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Ultrastructure of Two Oil-Degrading Bacteria Isolated from the Tropical Soil Environment

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ABSTRACT. Two oil-degrading bacteria identified as *Pseudomonas aeruginosa* and *Micrococcus luteus* were isolated from crude-oil-polluted soils in Nigeria. The organisms were grown on *n*-hexadecane and sodium succinate and then examined for the presence of hydrocarbon inclusions. Inclusion bodies were found in *n*-hexadecane-grown cells and were absent in succinate-grown cells. Formation of hydrocarbon inclusion bodies appears to be a general phenomenon among hydrocarbon utilizers.

A diverse group of bacteria and fungi called hydrocarbonoclastic microorganisms are capable of degrading petroleum hydrocarbons. More than 100 species representing about 30 genera have been shown to be capable of utilizing hydrocarbons including at least 22 genera of bacteria and 14 genera of fungi (Atlas 1992). The most important genera of such bacteria in soils include *Pseudomonas*, *Achromobacter*, *Micrococcus*, *Arthrobacter*, *Nocardia*, *Acinetobacter*, *Corynebacterium*, and *Flavobacterium* (Rojas-Avelizapa *et al.* 1999; Ilori 1999). Hydrocarbon utilizers are ubiquitous in soils and this confers a reasonable degree of hydrocarbon assimilative capacity to most soils (cf. Damborský *et al.* 2000).

Alkane-grown bacteria are characterized by unusual morphological features such as presence of intracellular cytoplasmic hydrocarbon inclusions and presence of intracytoplasmic membranes (Singer and Finnerty 1984). However, these features are absent in such organisms when grown on non-hydrocarbon substrates. Hydrocarbons have been shown to accumulate in an unmodified form in cells of *Acinetobacter* sp. HOI-N (Scott and Finnerty 1976*a*), *Flavobacterium* sp., *Brevibacterium* sp. (Atlas and Heintz 1973) and *Aeromonas* sp. (Floodgate 1984). Formation of inclusion bodies may therefore be a required step prior to alkane oxidation by most bacteria.

Most reports on ultrastructure of oil-degrading bacteria originated from work carried out on bacterial strains from the temperate environment while there is little or no report on the presence of similar structures in indigenous oil-degrading tropical bacteria. In this paper, we report the presence of hydrocarbon inclusions in two oil-degrading bacteria isolated from oil-polluted soils in Nigeria.

MATERIALS AND METHODS

Organisms. Two organisms capable of biodegrading crude oil were isolated from crude-oil-polluted soils in Nigeria by plate culture technique on minimal salts agar described by Mulkins-Phillips and Stewart (1974). The organisms were identified using the diagnostic schemes of Holt *et al.* (1994). Diagnostic properties used include Gram reaction, motility, colonial morphology, production of catalase, oxidase, indole and urease, starch hydrolysis, Methyl red test, Voges–Proskaeur test and sugar utilization tests.

Media and cultural conditions. The organisms were cultivated in the minimal salts medium using *n*-hexadecane (0.2 %, *V/V*) or sodium succinate (0.1 %, *W/V*) as sole carbon source. Incubation was carried out at room temperature for 3 d.

Thin-section electron microscopy. Preparation of the cell suspension for thin sectioning was carried out as described by Atlas and Heintz (1973). The cells were fixed in iced solutions of formaldehyde and glutaraldehyde in 0.1 mol/L cacodylate buffer and postfixed in 1 % OsO_4 in 0.1 mol/L cacodycate buffer (pH 7.0) on ice for 2 h. The cells were rinsed in buffer three times and then dehydrated by passing through increasing concentrations of ethanol followed by three washings in absolute ethanol. The cell samples were then embedded in Spurr's resin (*TAAB Laboratories*, Reading, Berks., UK). The cells were cut using an LKB Ultratome III (*LKB Products AB*, Bromma, Sweden) and mounted on copper grids. The sections were stained with uranyl acetate (60 °C, 30 min) followed by lead citrate (room temperature, 10 min). Sections were viewed under an electron microscope (Philips EM301).

RESULTS

The organism putatively identified as *Pseudomonas aeruginosa* was a Gram-negative rod and did not produce indole. However, it did produce pyocyanin and pyoverdin pigments and grew at 42 °C. The second organism was a gram positive coccus with golden yellow pigmentation. It was putatively identified as a strain of *Micrococcus luteus* (Table I).

Table I. Characterization of the isolates

Test	Organism *1*	Organism *2*
Elevation	raised	raised
Consistency	sticky	soft
Pigment	greenish	yellowish
Spores	absent	absent
Cell shape	rod	coccus
Gram reaction	–	+
Catalase	+	+
Motility	+	–
MR	–	–
VP	–	–
Indole	–	–
Citrate	+	–
Urease	–	–
Oxidase	+	+
Starch hydrolysis	–	–
Gelatine liquefaction	+	+
Utilization of glucose	+	+
xylose	–	+
raffinose	+	–
sucrose	+	+
lactose	–	–
mannose	+	–
maltose	+	–
salicin	–	–
Probable identity	*Pseudomonas aeruginosa*	*Micrococcus luteus*

Upon examination of a thin section of *n*-hexadecane-grown *P. aeruginosa* (Fig. 1 *left*), it was found to have subterminal electron-transparent spherical hydrocarbon inclusions bounded by thin intracytoplasmic membranes. A hydrocarbon-grown cell of *M. luteus* also had two pools of hexadecane inclusion bodies (Fig. 2 *left*). These inclusions were not seen in thin sections of succinate grown cells (Figs 1 *right* and 2 *right*).

DISCUSSION

Alkane uptake by bacteria has been reported to take place by dissolution of the hydrophobic oil into the aqueous phase after emulsification or by direct contact with oil droplets larger than the microbes or with oil globules, *i.e.* particles smaller than the bacteria (Floodgate 1984). Different bacteria may employ one or more of such routes of hydrocarbon assimilation depending on the prevailing environmental factor or genetic composition. What appears to be certain and common to most oil-degrading bacteria is the accumulation within the cell of the assimilated hydrocarbon. Two oil-degrading bacteria, *P. aeruginosa* (a Gram-negative organism) and *M. luteus* (a Gram-positive organism) isolated from oil-polluted soils in Nigeria showed the presence of intracellular inclusion bodies. Scott and Finnerty (1976*b*) reported similar hydrocarbon inclusions in hexadecane-grown *Arthrobacter* sp. 80, *Corynebacterium* sp., *Mycobacterium album* 7E4, *Nocardia rubra*, *Nocardia* sp. 72, *Mycobacterium vaccae*, *Candida tropicalis* and naphthalene-grown *Pseudomonas* sp. The inclusion bodies found in these organisms were clearly different from fixation artifacts which could be seen in the succinate-grown cells as previously reported by Dubochet *et al.* (1983).

According to earlier findings using gas chromatography (Muller *et al.* 1983), these inclusions were hexadecane pools. Hydrocarbon inclusions may contain unmodified alkanes (Kennedy *et al.* 1975). Isolated

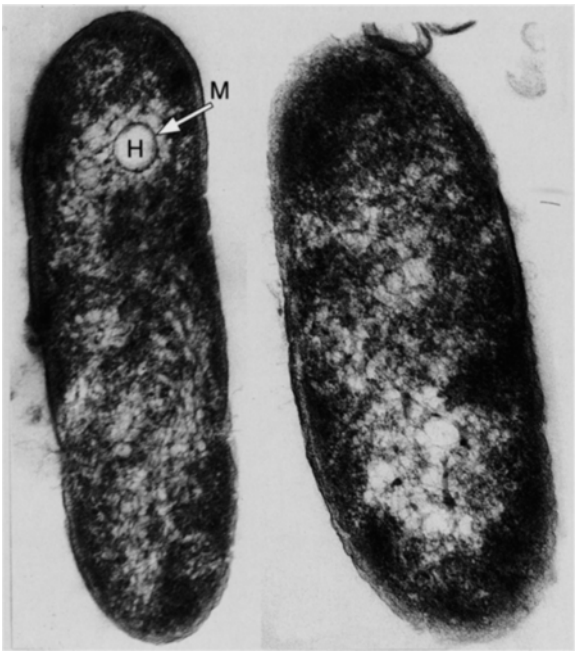

Fig. 1. *n*-Hexadecane- (*left*; ×48 000) and succinate-grown (*right*; ×50 000) *P. aeruginosa*; H – hydrocarbon inclusion, M – membrane.

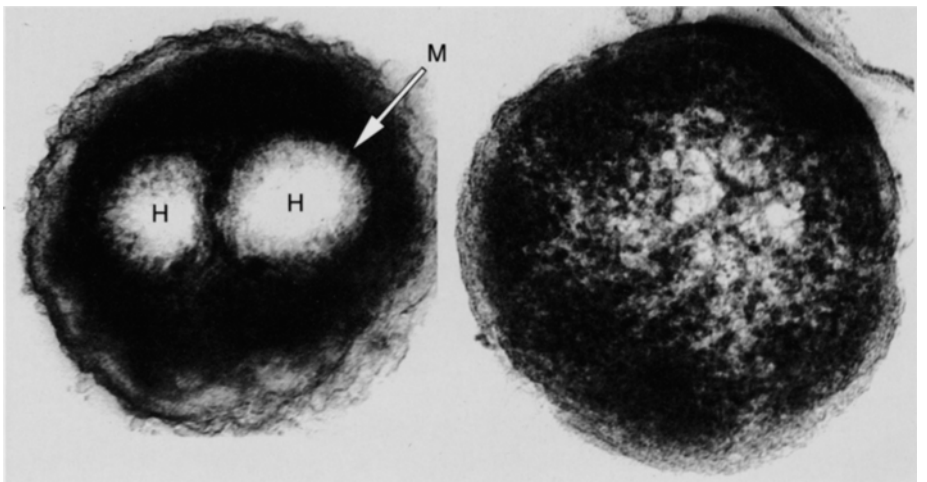

Fig. 2. *n*-Hexadecane- (*left*; ×59 000) and succinate-grown (*right*; ×58 000) *M. luteus*; H, M – *see* Fig. 1.

hydrocarbon inclusions, after chemical analysis were found to contain about 72 % hexadecane, 16 % protein, 9.5 % phospholipid and 3.2 % neutral lipid (Singer and Finnerty 1984). The intracytoplasmic membranes may play important roles in eventual metabolism of the hydrocarbon. The membrane aggregates are formed by repeated looping of the cytoplasmic membrane. This view was corroborated by Muller *et al.* (1983) that the membrane-bound NADP-dependent aldehyde dehydrogenase in *Acetobacter calcoaceticus* was localized at the surface of small vesicles containing hydrocarbons. The enzymes responsible for the initial oxidation of the hydrocarbon may be associated with the membranes and these intracytoplasmic membranes may provide continuous channels for hydrocarbon transfer to the intracellular site of oxidation (Ensley and Finnerty 1980).

The findings in this study show that formation of inclusion bodies may be a common phenomenon among hydrocarbon-degrading bacteria. To our knowledge, this is the first report of such inclusion bodies in a hydrocarbon-degrading *Micrococcus* spp.

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