50 Substrate Transport

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Abstract: Hydrocarbon compounds are known to passively diffuse across bacterial cytoplasmic membranes and this may be the primary mechanism of hydrocarbon entry into most bacteria. The participation of active transport systems has been suggested in some bacterial strains, but solid evidence for active transport is currently lacking. However, in Gram-negative bacteria, specific inducible outer membrane channels that allow entry of various aromatic hydrocarbon substrates have been identified.

1 Introduction

A particularly intriguing question about the utilization of hydrocarbons by microorganisms as sources of carbon and energy is how these cells manage the problem of growth with a toxic compound. Because hydrocarbons are highly hydrophobic and lipophilic molecules, they are capable of unregulated entry into cells through the cytoplasmic membrane and can cause intracellular toxicity (reviewed in (Sikkema et al., 1995)). Therefore, cells that utilize these toxic molecules must acquire sufficient amounts of hydrocarbons to allow growth but must somehow limit their concentrations to sub-toxic levels.

In Gram-negative cells, the outer membrane provides a significant barrier to hydrocarbon entry (reviewed in (Nikaido, 2003)). Structurally, the outer membrane is very different from the cytoplasmic membrane, primarily due to the presence of lipopolysaccharide (LPS) in the outer leaflet. The outer membrane prevents the diffusion of molecules because of the low fluidity of the tightly packed saturated fatty acids and strong lateral molecular interactions between LPS molecules. The polyanionic lipid of the LPS is further stabilized by divalent cations. These features make the outer membrane an extremely efficient permeability barrier for the cell. Passage of substrates across the outer membrane is accomplished via nonspecific porins and specific outer membrane protein channels, which allow the entry of nutrients (*Fig. 1*). Porins typically have substrate preferences based on the size and charge of the molecules (Nikaido, 2003).

The cytoplasmic membrane provides a critical permeability barrier to polar and charged molecules but hydrophobic compounds such as hydrocarbons can readily penetrate the lipid bilayer (Sikkema et al., 1995). At this time, there is no definitive answer to the question of whether hydrocarbons passively diffuse across bacterial cytoplasmic membranes or if they are actively transported. General arguments for the absence of hydrocarbon transporters are as follows: (1) the hydrophobic nature of hydrocarbons is expected to allow these molecules to permeate the cytoplasmic membrane; (2) hydrocarbon transport mutants have not been isolated; (3) genes encoding cytoplasmic transport proteins have not been identified in known gene clusters for hydrocarbon catabolism; and (4) expression and functional activity of genes for (substituted) hydrocarbon degradation in *E. coli* and other heterologous hosts do not require the introduction of transport genes, indicating that hydrocarbons gain entry into cells without expression of specific heterologous transport proteins. However, most of these generalizations are based on the absence of data rather than on any solid empirical evidence.

Due to the differences in cytoplasmic and outer membrane structures, there are potentially different requirements for the presence of transport mechanisms for hydrocarbons. Unfortunately, relatively few studies have been undertaken to characterize hydrocarbon uptake by bacteria across either membrane, and the available studies seem to present conflicting evidence. The purpose of this chapter is to review our current understanding of hydrocarbon uptake by bacterial cells.

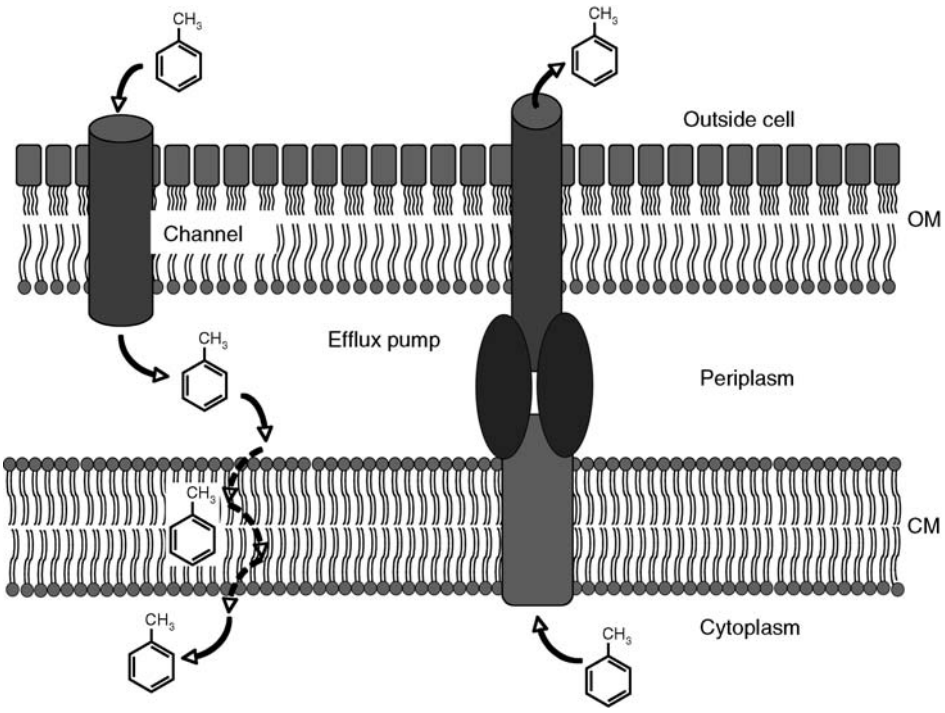

Figure 1

Cartoon of the Gram-negative bacterial cell envelope. Based on our current understanding of hydrocarbon transport, cells have either specific (e.g., TodX) or nonspecific outer membrane channels to allow passage of hydrocarbons across the outer membrane into the periplasm, but do not need transport proteins for passage of hydrocarbons across the cytoplasmic membrane. Some bacteria also have specific or nonspecific efflux pumps to reduce hydrocarbon concentrations within the cell and prevent toxic effects. OM, outer membrane; CM, cytoplasmic membrane.

2 Hydrocarbon Transport Across the Outer Membrane of Gram Negative Bacteria

The outer membrane of Gram-negative bacteria provides an effective permeability barrier, and all substrates must enter the Gram-negative cell through outer membrane channels (Nikaido, 2003). Several bacterial hydrocarbon degradation gene clusters have been found to contain a gene that encodes a putative outer membrane protein with homology to FadL, an outer-membrane protein that is required for long chain fatty acid transport in *E. coli* (Black, 1991; DiRusso and Black, 2004; van den Berg, 2005). The FadL protein functions to allow the passage of long chain fatty acids across the outer membrane. Crystal structures of FadL demonstrated that it is a monomeric β-barrel comprised of 14 antiparallel β-strands (van den Berg et al., 2004). It has low and high affinity binding sites for fatty acids, and a channel that allows these ligands to enter the periplasmic space. Available evidence suggests that the FadL homologs TodX, TbuX, TmoX, XylN, and StyE function as channels that allow aromatic hydrocarbons such as toluene, *m*-xylene, and styrene to cross the outer membrane (*Fig. 1*). In the toluene degrading strain

P. putida F1, *todX* is the first gene in the *tod* operon (Wang et al., 1995). This toluene-inducible operon encodes all of the enzymes required for toluene degradation in this organism (Lau et al., 1994; Menn et al., 1991; Wang et al., 1995; Zylstra and Gibson, 1989). When expressed in *E. coli*, TodX localized to the outer membrane (Wang et al., 1995), and a *todX* mutant of *P. putida* F1 had a decreased growth rate on low levels of toluene. Together, these data suggested that TodX facilitates entry of toluene into the periplasm of the cell (Wang et al., 1995). At higher toluene concentrations, toluene can efficiently cross the outer membrane in the absence of TodX, presumably through nonspecific channels or porins.

Several other *todX* homologs have been identified in various hydrocarbon degradation gene clusters. For example, in *Ralstonia pickettii* PKO1, which grows on toluene using a toluene 3-monooxygenase-mediated pathway (Olsen et al., 1994), the TodX homolog TbuX (34% amino acid identity) was shown to be required for toluene utilization and induction of the toluene degradation genes. Based on sequence analysis, TbuX was predicted to be an outer membrane protein and was postulated to facilitate passage of toluene across the outer membrane (Kahng et al., 2000). The *xylN* gene is another *todX* homolog. It is the last gene in the TOL pathway upper operon (for toluene and xylene degradation) and its product shares 38% amino acid identity with TodX (Kasai et al., 2001). XylN was localized to the outer membrane of wild-type *P. putida* carrying the TOL plasmid. Inactivation of *xylN* resulted in a reduced growth rate on *m*-xylene provided in the vapor phase. In contrast, when *m*-xylene was added directly to the medium, the *xylN* mutant entered exponential growth phase almost immediately, while the wild type had a $\sim$ 40 h lag phase. Together these data indicate that XylN allows *m*-xylene to cross the outer membrane, and makes the cell sensitive to *m*-xylene at high concentrations. In contrast, in *P. putida* CA-3, the StyE protein was required for growth of the strain on the aromatic hydrocarbon styrene (Mooney et al., 2006). StyE was localized to the outer-membrane of *P. putida* CA-3 and was proposed to facilitate the transport of styrene across the outer membrane. Other putative outer membrane hydrocarbon transporters (e.g., CymD, CumH, TmoX) were identified based on sequence analysis and gene location within characterized aromatic hydrocarbon catabolic operons (Eaton, 1997; Eaton and Timmis, 1986; Habe et al., 1996; Ramos-Gonzalez et al., 2002). Thus it appears that the entry of aromatic hydrocarbons into Gram-negative cells frequently involves the participation of a specific inducible outer membrane protein. However, none of the identified FadL homolog have been studied in detail and additional research will be required to characterize the role of these proteins in the entry of hydrocarbons into the periplasm.

The alkane-degrading *Pseudomonas putida* strains GPo1 and P1 carry *alkL* genes in alkane degradation gene clusters (van Beilen et al., 2001, 1994). The *alkL* gene encodes an outer membrane protein with homology to the *Vibrio cholerae* OmpW outer membrane protein of unknown function (van Beilen et al., 1992). AlkL has been proposed to be involved in uptake of alkane substrates; however, the deletion of *alkL* did not affect the metabolism of alkane substrates (van Beilen et al., 1994).

3 Hydrocarbon Transport Across the Cytoplasmic Membrane

A study of naphthalene uptake by *Pseudomonas putida* PpG1 concluded that naphthalene entered cells by passive diffusion across the cytoplasmic membrane based on several lines of evidence. Induction of *P. putida* PpG1 cells was not required for naphthalene accumulation, indicating that the production of a specific protein is not required for this process. In addition,

various inhibitors of ATP synthesis, or PMF generation had no effect on naphthalene uptake, and naphthalene uptake was not saturable (Bateman et al., 1986). Bugg et al. demonstrated that phenanthrene entry into cells by passive diffusion was counteracted by a chromosomally-encoded active efflux system in *P. fluorescens* LP6a (Bugg et al., 2000). The genetic location of the efflux system (chromosome) suggests that efflux and polycyclic aromatic hydrocarbon (PAH) catabolism (plasmid-encoded) are not linked, and that the efflux system may function to maintain sub-toxic levels of PAHs within the cell. Although the specific efflux protein was not identified, the efflux system was selective for phenanthrene, anthracene, and fluoranthene; it did not pump out naphthalene (Bugg et al., 2000). In contrast to these results, it was reported that naphthalene uptake by *Pseudomonas fluorescens* Uper-1 may involve an active transport system (Whitman et al., 1998). This study demonstrated that naphthalene transport only occurred in the wild-type naphthalene degrading strain; a mutant defective in the first enzyme in the naphthalene degradation pathway was incapable of naphthalene uptake. However, the transport assays were carried out over a period of hours rather than minutes, and significant growth and metabolism of ^{14}C-naphthalene was occurring during this period. Therefore, the results of this study remain inconclusive because it is impossible to separate uptake from metabolism under these conditions.

Two recent reports suggest that phenanthrene is actively transported by the Gram-positive isolates *Mycobacterium* sp. strain RJGII-135 (Miyata et al., 2004) and *Arthrobacter* sp. strain Sphe3 (Kallimanis et al., 2007). In both strains, phenanthrene appeared to enter uninduced cells by passive diffusion. However, induced cells showed saturable, energy-dependent phenanthrene uptake. Kinetic analyses suggested that phenanthrene is specifically bound to induced *Mycobacterium* sp. strain RJGII-135 cells (Miyata et al., 2004). In both studies, the bacteria were actively metabolizing phenanthrene; in fact essentially all of the ^{14}C-phenanthrene taken up by *Mycobacterium* sp. strain RJGII-135 was converted to metabolites within a minute. Therefore, the contribution of metabolism to the measured uptake cannot be ruled out, and further studies with catabolic mutants and/or the identification of specific transport proteins will be necessary to confirm that active transport of phenanthrene is occurring.

A Gram-positive strain, *Rhodococcus erythropolis* S+ 14He, which grows on linear alkanes, was reported to actively and selectively transport *n*-hexadecane (Kim et al., 2002); similarly hexadecane uptake by *Pseudomonas aeruginosa* PG201 was shown to be energy dependent (Beal and Betts, 2000). However, as in the other studies, cells were both accumulating and metabolizing the compounds during the assays. More importantly, no transport proteins for hydrocarbon uptake have been identified to date in any of these hydrocarbon-degrading bacteria. One study did identify a putative ABC transport system that was induced by *n*-tetradecane (Noda et al., 2003). When the *hcuABC* genes in *P. aeruginosa* were inactivated by random insertion of a transposon carrying desulfurization genes, the recombinant strain was able to desulfurize dibenzothiophene in aqueous culture but not in a two-phase system with the substrate in *n*-tetradecane. However, it seems equally likely that the *hcuABC* genes encode a tetradecane-inducible efflux pump that may prevent accumulation of toxic levels of hydrocarbons from the oil phase (see section on efflux pumps below).

4 Other Mechanisms to Enhance Hydrocarbon Acquisition

Bacteria have developed various strategies to enhance the uptake of hydrophobic substrates such as hydrocarbons. These adaptations seem to increase the solubility of poorly accessible

hydrocarbons or bring bacteria into direct physical contact with the substrates, thereby contributing to more efficient uptake and metabolism of these hydrophobic substrates.

For example, *Mycobacterium* sp. LB501T has been shown to attach to anthracene crystals, which are poorly soluble in aqueous solution, and form biofilms. The organism changes its cell surface properties when grown with anthracene in order to bind more efficiently to hydrophobic surfaces (Wick et al., 2002). Similarly, *P. putida* ATCC17514 was shown to adhere to solid polycyclic aromatic hydrocarbons (fluorene and phenanthrene) and it also produced more exopolysaccharide when grown with phenanthrene (Rodrigues et al., 2005). Cells of the alkane-degrading strain *P. aeruginosa* PG201 were more hydrophobic when grown with *n*-hexadecane than with glucose (Beal and Betts, 2000). Another alkane degrader, *Rhodococcus erythropolis* S+ 14He was highly hydrophobic and adhered to *n*-hexadecane when grown in liquid medium (Kim et al., 2002). This strain, as well as other alkane degraders have been shown to accumulate alkane substrates in internal membrane-bound inclusions (Kim et al., 2002; Scott and Finnerty, 1976).

Many hydrocarbon-degrading strains, such as *P. aeruginosa* PG201, produce rhamnolipids, which are biosurfactants that improve the bioavailability of poorly soluble hydrocarbon substrates. This strain was shown to be more efficient at hexadecane uptake than a mutant incapable of producing rhamnolipids. Evidence for a species-specific energy-dependent rhamnolipid-facilitated uptake system for hexadecane was reported for *P. aeruginosa* UG2 (Noordman and Janssen, 2002). Strain UG2 was also capable of controlling attachment to hydrocarbon substrates and had an active efflux system (see below) to prevent accumulation of toxic concentrations of hydrocarbons.

5 Hydrocarbon Efflux Pumps

Hydrocarbon-degrading bacteria must balance substrate acquisition with inherent hydrocarbon toxicity. To deal with this problem, many hydrocarbon-degrading bacteria have both specific and nonspecific efflux pumps (*Fig. 1*) to expel excess solvent from within cells (Ramos et al., 2002, 1997). The solvent efflux pumps that have been described to date are members of the resistance-nodulation-cell division (RND) family of transporters (Saier and Paulsen, 2001). These pumps function in direct opposition to entry of hydrocarbons into cells and are presumed to limit the accumulation of solvents to tolerable levels, thus reducing membrane toxicity (Sikkema et al., 1995). Efflux pumps for toluene, phenanthrene and anthracene have been described, suggesting that efflux pumps for toxic aromatic hydrocarbons may be widely distributed (Bugg et al., 2000; Kieboom et al., 1998; Phoenix et al., 2003; Rojas et al., 2001; Ramos et al., 1998). Genes encoding three-component pumps have been identified in several strains of *Pseudomonas putida*, including the highly solvent resistant strains *P. putida* S12 and DOT-T1E. Mutations in the efflux pumps resulted in reduced solvent resistance (reviewed in (Ramos et al., 2002)). Details about the mechanisms mediating solvent tolerance are presented in Vol. 2, Part 9.

6 Summary and Research Needs

In Gram-negative microorganisms that use hydrocarbons as a source of carbon and energy, specific channel proteins that are located in the outer membrane are utilized for effective entry of

these molecules into the periplasm. Proteins related to FadL appear to serve as channels for the passage of specific aromatic hydrocarbons across the outer membrane and these channels are inducible in the presence of aromatic substrates. By synthesizing proteins for hydrocarbon entry and metabolism concurrently, the cells are poised to immediately metabolize the aromatic molecule and prevent accumulation to toxic levels that can cause membrane damage. Relatively little detail is known about these channel proteins and even less is known about the possible role of the OmpW homolog AlkL in alkane uptake. Nothing is known about the rate of hydrocarbon passage through the channels, the range of substrates that can enter cells through specific channels, or the basis for hydrocarbon specificity. Presumably TodX and related proteins have structures similar to that of FadL, but this has not been demonstrated. Environmental conditions under which these channels are important (or essential) for hydrocarbon uptake are not well defined and appear to differ in the few strains that have been studied.

Although genes and proteins have been identified for passage of hydrocarbons across the outer membrane, there is relatively little compelling evidence for active transport of hydrocarbons across the cytoplasmic membrane. These studies are particularly difficult due to the volatile nature of hydrocarbons, and the proficiency of these molecules to adsorb to plastic and glassware. The available studies provide conflicting data, although strain-to-strain or substrate-to-substrate differences are certainly possible. However, experiments reported to date that claim active transport of hydrocarbons have failed to clearly separate transport from metabolism. Thus, careful studies with specific catabolic mutants must be undertaken in order to study transport in the absence of metabolism.

Based solely on the hydrophobic nature of the cytoplasmic membrane, it would appear that active transport of hydrocarbons is not needed. In aqueous solution, hydrocarbons preferentially partition into cell membranes. Therefore, it seems energetically and physiologically counterproductive to maintain active transport mechanisms that expend energy to accumulate chemicals that can freely pass through membranes and accumulate to potentially toxic levels. In fact, many cells actually expend energy to export excess hydrocarbons using efflux pumps in order to minimize the toxic effects of these chemicals. In addition, it is interesting that not one gene or protein has been identified and demonstrated to play a role in hydrocarbon transport. It seems likely that such genes would be located near the catabolic gene clusters (much like aromatic hydrocarbon outer membrane channel encoding genes), and would therefore have been identified through bioinformatics searches. With the vast array of genome and catabolic plasmid sequences available, it is noteworthy that such genes have not been found.

In no other metabolic situation is there such a fine line between substrate availability and toxicity. The essential functions of the cytoplasmic membrane in energy generation and maintenance, regulation of internal cellular pH, solute transport, and signaling can be disrupted by the accumulation of hydrocarbons in the bilayer. Various hydrocarbons have been shown to alter the membrane structure, causing leakage of ions and macromolecules, impairment of ATP synthesis, dissipation of the pH gradient across the membrane, and inability to control internal pH (reviewed in (Sikkema et al., 1995)). Hydrocarbons are generally poorly soluble in aqueous liquids and studies have shown that microbes are capable of utilizing hydrocarbons only when they are dissolved in the aqueous phase (Wodzinski and Bertolini, 1972; Wodzinski and Coyle, 1974). In some cases, the low bioavailability of hydrocarbons results in the rate of metabolism exceeding the rate of hydrocarbon entry into cells, thus limiting the growth rate, but also preventing toxicity (Volkering et al., 1992). Maintaining mechanisms for both hydrocarbon entry and efflux can also modulate the delicate balance between uptake and toxicity.

The absence of conclusive evidence for transport across the cytoplasmic membrane does not mean that such active transport mechanisms do not exist. However, the definitive studies to determine if cytoplasmic transport proteins are required for hydrocarbon accumulation will necessitate the isolation of transport mutants, and the identification of specific genes and proteins that are required for the process.

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