transport of *n*-octadecane by *Pseudomonas* sp. DG17 was assayed by measuring cellular ^{14}C *n*-octadecane in the presence of initial substrate concentrations ranging from 0.227 to 36.36 μmol/L (0.227, 0.454, 0.909, 1.515, 4.54, 9.09, 15.15, 18.18, 22.73, 36.36 μmol/L). A methanol solution of ^{14}C *n*-octadecane (25 μCi/ml) was added to 100-ml flasks containing 15 ml of a cell suspension (A_{600} at 0.45) in MSM at room temperature. All glassware was treated with nitric acid to minimize the adsorption of octadecane. Samples were collected at 1, 3, 5, 8, 11, 14, 17, and 20 min. At each time point, 1-ml aliquots were taken from the flask and applied to a Whatman GF/C glass fiber filter under a vacuum. The cells in the filter were washed under a vacuum using 1 ml of the phosphate buffer six times before being transferred into 2 ml of scintillation fluid (nonylphenolethoxylate, 9016-45-9, PerkinElmer Waltham, USA). The radioactivity was measured by a PerkinElmer liquid scintillation counter (Wallac Oy 1450 MicroBeta). This fraction of ^{14}C *n*-octadecane was taken as cellular *n*-octadecane (μmol/L). During the incubation, no significant loss of radioactivity due to volatilization of *n*-octadecane was observed.

Energy dependent trans-membrane transport assay

To examine whether the transport of n-octadecane by *Pseudomonas* sp. DG17 was energy dependent (active transport), transport assays were performed in the presence of 30 mmol/L of sodium azide, an inhibitor of the electron-flow chain in oxidative phosphorylation, or 0.1 mmol/L of protonophore DNP and 0.1 mmol/L of CCCP, an uncoupler of oxidative phosphorylation. The inhibitors were added at time zero and at 5 min, respectively. A control flask that was not treated with the inhibitors was also prepared. For the energy-dependent transport assay at a low substrate concentration, 15 μl and of methanol solution of ^{14}C *n*-octadecane (25 μCi/ml) was added to 100-ml flasks containing 15 ml of cells suspended in MSM with a final concentration of 0.36 μmol/L. For the energy-dependent transport assay at a high substrate concentration, 150 μl of a methanol solution of ^{14}C *n*-octadecane (100 μCi/ml) was added to 15 ml of cells suspend in MSM at a final of 15.15 μmol/L. The cell density at 600 nm (A_{600}) was approximately 0.5. The samples were analyzed as described above. During the incubation, biodegradation of ^{14}C *n*-octadecane in the culture broth was not observed.

Specific transport of *n*-octadecane

We investigated the specific trans-membrane transport of *n*-octadecane as described by Miyata *et al.* (2004) with some changes. A methanol solution of ^{14}C *n*-octadecane (25 μCi/ml,

454.54 µmol/L) was added to 100-ml flasks containing 15 ml of MSM at 30–35°C for 20 min. The final concentration of ^{14}C *n*-octadecane in the assay mixture was approximately 0.45 µmol/L. The flasks were supplemented with 0.45 µmol/L of non-labeled *n*-octadecane, *n*-hexacosane, and *n*-triacontane before the addition of ^{14}C *n*-octadecane. Transport was initiated by the addition of DG17 cells of (A_{600} was 0.42) grown on octadecane or glucose. Samples were collected at different time intervals, and the analysis was performed as described above.

Detection of the internalization and catabolism of ^{14}C *n*-octadecane

To verify the catabolic production of ^{14}C *n*-octadecane, $^{14}CO_2$ detection was performed as described by Whitman *et al.* (1998) with some modifications. Catabolism of ^{14}C was conducted in 16 mm×70 mm teflon-lined screw-capped culture tubes containing a 6 mm×50 mm Durham tube with 800 µl freshly prepared 10 mol/L NaOH simultaneously. Before the catabolism process, 5 ml of cells suspended in MSM (final A_{600} was 0.45) was added to the teflon-lined tubes. The final value of A_{600} was 0.45. The reaction began with different concentrations of ^{14}C *n*-octadecane ranging from 0.38 to 34.23 µmol/L. The reaction was stopped after 48 h of incubation at room temperature by injecting 20 µl 95% H_2SO_4. The tubes were kept for 30 min after the acid addition to allow the absorption of $^{14}CO_2$ by the NaOH. The method of analysis of the extracellular and cellular ^{14}C *n*-octadecane was the same as described above. For the $^{14}CO_2$ determination, 200 µl of the NaOH solution was transferred to scintillation vials, and the radioactivity was determined as described above. Cellular ^{14}C was determined as described above. Meanwhile, another 1-ml aliquot was directly removed for radioactivity analysis to determine the total ^{14}C *n*-octadecane. The biodegradability of ^{14}C *n*-octadecane was calculated as: (initial total ^{14}C in the broth-total ^{14}C in the broth after incubation for 48 h) / initial total ^{14}C in the broth ×100%. A control incubation without *Pseudomonas* sp. DG17 cells was conducted simultaneously to evaluate the total ^{14}C loss. The radioactivity of $^{14}CO_2$ and cellular ^{14}C *n*-octadecane could be used for kinetic biodegradation analysis. The standard deviations were calculated based on three samples for each experimental replicate. Previous studies have shown that the abiotic loss of *n*-octadecane for each sample was less than 7%.

Ultrastructure observation

Cellular octadecane was observed by TEM (Transmission Electron Microscopy). Cells grown on 400 mg/L of non-labeled *n*-octadecane were used for the ultrastructural studies. Meanwhile, cells grown on 100 mg/L glucose was taken as control group. Three days later, samples were collected and washed with 0.1 mol/L phosphate buffer (pH 7.2), fixed with 2.5% glutaraldehyde, washed with 0.1 mmol/L phosphate buffer pH 7.4, and post-fixed in 1% osmium tetroxide for 2 h. After several washes in the same buffer, the cells were dehydrated through a partial ethanol dehydration series [50, 70, 85, 95, and 100% (v:v) ethanol] to preserve the hydrophilic and hydrophobic interface. After removing the 100% ethanol treatment, the samples were embedded in 50 : 50 EPON 812 araldite: ethanol for 2 h, followed by 100% EPON 812 araldite (1 h) and a second 100% EPON 812 araldite overnight, along with dodecenyl succinic anhydride (DDSA) as a hardener and tri(dimethylaminomethyl) phenol (DMP-30) as a catalyst (All incubations were performed at 4°C). Ultrathin sections of 60–80 nm thickness were cut using an Ultracut E (Reichert Jung) ultramicrotome, and the sections were stained in alcoholic uranyl acetate (10 min) and subsequently in lead acetate (10 min) before examining the grids in TEM (JEM-1400, JEOL, Japan; Camera Name: BC3-2) operated at 80 kV, which gave better resolution at low magnification. Cells grown for three days on 50 mg/L of glucose in minimal medium were taken as controls and processed similarly.

Results

Trans-membrane transport properties of ^{14}C *n*-octadecane

The transport properties of *n*-octadecane by *Pseudomonas* sp. DG17 with the addition of different concentrations of octadecane is shown in Fig. 1. The immediate uptake was evaluated at 1 min. The cellular ^{14}C levels were 0.16±0.0024, 0.50±0.0044, 2.29±0.0088, 3.88±0.0102, 4.66±0.022 µmol/L, respectively, when the cells were incubated with 0.45, 1.52,

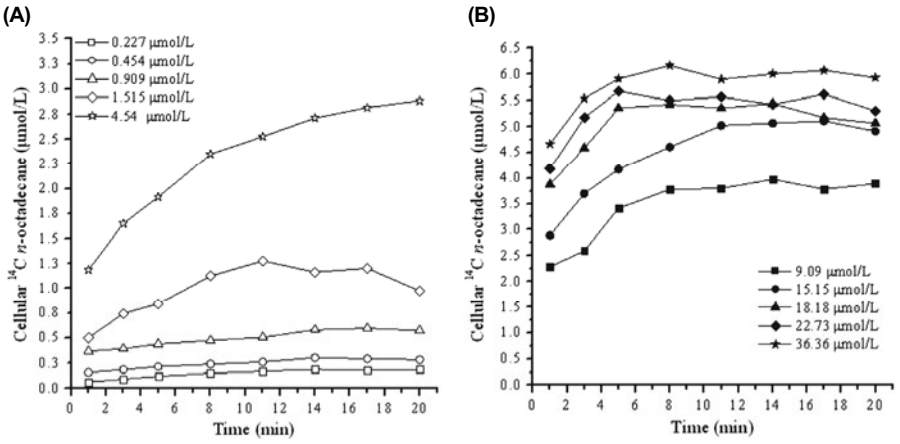

Fig. 1. Trans-membrane transport of ^{14}C *n*-octadecane by *Pseudomonas* sp. DG17 in the presence of different substrate concentrations. The cell protein concentration was 12 µg/ml after 20 min of incubation. The standard deviations were lower than 0.014 µmol/L.

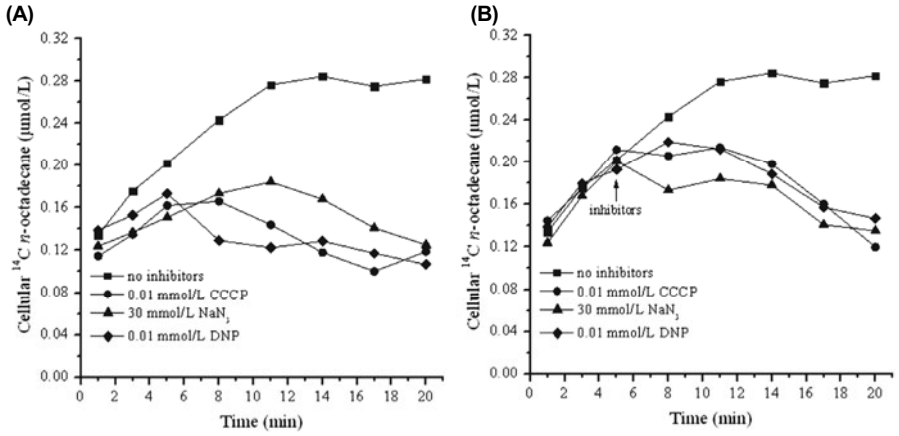

Fig. 2. Effect of different inhibitors on the trans-membrane transport of ^{14}C *n*-octadecane by *Pseudomonas* sp. DG17. The substrate concentration was 0.36 μmol/L. Inhibitors were added at time zero (A) and 5 min (B), respectively. The cell protein content was 15.11 μg/ml after incubation for 20 min. The standard deviations were lower than 0.0067 μmol/L.

9.09, 18.18, and 36.36 μmol/L ^{14}C *n*-octadecane, and this value increased along with the increase in the substrate concentration. As shown in Fig. 1A, when the substrate concentration ranged from 0.227 to 4.54 μmol/L, we found that the fraction of cellular ^{14}C still increased and accounted for over 50% of the total ^{14}C in the broth. After incubation for 8 min, the cellular ^{14}C *n*-octadecane was 0.22±0.0083, 0.47±0.0041, and 1.12±0.0072, 2.35±0.010 μmol/L, respectively, when the cells were incubated with 0.45, 0.91, 1.52, and 4.54 μmol/L ^{14}C *n*-octadecane. Accordingly, the fractions of cellular ^{14}C accounting for the total ^{14}C were 52.69, 52.01, 74.30, and 51.74%, respectively, which indicated that the levels of cellular ^{14}C *n*-octadecane were higher than the extracellular levels. In this case, the trans-membrane transport of *n*-octadecane could not be simple diffusion; DG17 cells must have transported extracellular *n*-octadecane across the membrane against the concentration gradient. The ^{14}C octadecane transport reached a steady-state level at approximately 14 min, and the cellular levels were 0.18±0.0055, 0.29±0.0058, 0.60±0.0042, 1.16±0.0094, 2.71±0.014 μmol/L, respectively, when the ^{14}C *n*-octadecane concentration ranged from 0.227 to 4.54 μmol/L. As shown in Fig. 1B, however, the cellular ^{14}C level also reached a steady state and the cellular ^{14}C *n*-octadecane level was always lower that the extracellular level when the substrate concentration was higher than 4.54 μmol/L. After incubation for 20 min, the cellular ^{14}C levels were 3.89±0.0132, 4.91±0.0096, 5.06±0.0115, 5.30±0.0083, and 5.94±0.0137 μmol/L, respectively, in the presence of 9.09, 15.15, 18.18, 22.73, and 36.36 μmol/L ^{14}C *n*-octadecane. In this case, the trans-membrane transport of ^{14}C *n*-octadecane by *Pseudomonas* sp. DG17 was always down the concentration gradient. Although the initial uptake of *n*-octadecane occurred quickly (within 1 min) and could be accelerated under higher substrate concentrations (from 9.09 to 36.36 μmol/L), the accumulation of *n*-octadecane might be saturable. In this study, the highest value of cellular *n*-octadecane was 6.17 μmol/L in the presence of 36.36 μmol/L octadecane.

Effect of inhibitors on *n*-octadecane uptake

The substrate concentration and cellular ATP levels were considered to investigate the mechanism of trans-membrane transport of *n*-octadecane. Figure 2 shows the extent of *n*-octadecane transport by cells of DG17 in the presence or absence of ATP inhibitors. As shown in Fig. 2A, it was found that ^{14}C *n*-octadecane trans-membrane transport was highly sensitive to the presence of either azide (30 mmol/L), 2, 4-dinitrophenol (0.1 mmol/L), or CCCP (0.1 mmol/L) when the cells were incubated with 0.36±0.056 μmol/L octadecane. In the absence of inhibitors, the amount of cellular ^{14}C *n*-octadecane was 0.13±0.037 μmol/L at 1 min. After incubation for 11 min, *n*-octadecane accumulated within the cells,

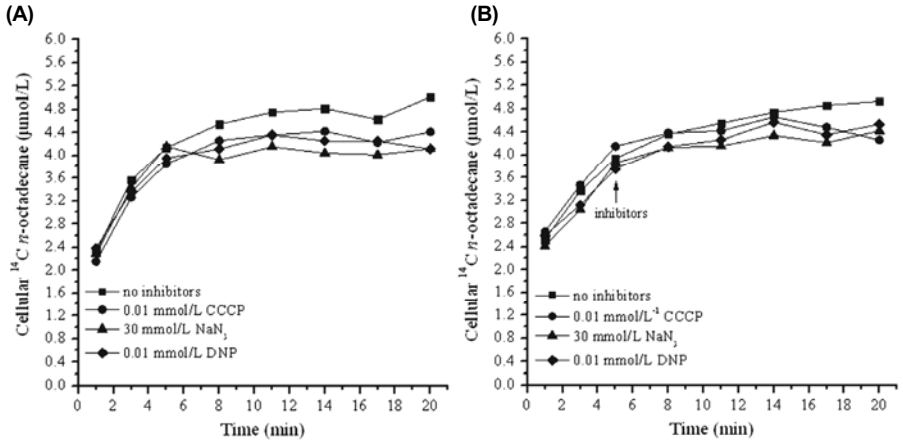

Fig. 3. Effect of different inhibitors on the trans-membrane transport of ^{14}C *n*-octadecane by *Pseudomonas* sp. DG17. The substrate concentration was 15.15 μmol/L. The inhibitors were added at time zero (A) and 5 min (B), respectively. The cell protein was 15.11 μg/ml after incubation for 20 min. Standard deviations were lower than 0.0082 μmol/L.

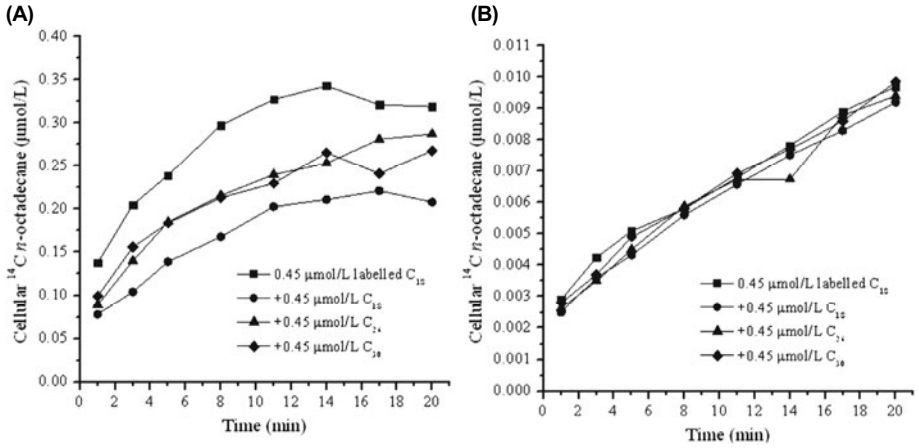

Fig. 4. Trans-membrane transport of ^{14}C *n*-octadecane by *Pseudomonas* sp. DG17 grown on octadecane (A) and glucose (B). The cell protein content was 15.11 μg/mL after incubation time for 20 min. The standard deviations were lower than 0.0053 μmol/L.

against the concentration gradient; the cellular concentration of *n*-octadecane was 0.29±0.042 μmol/L, which was higher than the extracellular concentration. However, the accumulation of *n*-octadecane was strongly inhibited in the presence of inhibitors added at time zero. For example, the cellular ^{14}C *n*-octadecane level at 20 min was decreased by 55.60, 62.13, and 60.32%, respectively, in the presence of NaN_3, DNP, and CCCP. Similarly, the addition of inhibitors at 5 min (Fig. 2B) also strongly prevented the cumulative transport of ^{14}C octadecane. The findings showed that *n*-octadecane travel across the cell membrane in *Pseudomonas* sp. DG17 cells occurred by an energy-dependent active transport mechanism. As shown in Fig. 3, however, in the presence of 15.15 μmol/L of octadecane, the transport of *n*-octadecane seemed less sensitive to the presence of ATP inhibitors. When NaN_3, DNP, and CCCP were added to the medium at time zero (Fig. 3A), the amounts of cellular substrate did not change during the first 8 min after the inhibitors were added. For instance, in the absence of the inhibitors, the initial cellular ^{14}C *n*-octadecane level in the DG17 cells was 2.32±0.084 μmol/L at 1 min. This finding indicated that the transport of *n*-octadecane during this time interval did not require energy. Subsequently, the cellular *n*-octadecane concentration increased to 5.02±0.067 μmol/L at 20 min. Meanwhile, the accumulation of ^{14}C *n*-octadecane also increased in the presence of ATP inhibitors at the later time points. After incubation for 20 min, the cellular ^{14}C *n*-octadecane levels were 4.41±0.032 μmol/L, 4.12±0.051 μmol/L, and 4.11±0.092 μmol/L, respectively, with the addition of CCCP, NaN_3, and DNP. Thus, the trans-membrane transport of octadecane by *Pseudomonas* sp. DG17 incubated with 15.15 μmol/L octadecane was less sensitive to the ATP inhibitors than that by cells incubated with 0.36 μmol/L octadecane. Furthermore, the cellular ^{14}C *n*-octadecane level was always lower than the extracellular level during the incubation time. Similar results were also found when the ATP inhibitors were added at 5 min (Fig. 3B). In this case, the results suggested that energy was only needed after incubation for 8 min, when the intra/extracellular substrate concentration gradient was reduced. The findings showed that *n*-octadecane can travel across cell membrane of DG17 cells, driven by a substrate concentration gradient. However, when intra/extracellular concentration gradient became lower, *Pseudomonas* sp. DG17 may require energy to transport extracellular ^{14}C *n*-octadecane.

Inhibitory effect of substrate analogues on the transport of ^{14}C *n*-octadecane

To verify whether ^{14}C *n*-octadecane transport is a specificity-induced process, an excess of non-labeled *n*-octadecane, *n*-hexacosane, and *n*-triacontane were supplemented in the culture medium in addition to labeled *n*-octadecane. As shown in Fig. 4A, the trans-membrane transport of ^{14}C *n*-octadecane by octadecane-grown cells was inhibited by an excess of non-labeled octadecane. When the cells were incubated with 0.45 μmol/L ^{14}C *n*-octadecane, cellular ^{14}C increased nonlinearly and reached a steady level at 0.32 ±0.0112 μmol/L after 20 min. However, the cellular ^{14}C decreased with the addition of excess non-labeled alkanes. For example, non-labeled 0.45 μmol/L *n*-octadecane, 0.45 μmol/L *n*-hexacosane, and 0.45 μmol/L *n*-triacontane reduced the cellular

Table 1. Distribution of ^{14}C *n*-octadecane radioactivity following incubation in the presence of *Pseudomonas* sp. DG17

Total ^{14}Cin the Tube /cpm	Clarifled Supernantant/cpm	Cell pellet/cpm	Trappedas $^{14}CO_2$/cpm	Abiotic lost/cpm
124360	37870	20650	64756	2300
444344	152120	81170	200589	10465
929512	245840	161370	488625	33677
1522928	497030	311140	642060	72098
3709576	2022184	559463	992783	135146
6242160	3732300	1010160	1113613	386087
11290160	7738710	1814960	1239472	497018

^{14}C *n*-octadeecane was added to the closed telfon tubes with *Pseudomonas* sp. DG17 cells. After incubation for 48 h, radioactivity was determined as described in materials and methods. Previous studies have shown that the abiotic loss of *n*-octadecane for each sample was lower than 7%.

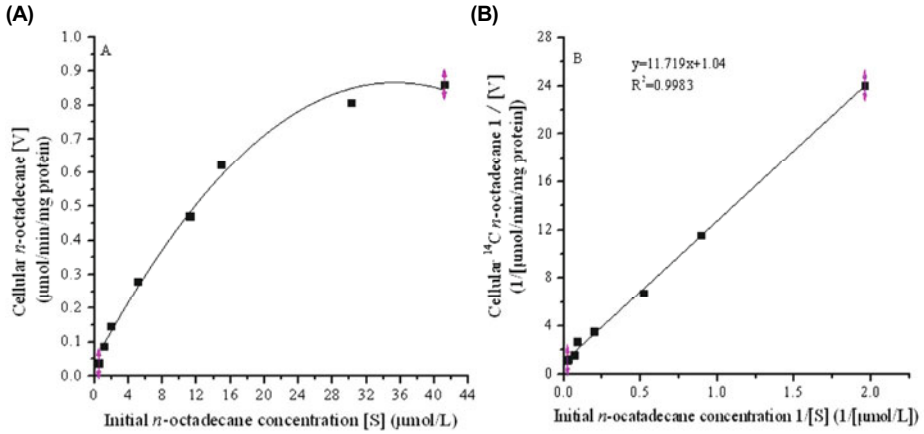

Fig. 5. Octadecane trans-membrane transport kinetic analysis. The relationship between cellular ^{14}C *n*-octadecane concentrations in the cells of *Pseudomonas* sp. DG17 and the initial *n*-octadecane concentrations (A); The solid line shows the nonlinear regression of the data for octadecane transembrane transport kinetic parameters K_t= 1.27 μmol/L and V_{max}=0.96 μmol/min/mg protein, R^2=0.9985 (B).

^{14}C *n*-octadecane level at 20 min to 0.21±0.0056, 0.29±0.0044, and 0.27±0.0081 μmol/L, respectively. In contrast, no significant decrease was observed when glucose-grown cells were incubated with 0.45 μmol/L ^{14}C *n*-octadecane and non-labeled alkanes (Fig. 4B). For example, the cellular ^{14}C level reached 0.0097±0.00054 μmol/L at 20 min with the addition of ^{14}C *n*-octadecane. Similarly, the amount of cellular ^{14}C *n*-octadecane increased to 0.0092±0.00073, 0.0094±0.00061, and 0.0099±0.00037 μmol/L when cells were treated with 0.45 μmol/L ^{14}C *n*-octadecane plus excess non-labeled 0.45 μmol/L *n*-octadecane, 0.45 μmol/L *n*-hexacosane, and 0.45 μmol/L *n*-triacontane, respectively. In this case, the non-labeled alkanes appeared not to influence the transport of labeled *n*-octadecane by glucose-grown cells. It should be noted that the cellular ^{14}C level increased to 0.32±0.0067 μmol/L at 20 min, which was approximately 30 times that of glucose-grown cells when the cells were incubated with 0.45 μmol/L labeled octadecane. Thus, it was inferred that *n*-octadecane could travel across the membrane of DG17 by an octadecane-inducible transport mechanism.

Detection of $^{14}CO_2$

To evaluate the uptake of ^{14}C *n*-octadecane by *Pseudomonas* sp. DG17 cells, ^{14}C *n*-octadecane was added at concentrations ranging from 0.5 to 20 μmol/L in closed tubes containing a NaOH trap for released $^{14}CO_2$. Following 48 h of incubation, the distribution of radioactivity ^{14}C in the cell pellet, clarified supernatant, and CO_2 trap, as well as the abiotic loss during the experiment, are shown in Table 1. It can clearly be seen that at lease 98.6% of the total radioactivity present in the incubation mixture was recovered following the 48 h incubation. When cells were incubated with 0.38, 1.35, 2.82, 4.62, 11.25, 18.92, or 34.23 μmol/L of ^{14}C *n*-octadecane, the proportion of radioactivity from $^{14}CO_2$ was 52.07, 45.14, 52.57, 42.16, 26.76, 17.84, 10.98%, respectively, and proportion of radioactivity in the cell pellet was 16.61, 16.02, 22.74, 20.43, 15.08, 16.18, and 16.08%, respectively. The abiotic loss of *n*-octadecane for each sample was lower than 7%. As an example, for 4.62 μmol/L ^{14}C *n*-octadecane, 62.58% of the radioactivity lost from the aqueous supernatant (67.36%) was recovered in the form of $^{14}CO_2$, only 30.33% of the radioactivity lost from the aqueous supernatant was recovered in the cell pellet, and the abiotic loss was 7.03%. The results indicated that the transport of octadecane involves concurrent catabolism of ^{14}C, leading to the generation of significant amounts of $^{14}CO_2$.

Trans-membrane transport kinetics of octadecane

The results of the kinetic analysis of the uptake of ^{14}C *n*-octadecane at external labeled *n*-octadecane concentrations ranging from 0.51 to 41.18 μmol/L as described above is shown in Fig. 5. After approximately 30 sec to 1 min, the uptake of cellular ^{14}C *n*-octadecane reached 0.037±0.031, 0.086±0.044, 0.15±0.057, 0.28±0.041, 0.47±0.052, 0.62±0.047, 0.81±0.066, 0.86±0.085 μmol/mg protein (Fig. 5A). The data obtained are presented in the form of a Lineweaver-Burk plot that showed Michaelis-Menten kinetics (R^2=0.9985) with a K_t value of 11.27 μmol/L and a V_{max} of 0.96 μmol/min/mg protein (Fig. 5B). Moreover, the partitioning of *n*-octadecane into bacterial cells (within 1 min) occurred quickly.

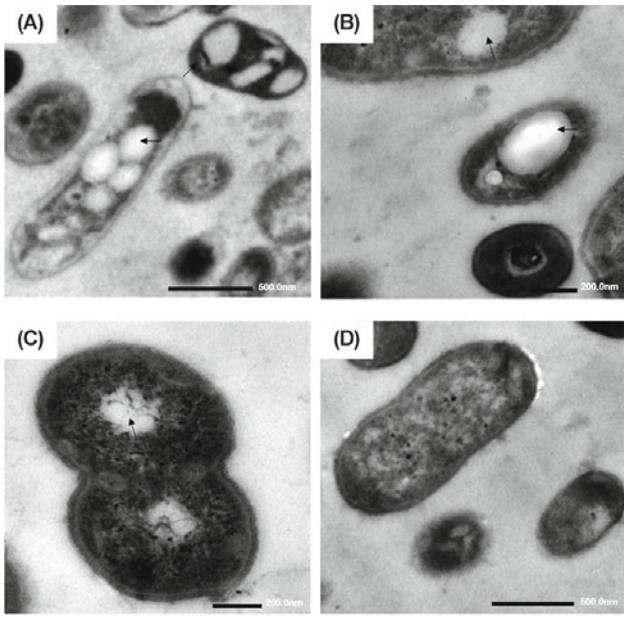

Fig. 6. Transmission Electron Microscopy of DG17 grown on 400 mg/L *n*-octadecane (A-C), and grown on 100 mg/L glucose (D). Arrows show inclusions of *n*-octadecane.

The ratio of extracellular and cellular ^{14}C *n*-octadecane indicated that this immediate uptake is caused by facilitated passive diffusion depending on the concentration gradient.

Transmission Electron Microscopy studies

Intracellular hydrocarbon inclusion bodies were observed by TEM in cells of *Pseudomonas* sp. DG17 grown on *n*-octadecane and glucose (Fig. 6). Results showed that cells grown on *n*-octadecane displayed rounded clear-vesicle type inclusions (Fig. 6A and 6C). Diameters of most of these inclusions were from 50 nm to 600 nm. None of these were observed in glucose grown cells (Fig. 6D).

Discussion

In previous reports, the trans-membrane transport of hydrocarbons has been investigated using a single substrate concentration. For example, when the substrate was 1.2 μmol/L, the transport of phenanthrene by the *Arthrobacter* sp. strain Sphe3 was linear between 15 and 60 sec, and it reached a steady-state level at 15 min, which was 3.12 nmol/mg dry weight of cells (Aristeidis *et al*., 2007). Similarly, when cells of the *Mycobacterium* sp. strain RJGII-135 were incubated with 0.06 μmol/L ^{14}C phenanthrene, the cellular ^{14}C increased linearly between 15 and 60 sec and reached a steady state after 90 sec. The amount of cellular ^{14}C at 90 sec was 5.0±0.5 nmol/mg of cells (Miyata *et al*., 2004). In our study, the accumulation of ^{14}C *n*-octadecane also reached at steady-state level when the cells were incubated with 0.909 μmol/L *n*-octadecane at 20 min, which was 47.50 nmol/mg of cells, approximately 15.22 times higher than cellular phenanthrene in the *Arthrobacter* sp. strain Sphe3 (Aristeidis *et al*., 2007). For *Pseudomonas fluorescens* Uper-1 incubated with 1.9 mg/L ^{14}C naphthalene, a 40% decrease was found in the level of naphthalene present in the clarified aqueous phase during the first 2 h of incubation (Whitman *et al*., 1998). Thus, it was indicated that the transport of ^{14}C naphthalene by *P. fluorescens* Uper-1 was down the intracellular/extracellular substrate gradient. This result was similar to transport of octadecane by *Pseudomonas* sp. DG17 incubated with 9.09 to 36.36 μmol/L substrate (high substrate concentrations). In this case, the concentration of cellular ^{14}C *n*-octadecane was always lower than the extracellular concentration. However, it seemed that the trans-membrane transport of ^{14}C *n*-octadecane in our study was not linear when the cells were incubated with either a high concentration of substrate or a low concentration.

A previous study also indicated that hydrocarbon transport across the cell membrane was determined by the substrate concentration. Shishido and Toda (1996) showed that phenol could enter the cells of degrading bacteria by active transport when the concentration of phenol was less than 50 mg/L, but at higher concentrations, the bacteria transported phenol into the cells by passive transport. It is evident that diverse adaptations for efficient PAH transport have evolved in PAH-utilizing bacteria, and different transport processes may play a role in the mineralization of low concentrations of cyclic hydrocarbons (Sikkema *et al*., 1995). Similarly, the larger the dose of CCCP, the larger the decrease in hexadecane uptake; in *Pseudomonas aeruginosa* PG201, 200, 100, and 50 mol/L CCCP reduced hexadecane uptake by 83.7, 63.7, and 49.5%, respectively, compared with the untreated control after 117 h of incubation. Thus, the mechanism for hexadecane uptake requires energy (Beal and Betts, 2000). For the *Arthrobacter* sp. Strain Sphe3, when azide or DNP was added, the rate of intracellular accumulation of labeled phenanthrene was reduced dramatically regardless of whether an inhibitor was present at the beginning of the assay or it was added 45 sec later (Aristeidis *et al*., 2007). Similar results were found for phenanthrene uptake by the *Mycobacterium* sp. strain RJGII-135 in the presence of cyanide and CCCP, and phenanthrene uptake was a saturable and energy-dependent process in induced cells (Miyata *et al*., 2004). ^{14}C naphthalene uptake by a *P. fluorescens* Uper strain was inhibited by 94% and 82%, respectively, when sodium azide or DNP was added to the medium (Whitman *et al*., 1998). Meanwhile, the concentration of ^{14}C alkane in the cytosol of *Cladosporium resinae* was always less than that at the cell surface, and a diffusion gradient could transfer alkane to the site of its metabolism in the cytosol. Thus, this transport could also occur by facilitated diffusion as well as by active transport (Lindley and Heydeman, 1986). However, studies on the uptake of methanol by the *Pseudomonas* sp. strain AM1 revealed that methanol is taken into the cells by a passive mechanism with K_m of 4.8 μmol/L and a V_{max} of 60.6 nmol/min/mg of cells, and the uptake of methanol was not completely curtailed by CCCP (Bellion *et al*., 1983). In this study, which was different from previous studies, the trans-membrane transport of octadecane was related to both the substrate concentration and intracellular ATP.

Efflux pump which is an energy-dependent and active process, is another trans-membrane transport process that will be blocked by ATP inhibitor. Many microorganisms can protect themselves from cell injury caused by a toxicant by flushing the toxicant out of the cell, and enables microorganisms to survive in an adverse environment (Isken and de Bont, 1996; Kieboom *et al*., 1998). In living cells, the transport of solutes across the cell membrane can be assisted by "pump" proteins that use metabolic energy to transport components against the local concentration gradient (Sonja *et al*., 1998). Fumiyasu *et al*. (2008) showed that a proton motive energy-dependent toluene-transporting system that could pump toluene out of the cytoplasmic membrane into the environment was present in toluene-adapted cells of *Pseudomonas putida*. Similarly, for *P. fluorescens* LP6a, phenanthrene partitioned into the cell membranes by passive diffusion and was transported back into the medium by an active efflux pump. The efflux pump maintained the intracellular concentration of phenanthrene below its equilibrium level. When this pump was inhibited, the phenanthrene concentrations in the pellet approached the equilibrium levels (Bugg *et al*., 2000). In this study, cellular ^{14}C octadecane increased to a steady level after incubation for approximately 11 min. If efflux of the substrate occurred during the incubation time, a decline in the levels of cellular ^{14}C octadecane would be observed, but this was not found in this study. Until now, little is known about the efflux process of octadecane, and whether an efflux process may affect the uptake of hydrocarbons should be studied in depth.

Our findings are consistent with previous reports. For the *Arthrobacter* sp. strain Sphe3, when the cells were incubated with 1.2 μmol/L ^{14}C phenanthrene plus 1.2, 2.4, or 4.2 μmol/L unlabeled phenanthrene, the cellular ^{14}C level was reduced by 45, 65, or 84%, respectively, whereas no significant decrease was obtained in glucose-grown cells (Aristeidis *et al.*, 2007). Meanwhile, studies by Whitman *et al.* (1998) found that the addition of excess non-labeled phenanthrene and non-phenanthrene PAHs in phenanthrene-grown cells resulted in a decrease in the cellular ^{14}C, apparently due to competition with radiolabeled phenanthrene. For the *Mycobacterium* sp. Strain RJGII-135, transport of ^{14}C phenanthrene into acetate-grown cells depends upon a non-saturable, linear partitioning process upon the addition of non-labeled phenanthrene. Nevertheless, the transport of phenanthrene into phenanthrene-grown cells was nonlinearly saturable (Miyata *et al.*, 2004). A model for partitioning and transport across the inner and outer membranes showed that the permeability of the outer membrane was rate-controlling (Gray and Bugg, 2001). The cell membrane acts to facilitate or restrict the uptake of chemicals into the bacterial cell, and the greatest selectivity in controlling the uptake of chemicals into the cells resides primarily with the plasma membrane, which contains numerous specific transport proteins (Folsom, 1997). For example, PcaK is a transporter and chemoreceptor protein from *Pseudomonas putida* that is encoded as part of the β-ketoadipate pathway regulon for aromatic acid degradation, and PcaK-mediated transport is driven by the proton motive force (Nichols and Harwood, 1997). However, understanding the mechanisms of membrane-based alkane uptake requires additional molecular biological studies. Until now, the role of membrane proteins in the transport of alkanes or other hydrocarbons has not been clear. Our studies present two modes by which DG17 cells mediate cellular *n*-octadecane transport: facilitated diffusion driven by intra/extra cellular substrate concentration and energy-dependent active transport.

The trans-membrane transport and metabolism of PAHs has also been studied in other reports. For instance, when cells of the *Arthrobacter* sp. strain Sphe3 were incubated with 9 μg of ^{14}C phenanthrene for 12 h, 20% of ^{14}C phenanthrene was detected in CO_2, 65% in the cell-free eluate, and 10.5% in the cells, indicating that phenanthrene is both internalized and catabolized within cells (Aristeidis *et al.*, 2007). At the same time, according to Beal and Betts (2000), mineralization was measured as $^{14}CO_2$ evolution from ^{14}C *n*-hexadecane. The total mineralization of ^{14}C *n*-hexadecane by *Pseudomonas aeruginosa* PG201 and *Pseudomonas aeruginosa* UO299 over 288 h was 49.92 and 34.43%, respectively.

Similar results were also found for the phenanthrene uptake by the *Mycobacterium* sp. stain RJGII-135 (Miyata *et al.*, 2004) and the *Arthrobacter* sp. strain Sphe3 (Aristeidis *et al.*, 2007) and for naphthalene uptake by *Pseudomonas fluorescens* Uper-1 (Whitman *et al.*, 1998). For example, for cells of *Arthrobacter* sp. Strain Sphe3 (Aristeidis *et al.*, 2007), the immediate uptake of phenanthrene followed saturation kinetics, indicating the specific binding of phenanthrene to the PAH-transport system, with an apparent K_d of 0.48 μmol/L and nonspecific linear partitioning with an N of 0.031. For cells of *Mycobacterium* sp. strain RJGII-135, the cellular uptake of phenanthrene was nonlinear regression with kinetic parameters K_t of 26±3 nmol/L, V_{max} of 4.2±0.1 nmol/min/mg of cells, and an N of 1.50±0.04 (Miyata *et al.*, 2004). Meanwhile, trans-membrane transport of naphthalene by *P. fluorescens* Uper-1 exhibited a K_t value of 10.5 μmol/L and a V_{max} of 17 nmol/h/mg dry weight of cells (Whitman *et al.*, 1998). Lindley and Heydeman (1986) found that *n*-dodecane was taken up by *Cladosporium resinae* by a mechanism that obeyed Michaelis-Menten saturation kinetics with K_t of 1 mmol/L and V_{max} of 12.1 nmol/min (mg/protein). Thus, the microorganism seems have a stronger uptake capability of alkanes than that of PAHs. Meanwhile, in these studies, nonspecific linear partitioning was considered to be a process in which the non-labeled hydrocarbons would not influence the influx of labeled hydrocarbon. Cellular labeled hydrocarbon increased linearly and was not influenced by any of the ATP inhibitors. In this study, it was assumed that if the uptake of *n*-octadecane was just a linear passive diffusion process, the cellular n-octadecane would increase linearly and not be influenced by sodium azide or other inhibitors. However, our data showed an accumulative nonlinear concentration increase of cellular *n*-octadecane.

As a substrate, petroleum could typically be incorporated into the cells by a specific and inducible transport system, which was particularly important for oil biodegradation. Microorganisms have developed a variety of strategies allowing their survival in these variable environments, and the accumulation of storage lipids is only one strategy (de Andres *et al.*, 1991; Sonja *et al.*, 1998). For example, for cells of *P. aeruginosa* PG201, clear vesicles were present in the body of cells utilizing n-hexadecane as their sole carbon source; these vesicles were not present in any of the cells cultivated in Nutrient Broth (Beal and Betts, 2000). Similarly, *Rhodococcus* sp. formed inclusions during growth on diesel fuel but not on glucose plus acetate (Whyte *et al.*, 1999). Meanwhile, the types of electron-transparent inclusions were determined by the substrate in cells of *Rhodococcus erythropolis* S+14He. Cells grown on n-hexadecane displayed rounded inclusions, whereas the cells grown on *n*-octadecane and pristane displayed a mixture of rounded and rugose inclusions (Kim *et al.*, 2002). Although the chemical composition of the inclusion bodies was not determined completely in the current study, studies have shown that composition of the lipid inclusions depended on the compounds provided as a carbon source (Alvarez *et al.*, 1997). For example, this inclusion body was a characteristic feature observed with the growth of *Acinetobacter* sp. on a homologous set of alkanes varying in chain length from 12 to 20 carbon atoms, respectively (Kennedy and Finnerty, 1975). Barabas *et al.* (2001) found that alkane-utilizing microorganisms tended to accumulate fatty acids with chains equivalent in length to those of the alkane substrate. Thus, it is known that hydrocarbons are taken up in an unaltered form by microorganisms and are subsequently oxidized intracellularly (Puntus *et al.*, 2005). Once inside the cell, the hydrocarbon droplet appeared to be broken down by means of internal cellular projections, possibly to increase the surface area for further hexadecane metabolism (Swaranjit and Pooja, 2009).

Acknowledgements

This paper is sponsored by the National Natural Science Foundation of China (NO. 41072177).

References

Alexander, W., Trond, E.E., Hans-Kristian, K., Sergey, B.Z., and Mimmi, T.H. 2007. Bacterial metabolism of long-chain *n*-alkanes. *Appl. Microbiol. Biotechnol.* **76**, 1209–1221.

Alvarez, H.M., Mayer, F., Fabritius, D., and Steinbuchel, A. 1996. Formation of intracytoplasmic lipid inclusions by *Rhodococcus opacus* strain PD630. *Arch. Microbiol.* **165**, 377–386.

Alvarez, H.M., Pucci, O.H., and Steinbuchel, A. 1997. Lipid storage compounds in marine bacteria. *Appl. Microbiol. Biotechnol.* **47**, 132–139.

Barabas, G., Vargha, G., and Szabo, I.M. 2001. n-Alkane uptake and utilization by *Streptomyces* strains. *Antonie van Leeuwenhoek* **79**, 269–276.

Bateman, J.N., Speer, B.L., Feduik, L., and Hartline, R.A. 1986. Naphthalene association and uptake in *Pseudomonas putida*. *J. Bacteriol.* **166**, 155–161.

Beal, R. and Betts, W.B. 2000. Role of rhamnolipid biosurfactants in the uptake and mineralization of hexadecane in *Pseudomonas aeruginosa*. *J. Appl. Microbiol.* **89**, 158–168.

Bellion, E., Kent, E.M., Aud, J.C., Alikhan, M.Y., and Bolbot, J.A. 1983. Uptake of methylamine and methanol by *Pseudomonas* sp. strain AM1. *J. Bacteriol.* **154**, 1168–1173.

Bouchez-Naitali, M., Rakatozafy, H., Marchal, R., Leveau, J.Y., and Vandecasteele, J.P. 1999. Diversity of bacterial strains degrading hexadecane in relation to the mode of substrate uptake. *J. Appl. Microbiol.* **86**, 421–428.

Bugg, T., Foght, J.M., Pickard, M.A., and Gray, M.R. 2000. Uptake and active efflux of polycyclic aromatic hydrocarbons by *Pseudomonas fluorescens* LP6a. *Appl. Environ. Microbiol.* **66**, 5387–5392.

de Andres, C., Espuny, M.J., Robert, M., Mercad, M.E., Manresa, A., and Guinea, J. 1991. Cellular lipid accumulation by *Pseudomonas aeruyinosa* 44T1. *Appl. Microbiol. Biotechnol.* **35**, 813–816.

Folsom, B.R. 1997. Characterization of 2-nitrophenol uptake system of *Pseudomonas putida* B2. *J. Indian Microbiol. Biotechnol.* **19**, 123–129.

Fumiyasu, F., Hisako, H., and Hideto, T. 2008. Isolation and transposon mutagenesis of a *Pseudomonas putida* KT2442 toluene-resistant variant:involvement of an efflux system in solvent resistance. *Extremophiles* **2**, 395–400.

Gray, M.R., and Bugg, T. 2001. Selective biocatalysis in bacteria controlled by active membrane transport. *Ind. Eng. Chem. Res.* **40**, 5126–5131.

Harwood, C.S. and Gibson, J. 1986. Uptake of benzoate by *Rhodopseudomonas palustris* grown anaerobically in light. *J. Bacteriol.* **165**, 504–509.

Hua, F. and Wang, H.Q. 2011. Uptake modes of octadecane by *Pseudomonas* sp. DG17 and synthesis of biosurfactant. *J. Appl. Microbiol.* **112**, 25–37.

Isken, S. and De Bont, J.A.M. 1996. Active efflux of toluene in a solvent-resistant bacterium. *J. Bacteriol.* **178**, 6056–6058.

Kahng, H.Y., Byrne, A.M., Olsen, R.H., and Kukor, J.J. 2000. Characterization and role of tbuX in utilization of toluene by *Ralstonia pickettii* PKO1. *J. Bacteriol.* **182**, 1232–1242.

Kallimanis, A., Frillingos, S., Drainas, C., and Koukkou, A.I. 2007. Taxonomic identification, phenanthrene uptake activity, and membrane lipid alterations of the PAH degrading *Arthrobater* sp. strain Sphe3. *Appl. Microbiol. Biotechnol.* **76**, 709–717.

Kennedy, R.S. and Finnerty, W.R. 1975. Microbial assimilation of hydrocarbons I. The fine-structure of a hydrocarbon oxidizing *Acinetobacter* sp. *Arch. Microbiol.* **102**, 75–83.

Kieboom, J., Dennis, J.J., Zylstra, G.J., and de Bont, J.A.M. 1998. Active efflux of organic solvents by *Pseudomonas putida* S12 is induced by solvents. *J. Bacteriol.* **180**, 67–72.

Kim, I.S., Julia, M.F., and Gray, R.M. 2002. Selective transport and accumulation of alkanes by *Rhodococcus erythropolis* S+14He. *Biotechnol. Bioeng.* **80**, 650–659.

Lindley, N.D. and Heydeman, M.T. 1986. Mechanism of dodecane uptake by whole cells of *Cladosporium resinae*. *J. Gen. Microbiol.* **132**, 751–756.

Locher, H.H., Poolman, B., Cook, A.M., and Konings, W.N. 1993. Uptake of 4-toluene sulfonate by *Comamonas testosterone* T-2. *J. Bacteriol.* **175**, 1075–1080.

Merkel, S.M., Eberhard, A.E., Gibson, J., and Harwood, C.S. 1989. Involvement of coenzyme A thioesters in anaerobic metabolism of 4-hydroxybenzoate by *Rhodopseudomonas palustris*. *J. Bacteriol.* **171**, 1–7.

Miyata, N., Iwahori, K., Foght, J.M., and Gray, M.R. 2004. Saturable, energy-dependent uptake of phenanthrene in aqueous phase by *Mycobacterium* sp. strain RJGII-135. *Appl. Environ. Microbiol.* **70**, 363–369.

Murielle, B.N. and Vandecasteele, J.P. 2008. Biosurfactants, a help in the biodegradation of hexadecane? The case of *Rhodococcus* and *Pseudomonas* strains. *World J. Microbiol. Biotechnol.* **24**, 1901–1907.

Nichols, N.N. and Harwood, C.S. 1997. PcaK, a high-affinity permease for the aromatic compounds 4-hydroxybenzoate and protocatechuate from *Pseudomonas putida*. *J. Bacteriol.* **179**, 5056–5061.

Puntus, I.F., Sakharovsky, V.G., Filonov, A.E., and Boronin, A.M. 2005. Surface activity and metabolism of hydrocarbon-degrading microorganisms growing on hexadecane and naphthalene. *Process Biochem.* **40**, 2643–2648.

Rosenberg, E. 1993. Exploiting microbial growth on hydrocarbon: New markets. *Trends Biotechnol.* **11**, 419–424.

Sharma, S.L. and Pant, A. 2000. Biodegradation and conversion of alkanes and crude oil by a marine *Rhodococcus*. *Biodegradation* **11**, 289–294.

Shishido, M. and Toda, M. 1996. Apparent zero-order kinetics of phenol biodegradation by substrate-inhibited microbes at low substrate concentrations. *Biotechnol. Bioeng.* **50**, 709–717.

Sikkema, J., de Bont, J.A.M., and Poolman, B. 1995. Mechanisms of membrane toxicity of hydrocarbons. *Microbiol. Rev.* **59**, 201–222.

Sonja, I., Jan, A.M., and de Bont, J.A.M. 1998. Bacteria tolerant to organic solvents. *Extremophiles* **2**, 229–238.

Stahmann, K.P., Kupp, C., Feldmann, S.D., and Sahm, H. 1994. Formation and degradation of lipid bodies found in the riboflavin-producing fungus *Ashbya gossypii*. *Appl. Microbiol. Biotechnol.* **42**, 121–127.

Swaranjit, S.C. and Pooja, S. 2009. Synthesis of rhamnolipid biosurfactant and mode of hexadecane uptake by *Pseudomonas* species. *Microb. Cell Fact.* **8**, 1–7.

Throne-Holst, M., Markussen, S., Winnberg, A., Ellingsen, T.E., Kotlar, H.K., and Zotchev, S.B. 2006. Utilization of *n*-alkanes by a newly isolated strain of *Acinetobacter venetianus*: the role of two AlkB-type alkane hydroxylases. *Appl. Microbiol. Biotechnol.* **72**, 353–360.

Yuroff, A.S., Sabat, G., and Hickey, W.J. 2003. Transporter-mediated uptake of 2-chloro- and 2-hydroxybenzoate by *Pseudomonas huttiensis* strain D1. *Appl. Environ. Microbiol.* **69**, 7401–7408.

Whitman, B.E., Lueking, D.R., and Mihelcic, J.R. 1998. Naphthalene uptake by a *Pseudomonas fluorescens* isolate. *Can. J. Microbiol.* **44**, 1086–1093.

Whyte, L.G., Slagman, S.J., Pietrantonio, F., Bourbonniere, L., Koval, S.F., Lawrence, J.R., Inniss, W.E., and Greer, C.W. 1999. Physiological adaptations involved in alkane assimilation at a low temperature by *Rhodococcus* sp. strain Q15. *Appl. Environ. Microbiol.* **65**, 2961–2968.