5 Hydrocarbon-Oxidizing Bacteria

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Abstract
Hydrocarbon-oxidizing bacteria have been isolated from a variety of terrestrial and aquatic environments, using both enrichment and direct plating techniques. Although bacteria able to grow on aliphatic and aromatic hydrocarbons are found in many genera, the genera Alcanivorax appear to be special because these bacteria are specialized for growth on hydrocarbons. The initial step in the bacterial degradation of hydrocarbons is the introduction of oxygen into the molecules by group-specific oxygenases. Since these oxygenases are membrane bound, the cell must come into direct contact with their water-insoluble substrate. Hydrocarbon-oxidizing bacteria have potential applications in bioremediation of oil pollution, enhanced oil recovery, production of surface-active agents, and in the use of hydrocarbons as substrates for industrial fermentation processes.

1. Microbial spoilage of petroleum products
2. Treatment of oil spills and disposal of petroleum wastes
3. Enhanced oil recovery
4. Production of surface-active agents
5. Hydrocarbons as substrates in industrial fermentation processes

Introduction

Periodic ecological disasters caused by large oil spills call attention, in a dramatic manner, to the toxicity of petroleum. The fact that hydrocarbons persist for months and even years following major oil spills indicates that hydrocarbon biodegradation is slow in most natural environments. To the microbiologist, the fundamental questions are the following: What are the biochemical mechanisms of hydrocarbon degradation? Which microorganisms are involved? What are their special properties? What limits the rate of hydrocarbon degradation in the environment? And from an applied point of view, what (if anything) can be done to accelerate this rate? Several decades of research on hydrocarbon-oxidizing bacteria have provided considerable data relevant to these questions. This chapter will discuss the distribution, nutritional requirements, enumeration, isolation, identification, special physiologic characteristics, and potential applications of hydrocarbon-degrading bacteria. The specific class of methane oxidizers will be presented in a separate chapter.

Habitats

Hydrocarbons are a ubiquitous class of natural compounds. Not only are they found in petroleum-polluted areas, but chemical analyses have revealed the presence of significant quantities of aliphatic and aromatic hydrocarbons in most soils and sediments (Giger and Blumer 1974; Stevenson 1966). The most probable origin of the low concentrations of widely distributed

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hydrocarbons is ongoing biosynthesis by certain plants and microorganism (Fehler and Light 1970; Hardwood and Russel 1984; Hunt et al. 1980; Juttner 1976; Kolattukudy et al. 1972; Mikkelson and von Wettstein-Knowles 1978; Winters et al. 1969). Hydrocarbons are produced by reduction of fatty acyl-CoA by enzymes which utilize NADH or NADPH. Other sources of hydrocarbons are natural seeps on the ocean floor and unburned fuel from oil-burning engines (Floodgate 1984). Since hydrocarbons are natural products as well as pollutants, it is not surprising that hydrocarbon-oxidizing bacteria are widely distributed in nature. A sample of ecological studies on hydrocarbon-degrading bacteria is shown in *Table 5.1*.

It can be seen that hydrocarbon oxidizers are located in virtually all natural areas, although with large variations in cell concentration. As would be expected, the ratio of hydrocarbon-oxidizing bacteria to the total population of heterotrophic bacteria, as well as the variety of hydrocarbon-degrading microorganisms found in a particular ecosystem, may change according to the time of sampling or the extent of oil pollution (Geiselbrecht et al. 1996). Atlas (1981) has discussed many of the factors that limit the growth of hydrocarbon-oxidizing bacteria in nature. These include physical constraints, such as temperature, availability of oxygen, salinity, pH, and the extent to which the particular habitat is an open or closed ecosystem. Nutritional factors are also important and include the availability of utilizable sources of nitrogen, phosphorus, and other elements; the nature of the hydrocarbon substrate and its effective concentration; and the possible presence of toxic substances either in the petroleum product or in the environment itself.

Effect of Oil Pollution

The localization of hydrocarbon-oxidizing bacteria in natural environments has received considerable attention because of the possibility of utilizing their biodegradation potential in the treatment of oil spills. Because of the enormous quantities of crude and refined oils that are transported over long distances and consumed in large amounts, the hydrocarbons have now become a very important class of potential substrates for microbial oxidation. It is not surprising, therefore, that hydrocarbon-oxidizing microorganisms have recently been isolated in large numbers from a wide variety of natural aquatic and terrestrial environments. Several investigators have demonstrated an increase in the number of hydrocarbon-oxidizing bacteria in areas that suffer from oil pollution (*Table 5.1*). Walker and Colwell (1976a, b) observed a positive correlation between the percentage of petroleum-degrading bacteria in the total population of heterotrophic microorganisms and the amount of heptane-extractable material in sediments of Colgate Creek, a polluted area of Chesapeake Bay. In contrast, no correlation was found when total numbers of hydrocarbon oxidizers (rather than percentages) were compared to hydrocarbon levels. Horowitz and Atlas (1977b) observed shifts in microbial populations in an Arctic freshwater lake after the accidental spillage of 55,000 gal of leaded gasoline. The ratio of hydrocarbon-utilizing to total heterotrophic bacteria was reported to be an indicator of the gasoline contamination. These investigators also studied shifts in microbial populations in Arctic coastal water using a continuous flow-through system, following the introduction of an artificial oil slick (Horowitz and Atlas 1977a). The addition of the oil appeared to cause a shift to a greater percentage of petroleum-degrading bacteria. Atlas and Bartha (1973b) found similar results in an oil-polluted area in Raritan Bay off the coast of New Jersey. Hood et al. (1975) compared microbial populations in sediments of a pristine salt marsh with those of an oil-rich marsh in southeastern Louisiana. These investigators also found a high correlation between the percentage of hydrocarbon oxidizers and the level of hydrocarbons in the sediments. Significant increases in the number of hydrocarbon-utilizing microorganisms were found in field soils following the addition of several different oil samples (Raymond et al. 1976). No estimate of the ratio of hydrocarbon oxidizers to the total heterotrophic population was presented.

From the studies discussed above, it is clear that the presence of hydrocarbons in the environment frequently brings about a selective enrichment in situ for hydrocarbon-utilizing microorganisms. Evidence also has been presented suggesting that the supplementation of certain ecosystems, particularly oil-polluted marine environments with nitrogen and phosphorus, may increase the relative number of hydrocarbon oxidizers (Atlas and Bartha 1973a; Gutnick and Rosenberg 1977; Reisfeld et al. 1972; Song and Bartha 1990).

Isolation and Enumeration

The use of hydrocarbons as substrates for bacterial growth presents special problems both to the microorganism using them as a source of carbon and energy and to the investigators in the field of hydrocarbon microbiology. Depending on the solubility of the particular hydrocarbon in water, its physical state (solid, liquid, or gas), and toxicity, different isolation methods must be employed. In all cases, the heterogeneity of the system complicates sampling, enumeration, and growth measurement procedures. After a discussion of general nutritional requirements for hydrocarbon-degrading bacteria, several specific procedures for the selective enrichment and isolation of the different hydrocarbon degraders will be presented.

General Nutritional Requirements

In addition to the requirements for suitable cell-hydrocarbon interactions and the specific genetic potential of the organism for hydrocarbon oxidation, a number of general nutritional conditions must be fulfilled for bacteria to utilize hydrocarbons. These nutritional requirements depend on the fact that hydrocarbons, as the name denotes, are compounds composed solely of carbon and hydrogen atoms. Thus, all other elements essential for cell growth must be available in the growth medium. These

Table 5.1
Sample habitats and characteristics of hydrocarbon-utilizing microorganisms

Location	Source	Carbon source	Cell concentration	References
Prince William Sound	Surface	Hexadecane	2–12 × 10^3 per g	Lindstrom et al. (1991)
Alaska, beach gravel	Subsurface	Hexadecane	1–12 × 10^3 per g	
Tyrolean Alps	Subsoil	Diesel	0.2–3 × 10^4 per g	Margesin and Schinner (1997)
Bayway Refinery, New Jersey	Surface	Jet fuel	1–4 × 10^3 per g[a]	Song and Bartha (1990)
	Surface	Jet fuel	1–6 × 10^3 per g[a]	
Prince William Sound	Surface	Fuel oil	10^3–10^4 per g	Haines et al. (1996)
Alaska, Duck Island	Sand	–		
Puget Sound, Washington	Contaminated sediment	Phenanthrene	10^4–10^3 per g	Geiselbrecht et al. (1996)
	Uncontaminated sediment	Phenanthrene	10^3–10^4 per g	
Barataria Bay (Louisiana Coast)	Over 200 stations along the coast	Lightweight paraffin oil	10^3–10^4 per g mud	ZoBell and Prokop (1966)
Chesapeake Bay	Eastern Bay (water)	Nondetergent motor oil	0.5–6 × 10^3 per ml	Walker and Colwell (1976b)
	Eastern Bay (sediment)	Nondetergent motor oil	8–99 × 10^3 per g	Walker and Colwell (1976b)
	Colgate Creek (water)	Nondetergent motor oil	90–4.4 × 10^3 per ml	Walker and Colwell (1976b)
	Colgate Creek (sediment)	Nondetergent motor oil	10–9.0 × 10^3 per g	Walker and Colwell (1976b)
Atlantic Ocean sediment off the North Carolina coast	250 m off shore (depth, 9 m)	Model petroleum substrate	1.5–1.2 × 10^2 per ml	Walker and Colwell (1976b)
	50 km from shore, continental shelf (depth, 62 m)	Model petroleum substrate	3 × 10^2 to 3 × 10^3	Walker et al. (1976)
	375 km from shore (depth, 5,000 m)	Model petroleum substrate	4 × 10^4 per ml	Walker et al. (1976)
Alaskan waters	Chukchi Sea	Crude oil	10^3–10^4 per ml	Horowitz and Atlas (1977b)
	Port Valdez	Crude oil	3 × 10^2 per liter	Robertson et al. (1973)
	Prudhoe Bay	Crude oil	7 × 10^2 per liter	Atlas and Schofield (1975)
	Cape Simpson oil	Crude oil	3 × 10^6 per g soil	Atlas and Schofield (1975)
Southern Louisiana marsh sediments	Airplane Lake	Crude oil	10^3–10^2 per g sediment	Crow et al. (1975)
	Martigan Point	Crude oil	10^3–10^2 per g sediment	Crow et al. (1975)
Field plots	Marcus Hook, PA	Hexadecane	4.4–11 × 10^4 per g	Raymond et al. (1976)
	Tulsa, OK	Hexadecane	1–5 × 10^2 per g	Raymond et al. (1976)
	Corpus Christi, TX	Hexadecane	3–66 × 10^4 per g	Raymond et al. (1976)
Lake Mendota, WI	Surface water	Hexadecane	10^2–8 × 10^3 per ml	Ward and Brock (1976)
Athabasca oil sands	River sediment	Hexadecane	7 × 10^4 per ml	Wyndham and Costenon (1981)
		Naphthalene	1 × 10^3 per ml	

[a]Determined by FDA (fluorescein diacetate) epifluorescence

include molecular oxygen, utilizable forms of nitrogen, phosphorus, sulfur, metals, and trace components. The requirement for molecular oxygen has been given much attention, particularly with respect to maximum production of single-cell protein by hydrocarbon-degrading microorganisms (Mimura et al. 1973; Schocken and Gibson 1984). The limitation for oxygen is easily overcome in small-scale laboratory studies or in open aqueous systems where the oil–water interface is in direct contact with air at all times. The possibility of anaerobic decomposition of hydrocarbons has received considerable

attention (Hollinger and Zehnder 1996). Although hydrocarbon utilization by strictly anaerobic sulfate-reducing bacteria (e.g., Rosenfeld 1947) has been reported, evidence that pure cultures of sulfate-reducing bacteria can attack hydrocarbon in the absence of additional sources of organic carbon is not definite. However, a few microbial species appear to be able to grow on pure alkane in the absence of molecular oxygen, if provided with nitrate as an electron acceptor (Senez and Azoulay 1961; Mihelcic and Luthy 1988) or sulfate (Rueter et al. 1994; Rabus et al. 1999).

The nitrogen and phosphorus requirements for maximum growth of hydrocarbon oxidizers can generally be satisfied by ammonium phosphate. Alternatively, these requirements can be met with a mixture of other salts, such as ammonium sulfate, ammonium nitrate, ammonium chloride, potassium phosphate, sodium phosphate, and calcium phosphate. When ammonium salts of strong acids are used, the pH of the medium generally decreases with growth. This problem can often be overcome by using urea as the nitrogen source. In theory, approximately 150 mg of nitrogen and 30 mg of phosphorus are consumed in the conversion of 1 g of hydrocarbon to cell material. In open systems, the high water solubility of most utilizable sources of nitrogen and phosphorus reduces their effectiveness because of rapid dilution. In principle, this problem can be solved by using oleophilic nitrogen and phosphorus compounds with low C/N and C/P ratios. It was found that a combination of paraffinized urea and octyl phosphate was able to replace nitrate and inorganic phosphate, respectively (Atlas and Bartha 1973a). A more economical way may be to add water-insoluble controlled-release nitrogen and phosphorus fertilizers. This technology has been successfully demonstrated in laboratory and field experiments (Rosenberg et al. 1996). Another practical source of hydrophobic N and P is guano. One intriguing possibility to obviate the need for addition of nitrogen compounds to the medium is to use a bacterium that is capable of both hydrocarbon degradation and nitrogen fixation. Such microorganisms were reported following enrichment on hydrocarbon media lacking nitrogen salts (Coty 1967).

In addition to utilizable sources of nitrogen and phosphorus, the mineral requirements of hydrocarbon-degrading bacteria can be met by the addition of K^+, Mg^{2+}, Fe^{2+}, and SO_4^{2-} to purified media. All other inorganic ions required by bacteria to obtain optimum growth are commonly present in sufficient concentration as contaminants in these salts. For most marine hydrocarbon degraders, artificial seawater (or filtered seawater), supplemented simply with phosphate, a nitrogen source, and the hydrocarbon, serves as an adequate medium for enrichment culture studies. In certain aquatic environments under conditions in which the water was supplemented with nitrogen and phosphorus, a high concentration of iron may limit oil biodegradation (Dibble and Bartha 1976). Under these conditions, an encapsulated oleophilic iron compound, ferric octoate, was found to be as effective in stimulating biodegradation as various water-soluble iron derivatives, such as ferric ammonium citrate.

Enumeration of Hydrocarbon-Degrading Bacteria

The determination of the concentration of hydrocarbon-degrading bacteria is one of the methods commonly used for monitoring oil pollution in the environment. Theoretical difficulties associated with the interpretation of these data have been discussed elsewhere (Floodgate 1973). The enumeration of hydrocarbon-degrading bacteria presents two special technical problems, sampling and choice of carbon source. Petroleum-degrading bacteria tend to adhere to hydrophobic materials (*Fig. 5.1*). Thus, unless the bacteria are removed from the material and dispersed prior to enumeration, only minimum cell numbers can be obtained. The choice of a carbon source is an even more serious problem. Petroleum is an extremely complex mixture of hydrocarbons. Because certain bacteria may grow only on minor components in oil, it would be necessary to incorporate large quantities of petroleum into the growth medium to ensure sufficient substrate for these bacteria to grow well. However, high concentrations of petroleum and mixtures of hydrocarbons cannot be used because they are toxic to bacteria (Vestal et al. 1984). Thus, the enumeration of hydrocarbon-degrading bacteria using petroleum as the carbon source selects primarily for bacteria that can degrade major components of the oil mixture. Often, pure hydrocarbons and mixtures of pure hydrocarbons and fractions of crude oil can be used to advantage in replacing petroleum as the carbon source in the isolation medium. The following four methods have been used to enumerate hydrocarbon-degrading bacteria in the marine, estuarine, and freshwater environments.

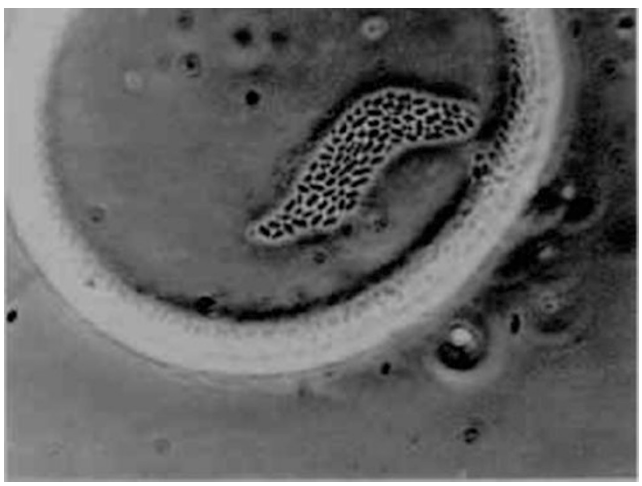

Fig. 5.1
Phase contrast photomicrograph of bacterial strain UP-2 (Horowitz et al. 1975) growing on supplemented 0.1 % hexadecane-seawater medium. During exponential growth, most of the cells appear to be in the form of microcolonies tightly bound to oil droplets. Diameter of oil droplets approximately 150 μm

Enumeration of Hydrocarbon-Degrading Bacteria in Marine Material Not Miscible with Water

1. Approximately 2 g of the material to be examined is placed in a sterile bottle containing 100 ml sterile seawater or salts medium (Gunkel and Trekel 1967).
2. After 1 ml of a sterile, nontoxic, nonionic emulsifier and 1 drop of an antifoam agent are added to the sample, the mixture is homogenized to disperse and break up the bacterial aggregates (e.g., an Ultra Turrax homogenizer run at 24,000 rpm for 30 s).
3. The homogenized sample is then diluted serially in steps of 1:10 in sterile seawater or salts medium.
4. One-milliliter samples of the appropriate dilutions are then inoculated into bottles or tubes containing the following sterile medium:

Aged seawater	750 g
Distilled water	250 ml
NH_4Cl	0.5 g
K_2HPO_4	0.5 g
NaH_2PO_4	1.0 g

5. After addition of one drop of sterile hydrocarbon, the samples are incubated for 2–6 weeks, depending on the temperature of incubation.
6. Bottles remaining turbid after addition of 1 ml HCl to dissolve inorganic salts are scored, and the most probable number is calculated from tables published in Standard Methods (American Public Health Association 1995).

Enumeration of Hydrocarbon-Utilizing Bacteria by Direct Plating of Estuarine Water and Sediment Samples

Estuarine salts solution (Colwell et al. 1973):

Distilled water	1 l
NaCl	10 g
$MgCl_2$	2.3 g
KCl	0.3 g

Oil powder: 10 g of hydrocarbon dissolved in 30 ml of diethyl ether is mixed with 10 g of silica gel, allowed to evaporate, and then added to the following basal medium prior to autoclaving.

Oil agar medium:

Distilled water	1 l
NaCl	10 g
$MgSO_4$	0.5 g
NH_4NO_3	1.0 g
$FeCl_3$ (25 g · ml^{-1})	1 drop
Purified agar (Difco)	20 g
Oil powder	10 g
KH_2PO_4 (10 g · 100 ml^{-1})[a]	3.0 ml
K_2HPO_4 (10 g · 100 ml^{-1})[a]	7.0 ml
Fungizone[a]	10 mg

[a]Added after autoclaving

Enumeration of Hydrocarbon-Degrading Bacteria in Freshwater

Basal medium (Ward and Brock 1976):

Distilled water	1 l
NaCl	0.4 g
NH_4Cl	0.5 g
$MgSO_4 \cdot 7H_2O$	0.5 g
$NaHPO_4 \cdot 7H_2O$[a]	0.05 g
KH_2PO_4[a]	0.05 g

[a]Added after autoclaving

Serial dilutions are made in the basal medium. One drop of sterile hydrocarbon is added to 10 ml of basal medium. After incubation at the appropriate temperature, growth is detected by pellicle formation at the surface of the oil droplet. Most probable number is determined from the tables published in Standard Methods (American Public Health Association 1995).

Enumeration of Hydrocarbon-Degrading Bacteria by a 96-Well Plate Procedure

The 96-well plates were processed with a Beckman Biomek 1000 laboratory robot (Beckman Instruments, Fullerton, CA, USA) which filled the wells with medium, performed tenfold serial dilutions of the sample, and added oil to the inoculated wells (Haines et al. 1996). The robot added 180 ml of BH (Bushnell-Haas medium, Difco) to each well in 11 of the 12 rows, leaving the first row empty. It transferred 200 ml of undiluted sample to the wells in the first row, mixed their contents, and then transferred 20 ml to each well in the second row. The contents of the second row were mixed, and 20 ml was transferred to each well in the third row. This procedure of mixing and transfer was carried out for all except the last row, which served as a sterile control. Sterile pipet tips were used for each transfer. After the dilutions were completed, 2 ml of oil was added to each well as the growth substrate. The plates were sealed in plastic bags and incubated for 14 days at 20 °C. Positive wells were scored in one of two ways. When F2 (number 2 fuel oil) was the carbon source, 50 ml of a sterile solution (3 g · l^{-1}) of INT (iodonitrotetrazolium violet;

Research Organics, Cleveland, OH, USA) was added to each well. INT competes with O_2 for electrons from the respiratory electron transport chain, and it is reduced to an insoluble formazan that deposits as a red precipitate in the presence of active respiring microorganisms. Red or pink wells were scored as positive. When a crude oil was used as the carbon source, a smooth oil slick developed in each well. Positive wells were scored by emulsification or dispersion of this oil slick. INT cannot be used effectively with crude-oil substrates because their dark color interferes with detection of formazan deposition.

Enrichment Culture for Hydrocarbon-Degrading Bacteria

Since hydrocarbons are natural products that are widely distributed in nature, it is not surprising that bacteria able to degrade hydrocarbons can easily be isolated by standard enrichment culture procedures. By varying parameters, such as temperature, pH, hydrocarbon concentration, and basal medium, a wide variety of different hydrocarbon-degrading and emulsifying bacteria can be obtained from either aquatic or terrestrial environments. In most studies, crude oi1 or a petroleum distillate was used as the sole carbon and energy source in the enrichment culture procedure. Under those conditions, bacteria that specialize in the oxidation of low-molecular-weight n-alkanes are generally obtained. Bacteria that grow more slowly or oxidize minor components of crude oil never increase much in batch enrichments, although the activity of these microorganisms may be of special significance in natural environments. To overcome this difficulty, enrichment culture procedures have to be employed using different carbon sources. The following examples represent only a few of the possible variations.

Enrichment of Crude Oil-Degrading Bacteria in Supplemented Seawater

To 20 ml of unsterilized seawater in a 125-ml flask was added 155 mg unsterilized crude oil, 0.056 mM KH_2PO_4, and 7.6 mM $(NH_4)_2SO_4$ (Reisfeld et al. 1972). After inoculation with about 1 g beach tar or oily sand, the flask was incubated at 30 °C with shaking. After about 1 week, the oil became evenly dispersed throughout the liquid. One milliliter of this culture was then transferred to 20 ml sterile seawater supplemented with 0.056 mM KH_2PO_4, 7.6 mM $(NH_4)_2SO_4$, and 1 drop of sterile crude oil. (The crude oil was sterilized by filtration through a Millipore 0.45 mm membrane filter.) After one passage, the oil became emulsified in 2–4 days. Such mixed cultures were maintained by serial transfers to fresh media at 3–4-day intervals. Pure cultures were obtained by streaking the enrichment culture either onto the above medium solidified with 1.5 % agar (Difco) or nutrient agar (Difco) prepared with filtered seawater. Isolated colony types were found to grow both on nutrient- and oil-containing media.

Enrichment culture procedures used to isolate crude oil-degrading bacteria, such as that described in the preceding paragraph, yield a mixture of several different strains even after several transfers. One reason for this is the heterogeneity of the carbon source. Low-molecular-weight paraffin oxidizers (C_{10} to C_{25}) generally dominate the cultures because of their more rapid growth rate. To isolate bacteria that could utilize other fractions of crude oil, the following sequential enrichment culture can be employed:

Sequential Enrichment of Hydrocarbon-Degrading Bacteria on Crude Oil in Supplemented Seawater

Inoculate the following sterile medium with a pure culture of an n-paraffin-oxidizing bacterium (which can be obtained by standard enrichment culture procedures): 1 l filtered water, 10 mg $K_2HPO_4 \cdot 3H_2O$, 450 mg urea, and 0.7 ml crude oil (Horowitz et al. 1975). After 3 days incubation with shaking at 30 °C, the residual oil is extracted with 1 l of benzene-pentane-ether (3:1:1, v/v/v). The oil remaining after evaporation of the organic solvent in vacuo is referred to as "bacteria-depleted oil." An enrichment culture is now carried out using the "bacteria-depleted oil" in place of crude oil as the sole source of carbon and energy.

The general failure of investigators to isolate microorganism on highly water-insoluble, solid hydrocarbons, such as anthracene, may be due to the fact that the cells remain firmly bound to the substrate. Thus, a standard enrichment culture procedure in which a portion of the bulk water phase is transferred would select against rather than for these specific microorganisms. It may be that for successful enrichments, the solid phase should be used as the inoculum during the sequential transfers.

Enrichment of Hydrocarbon-Degrading Bacteria on Bunker C Fuel Oil in Minimal Salts Medium

One gram of beach sand sample or 1 ml of a water sample was added to the following minimal medium containing 0.125 % Bunker C oil (steam-sterilized at 121 °C and 15 psi for 15 min in tightly capped flasks to prevent evaporation) (Mulkins-Phillips and Stewart 1974a):

Minimal salts medium:

Distilled water	1 l
NaCl	28.4 g
K_2HPO_4	4.74 g
KH_2PO_4	0.56 g
$MgSO_4$	0.50 g
$CaCl_2$	0.1 g
NH_4NO_3	2.5 g
Trace element stock (pH 7.1)	1 ml

Flasks were incubated at 20 °C for 14 days and 120 rpm on a refrigerated gyratory shaker bath. Pure cultures of hydrocarbon-utilizing bacteria were isolated from the enrichment culture by streaking onto minimal salts medium to which 2 % washed Ionagar no. 2 (Oxoid) was added. The carbon source consisted of 0.5 ml of the following hydrocarbon mixture added to sterile filter paper secured in the lids of the petri dishes. The dishes were then inverted and incubated at the appropriate temperature for 1–3 weeks.

Hydrocarbon mixture:

Naphthalene	0.1 g
Anthracene	0.1 g
Dibenzothiophene	0.1 g
Decalin	5 ml
Hexadecene-1	5 ml
Hexadecane	5 ml
Octadecane	0.1 g
Dodecane	5 ml
Isooctane	5 ml

Enrichment of Polyaromatic Hydrocarbon-Degrading Bacteria (PAHs)

Small amounts of fresh sediment known to be contaminated with PAHs were inoculated into the following mineral salts medium (g/l) (Churchill 1999):

$(NH_4)2SO_4$	10
KH_2PO_4	5.0
$MgSO_4 \cdot 7H_2O$	0.1
$Fe(NH)_2(SO_4)_2$	0.005
Pyrene	40

Trace metals (Beauchop and Elsden 1960)

After adjusting the pH to 7.0 with NaOH, the flasks were shaken for 1 week. Pyrene-degrading bacteria were detected on pyrene-coated mineral medium (as above) agar plates. Zones of clearing around colonies indicated pyrene degradation. The same procedure can be used with other PAHs replacing the pyrene.

Enrichment on Liquid Aromatic Hydrocarbons

Liquid aromatic hydrocarbons, such as benzene, toluene, and ethylbenzene, are toxic to bacteria when present in the liquid phase (Gibson 1971). However, if these carbon sources are introduced in the vapor phase, good growth can be obtained. *Figure 5.2* illustrates two methods that can be used for growing bacteria on volatile toxic hydrocarbons. Since the liquid hydrocarbons do not come in direct contact with the salts medium, they need not be sterilized. When the reservoir of volatile hydrocarbons is exhausted, it can easily be refilled with a Pasteur pipette.

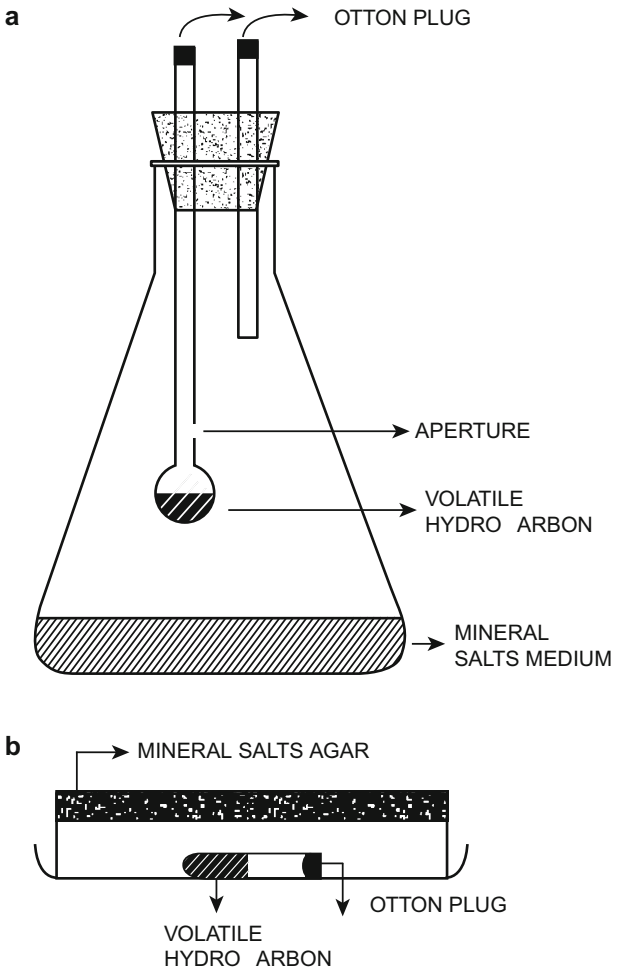

Fig. 5.2
Two methods for the growth of bacteria on volatile hydrocarbons using (a) liquid media and (b) solid media

Enrichment for Nitrogen-Fixing Hydrocarbon Oxidizers

Hydrocarbon-oxidizing bacteria able to grow in the absence of added nitrogen compounds were isolated by addition of 0.1 g soil to 25 ml of mineral salts medium of the following composition (g/l) (Coty 1967):

Na_2HPO_4	0.3
KH_2PO_4	0.2
$MgSO_4 \cdot 7H_2O$	0.1
$FeSO_4 \cdot 7H_2O$	0.005
$Na_2MoO_4 \cdot 2H_2O$	0.002

The containers were incubated in an atmosphere of air and hydrocarbon vapors. After turbidity developed, the cultures were streaked and reincubated on the above mineral salts medium containing 1.5 % washed agar. Purification was achieved after several restreakings and culturing on nitrogen-free mineral salts agar medium. Bacteria able to utilize atmospheric nitrogen on addition of naphthenic acid, n-butane, n-tetradecane, or sodium cyclohexane carboxylate were reported to be isolated by this procedure.

Enrichment for Solid Hydrocarbon-Degrading Bacteria

A slurry of soil in 0.1 M phosphate buffer, pH 7.0, with 0.1 % octadecane was incubated for 1 week, with shaking (Miller and Bartha 1989). This enrichment culture was transferred (1:100 ratio) to the following medium (g/l):

Na_2HPO_4	0.4
KH_2PO_4	0.15
NH_4Cl	0.1
$MgSO_4 \cdot 7H_2O$	0.02
Iron ammonium citrate	0.005
$CaCl_2$	0.001
Octadecane	0.1

To obtain pure cultures, the enrichment was streaked on the above medium solidified with 2 % agar. The *Pseudomonas* sp. that was isolated grew on solid alkanes such as hexatriacontane (C_{36}).

Identification

The variation in bacterial populations isolated by enrichment culture depends largely on the hydrocarbon substrate used in the enrichment, the culture conditions, and the source of the inoculum. Many species capable of hydrocarbon degradation have been isolated (Table 5.2). The most frequently isolated bacterial genera are *Pseudomonas*, *Acinetobacter*, *Flavobacterium*, *Corynebacterium*, *Alcanivorax*, and *Arthrobacter*. Most of the investigations on the degradation of aromatic hydrocarbons have been carried out using *Pseudomonas putida* and species of *Beijerinckia* and *Nocardia* (Gibson 1971). Westlake et al. (1974) studied the effect of oil quality and incubation temperature on the genetic composition of hydrocarbon-decomposing populations isolated from an area in British Columbia that had been exposed to chronic pollution with diesel fuel. All of the populations consisted predominantly of Gram-negative rods, including species of *Pseudomonas*, *Acinetobacter*, *Xanthomonas*, *Arthrobacter*, and *Alcaligenes*.

An extensive study of petroleum-degrading bacteria isolated from Chesapeake Bay waters and sediments was carried out by Austin et al. (1977a, b). A total of 99 strains were examined for

Table 5.2
Genera of hydrocarbon-degrading bacteria

Genus	References[a]	
	From soil	From aquatic environment
Achromobacter	9, 12, 27	5, 8, 10, 20, 21, 25, 28
Acinetobacter	9, 23, 27, 34	5, 8, 17, 24, 26
Actinomyces	12	4, 28, 31
Aeromonas		7, 26
Alcaligenes	9, 16, 23, 27	6, 8, 9
Alcanivorax		36
Arthrobacter	9–12, 14, 15, 18	5, 7, 10, 25
Bacillus	14, 23	4, 7, 10, 20, 28
Beneckea		10
Brevibacterium	10	5
Corynebacterium	12, 14, 15, 22, 27	4, 5, 7, 10, 25, 26, 28
Cycloclasticus	30	
Cytophaga	9, 27	
Erwinia	1	4
Flavobacterium	1, 13, 15, 22, 27	2, 7, 9, 10, 20, 21
Klebsiella		2
Lactobacillus		2
Leucothrix	1	2
Micrococcus	13, 14, 22	
Moraxella		2
Mycobacterium		29
Myxobacterium	3, 14, 23, 32	28
Nocardia	14, 15, 23	2, 4, 5, 10, 21, 26
Peptococcus		7
Pseudomonas	1, 9, 11–14, 16, 18, 19, 23, 27, 33	2, 4, 5, 10, 20, 21, 26, 28
Rhodococcus	35	
Sarcina	14, 22	20
Serratia	1	
Sphaerotilus		4
Spirillum	10	20
Vibrio	1, 30	4, 5, 10, 20, 21, 26, 28
Xanthomonas	9, 10	4

[a]Key to references: 1, Atlas et al. (1978); 2, Atlas and Bartha (1972); 3, Antoniewski and Schaefer (1972); 4, Austin et al. (1977a, b); 5, Bartha and Atlas (1977); 6, Bertrand et al. (1976); 7, Buckley et al. (1976); 8, Byrom et al. (1970); 9, Cook et al. (1973); 10, Cundell and Traxler (1973a, b, 1976); 11, Jensen (1975a); 12, Jensen (1975b); 13, Jobson et al. (1972); 14, Jones and Edington (1968); 15, Kincannon (1972); 16, Kiyohara et al. (1982); 17, Makula et al. (1975); 18, McKee et al. (1972); 19, Miller and Bartha (1989); 20, Mironov (1970), Mironov and Lebed (1972); 21, Mulkins-Phillips and Stewart (1974b); 22, Odu (1978); 23, Perry (1977); 24, Reisfeld et al. (1972); 25, Soli (1973); 26, Walker and Colwell (1974), Walker and Colwell (1975), Walker et al. (1976b); 27, Westlake et al. (1974); 28, ZoBell (1964). Adapted from Floodgate (1984) and Bossert and Bartha (1984). 29, Churchill (1999); 30, Geiselbrecht et al. (1996); 31, Barabas et al. (1995); 32, Burback and Perry (1993); 33, Griffol et al. (1994); 34, Ratajczak et al. (1998); 35, Whyte et al. (1998); 36, Cappello and Yakimov (2010)

48 biochemical, cultural, morphological, and physiological characteristics. A statistical analysis revealed 14 phonetic groups, comprising about 85 % of the hydrocarbon-degrading bacteria. These groups were characterized as actinomycetes; coryneforms; *Enterobacteriaceae*; *Klebsiella aerogenes*; species of *Micrococcus*, *Nocardia*, and *Pseudomonas*; and *Sphaerotilus* natans.

Special mention should be made of the genus *Alcanivorax* (Cappello and Yakimov 2010) because these bacteria seem to play a particularly pivotal role in the oil-spill bioremediation (Schneiker et al. 2006). This obligate hydrocarbonoclastic bacterium (petroleum hydrocarbons serve almost exclusively as its source of carbon and energy) is cosmopolitan and found in various marine environments.

Physiological Properties

There are two essential characteristics that define hydrocarbon-oxidizing bacteria: (1) hydrocarbon-group-specific oxygenases and (2) mechanisms for optimizing contact between the bacterium and the hydrocarbon.

Group-Specific Oxygenases

Several reviews have appeared on the microbial metabolism of straight-chain and branched alkanes (Asperger and Kleber 1991; Singer and Finnerty 1984), cyclic alkanes (Perry 1984), and aromatic hydrocarbons (Gibson 1977; Cerniglia 1984; Pérez-Pantoja et al. 2010). It has been established that the first step in the degradation of hydrocarbons by bacteria is the introduction of both atoms of molecular oxygen into the hydrocarbon. In the case of aromatic hydrocarbons, ring fission requires a dihydroxylation reaction, the introduction of two atoms of oxygen, and the subsequent formation of a *cis*-dihydrodiol (Gibson 1968; Simon et al. 1993). This reaction is catalyzed by a dioxygenase which is a multicomponent, membrane-bound enzyme system (Cerniglia 1992). Further oxidation of the *cis*-dihydrodiol leads to the formation of catechols that are substrates for another dioxygenase that catalyzes ring fission (Evans et al. 1965).

It is important to emphasize that the biochemical mechanism of aromatic hydrocarbon oxidation in prokaryotes is fundamentally different from that of eukaryotes. Fungi and mammalian cells metabolize aromatics using the cytochrome P-450 monooxygenase system, which leads to the formation of arene oxides. These active epoxides can form covalent bonds with nucleophilic sites in DNA, leading to mutations and carcinogenesis. Aromatic hydrocarbons that have been shown to serve as substrates for bacterial oxygenases include benzene, toluene, xylene, naphthalene, phenanthrene, anthracene, benz(a)anthracene, biphenyl, and several of their methylated derivatives. The enzymes necessary for aromatic hydrocarbon degradation are specified, in part, by degradative catabolic plasmids. Enzymes capable of monooxygenating benzene/toluene to phenol/methylphenol and phenols to catechols belong to an evolutionary related family of soluble di-iron monooxygenases (Leahy et al. 2003), which are enzyme complexes consisting of an electron transport system comprising a reductase (and in some cases a ferredoxin), a catalytic effector protein which contains neither organic cofactors nor metal ions and is assumed to play a role in assembly of an active oxygenase (Powlowski et al. 1997), and a terminal hydroxylase with a $(\alpha\beta\gamma)_2$ quaternary structure and a di-iron center contained in each α subunit. These monooxygenases are classified according to their α-subunits, which are assumed to be the site of substrate hydroxylation, into four different phylogenetic groups: the soluble methane monooxygenases, the alkene monooxygenase of *Rhodococcus corallinus* B-276, the phenol hydroxylases, and the four-component alkene/aromatic monooxygenases (Leahy et al. 2003).

In general, alkanes are terminally oxidized to the corresponding alcohol, aldehyde, and fatty acid (Asperger and Kleber 1991). The hydroperoxides may serve as unstable intermediates in the formation of the alcohol (Singer and Finnerty 1984). Fatty acids derived from alkanes are then further oxidized to acetate and propionate (odd-chain alkanes) by inducible β-oxidation systems. The group specificity of the alkane oxygenase system is different in various bacterial species. For example, *Pseudomonas putida* PpG6 (oct) grows on alkanes of 6–10 carbons in chain length (Nieder and Shapiro 1975), whereas *Acinetobacter* sp. HOI-N is capable of growth on long-chain alkanes (Singer and Finnerty 1984). The ability of *P. putida* to grow on C^6–C^{10} alkanes was shown to be plasmid encoded (Chakrabarty et al. 1973). In contrast, all activities necessary for growth of *Acinetobacter* sp. HOI-N and *A. calcoaceticus* BD413 appear to be coded by chromosomal genes (Singer and Finnerty 1984).

Subterminal alkane oxidation apparently occurs in some bacterial species (Markovetz 1971). This type of oxidation is probably responsible for the formation of long-chain secondary alcohols and ketones. Pirnik (1977) and Perry (1984) have reviewed the microbial oxidation of branched and cyclic alkanes, respectively.

Physical Interactions Between Bacteria and Hydrocarbons: Adhesion, Desorption, and Emulsification

The low solubility of hydrocarbons in water, coupled to the fact that the first step in hydrocarbon degradation involves a membrane-bound oxygenase, makes it essential for bacteria to come into direct contact with their hydrocarbon substrates. Two general biological strategies have been suggested for enhancing contact between bacteria and water-insoluble hydrocarbons: (1) specific adhesion mechanisms and (2) emulsification of the hydrocarbon.

To understand the special cell-surface properties of bacteria that allow them to grow on hydrocarbons, it is necessary to consider the dynamics of petroleum degradation in natural environments (Rosenberg et al. 1992). Following an oil spill in the sea, the hydrocarbons rise to the surface and come into contact with

air. Some of the low-molecular-weight hydrocarbons volatilize; the remainder are metabolized relatively rapidly by microorganisms, such as *Pseudomonas* sp., which take up soluble hydrocarbons. These bacteria do not adhere to oil and do not have a high cell-surface hydrophobicity (Rosenberg and Rosenberg 1985). The next stage of degradation involves microorganisms with high cell-surface hydrophobicity, which can adhere to the residual high-molecular-weight hydrocarbons. In the case of *A. calcoaceticus* RAG-1, this adherence is due to thin hydrophobic fimbriae (Rosenberg et al. 1982). Mutants lacking these fimbriae failed to adhere to hydrocarbons and were unable to grow on hexadecane. Other bacteria exhibit high cell-surface hydrophobicity as a result of a variety of fimbriae and fibrils, outer-membrane and other surface proteins and lipids, and certain small cell-surface molecules, such as gramicidin S (Rosenberg et al. 1985) and prodigiosin (Rosenberg et al. 1989). Bacterial capsules and other anionic exopolysaccharides appear to inhibit adhesion to hydrocarbons (Rosenberg et al. 1983).

Desorption from the hydrocarbon is a critical part of the growth cycle of petroleum-degrading bacteria. Petroleum is a mixture of thousands of different hydrocarbon molecules. Any particular bacterium is only able to use a part of the petroleum. As the bacteria multiply at the hydrocarbon/water interface of a droplet, the relative amount of nonutilizable hydrocarbon within the droplet continually increases until the cells can no longer grow. For bacteria to continue to multiply, they must be able to move from the depleted droplet to a fresh oil droplet. *A. calcoaceticus* RAG-1 has an interesting mechanism for desorption and for ensuring that it only reattaches to a droplet of fresh oil. When cells become starved on the "used" hydrocarbon drop or tar ball, they release their capsule. The capsule is composed of an anionic heteropolysaccharide, with fatty acid side chains, referred to as emulsan (Rosenberg 1986). The extracellular, amphipathic emulsan attaches avidly to the hydrocarbon/water interface, thereby displacing the cells to the aqueous phase. Each "used" oil droplet or tar ball is then covered with a monomolecular film of emulsan. The hydrophilic outer surface of the emulsan-coated hydrocarbon prevents reattachment of the RAG-1 cells. The released capsule-deficient bacteria are hydrophobic and readily adhere to fresh hydrocarbon substrate.

Many hydrocarbon-degrading microorganisms produce extracellular emulsifying agents (Desai and Banat 1997; Rosenberg and Ron 1997). In some cases, emulsifier production is induced by growth on hydrocarbons (Hisatsuka et al. 1971). Mutants that do not produce the emulsifier grow poorly on hydrocarbons (Itoh and Suzuki 1972). Pretreatment of oil with emulsifying agents can both inhibit and stimulate oil biodegradation (e.g., Foght et al. 1989; Nakahara et al. 1981; Tiehm 1994; Thibault et al. 1996; Liu et al. 1995; Zhang and Miller 1994). As discussed above, emulsification may be a by-product of a cell/hydrocarbon detachment process. An entire chapter of this book is devoted to bioemulsifiers (Rosenberg and Ron 1997).

Acinetobacter sp. HOI-N accumulates extracellular membrane vesicles of 20–50 nm in diameter when grown on hexadecane (Kappeli and Finnerty 1980). The isolated vesicles partition exogenously supplied hydrocarbons in the form of a microemulsion. These vesicles appear to play a role in the uptake of alkanes. Miller and Bartha (1989) have been able to overcome the difficulties involved in the transport of water-insoluble, solid hydrocarbons by using unilamellar vesicles. A *Pseudomonas* isolate grew on octadecane (C_{18}) and hexatriacontane (C_{36}) with K_s values of 2,450 and 2,700 $mg \cdot l^{-1}$, compared to 60 and 41 $mg \cdot l^{-1}$, respectively, when the hydrocarbon was presented in the form of liposomes. The data clearly demonstrate the importance of transport in the microbial metabolism of recalcitrant hydrocarbons.

Applications

Petroleum microbiology began as an applied subject, and the applied aspects continue to provide the primary impetus for research in this field. Current areas of applied interest are:

1. Microbial spoilage of petroleum products
2. Treatment of oil spills and disposal of petroleum wastes
3. Enhanced oil recovery
4. Production of surface-active agents
5. Hydrocarbons as substrates in industrial fermentation processes

Biodeterioration of petroleum products, such as fuels, lubricating oils, and oil emulsions, has obvious economic implications. Genner and Hill (1981) have reviewed the data on the microbial spoilage of petroleum products and emphasized that spoilage only occurs when the petroleum products come in contact with water. In addition to avoiding water, spoilage can sometimes be retarded by the use of biocides (Rogers and Kapian 1968) or membrane filtration.

In considering the microbial treatment of oil spills, it is essential to distinguish between open systems (e.g., the ocean) and closed ones (e.g., oil storage tanks). In the latter case, it is possible to supplement the system with appropriate sources of nitrogen, phosphates, oxygen, and seed bacteria to enhance microbial growth and petroleum degradation, emulsification, or both. Two early published accounts of the use of these fundamental microbiological principles to enhance oil conversion in a restricted area are the treatment of oily ballast water from an oil tanker (Gutnick and Rosenberg 1977) and of contaminated soil (Raymond et al. 1976). More recently, petroleum pollution has been treated by composting (Kirchmann and Ewnetu 1998), by thermophilic bacteria (Mueller and Nielsen 1996), in soil-water slurries (Zhang and Bouwer 1997), and by using water-insoluble fertilizers (Rosenberg et al. 1996; Knezevich et al. 2006). In an open system, such as the sea, the ability of resident bacteria to extensively degrade a large oil slick is limited primarily by the concentration of nitrogen and phosphorus. Since there is no economical technology for overcoming these nutrient limitations in an open system, there is at present no practical microbial solution for oil spills at sea.

The use of microorganism in tertiary oil recovery has been the subject of several international conferences and literature reviews (e.g., Westlake 1984; Moses and Springham 1982). After primary and secondary recovery (waterflooding) processes, approximately 70 % of the reservoir oil remains underground, trapped in pore spaces and bound to inorganic minerals. The potential use of microorganisms in situ to release this oil depends on the anaerobic production of organic solvents, such as ethanol and butanol, gasses, such as methane and carbon dioxide, and organic acids. These materials can help overcome the physical forces holding the oil in the reservoir. Also, acid production can dissolve carbonates thus increasing the permeability of the reservoir. In addition, microbial products could enhance oil recovery by producing surface-active material and viscosity-altering polymers. Although the evidence for the positive role of microorganism in enhanced oil recovery is limited to a few poorly controlled experiments (Hitzman 1983), the enormous potential of this technology warrants further investigation. In recent years, interest in bioemulsifiers and other microbial surface-active agents has been growing. Many of these compounds are produced by hydrocarbon-degrading microorganisms (Rosenberg 1986; Rosenberg and Ron 1997; Desai and Banat 1997). The advantages of microbially produced surfactants include (1) biodegradability and controlled inactivation, (2) diversity of structure and function for different applications, (3) selectivity for specific hydrocarbon/water interfaces, and (4) characteristic surface modifications.

The use of hydrocarbons as inexpensive raw materials for the production of single-cell protein (SCP) was stimulated by the publications of Champagnat and Llewelyn (1962) and Champagnat et al. (1963). During the 1960s, many large oil and fermentation companies were involved in large-scale research and development projects for the conversion of petroleum fractions into SCP. Although the anticipated market for SCP in human and animal nutrition was not realized, these technological developments have provided a rich source of information about how bacteria grow on petroleum, how a continuous process can be scaled-up, and how bulk products can be recovered economically. In the 1970s, several fermentation plants were operating with capacities of 100,000 t of SCP per year. These were the largest biotechnology plants ever built. Because of the increased cost of hydrocarbon feedstock and more stringent governmental regulations governing its use in fermentation industry, there are presently no large-scale commercial fermentation processes based on hydrocarbon substrates. There are, however, a number of excellent microbial processes that have already been developed; these could be activated under the right set of economic conditions. These include processes for producing alcohols, organic acids, and ketones from specific alkanes; single-cell (food) oil from mixed n-paraffins; and large numbers of microbiological metabolites, including vitamins, amino acids, pigments, polysaccharides, enzymes, and alkanes.

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