RESEARCH LETTER

Microorganisms form exocellular structures, trophosomes, to facilitate biodegradation of oil in aqueous media

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Abstract

Cytochemical staining and microscopy were used to study the trophic structures and cellular morphotypes that are produced during the colonization of oil–water interfaces by oil-degrading yeasts and bacteria. Among the microorganisms studied here, the yeasts (*Schwanniomyces occidentalis*, *Torulopsis candida*, *Candida tropicalis*, *Candida lipolytica*, *Candida maltosa*, *Candida paralipolytica*) and two representative bacteria (*Rhodococcus* sp. and *Pseudomonas putida*) produced exocellular structures composed of biopolymers during growth on petroleum hydrocarbons. Four of the yeasts including *S. occidentalis*, *T. candida*, *C. tropicalis* and *C. maltosa* excreted polymers through modified sites in their cell wall ('canals'), whereas *C. lipolytica* and *C. paralipolytica* and the two bacterial species secreted polymers over the entire cell surface. These polymers took the form of fibrils and films that clogged pores and cavities on the surfaces of the oil droplets. A three-dimensional reconstruction of the cavities using serial thin sections showed that the exopolymer films isolated the ambient aqueous medium together with microbial cells and oil to form both closed and open granules that contained pools of oxidative enzymes utilized for the degradation of the oil hydrocarbons. The formation of such granules, or 'trophosomes,' appears to be a fundamental process that facilitates the efficient degradation of oil in aqueous media.

Introduction

Biodegradation of petroleum hydrocarbons in marine and freshwater environments is constrained by the ability of microorganisms to access the hydrophobic surfaces of oil droplets. A key process for attachment to oil droplets involves the production of surface active agents (Horowitz *et al.*, 1975), which is further accompanied by changes in the properties of the cell envelope. One of the most notable features is the formation of canals in the cell wall, which appears to enable the transport of nanometer-sized droplets into the surface of the interior cell membrane (Southam *et al.*, 2001). The first step involving the secretion of surface active agents includes the production of relatively low-molecular-weight surfactants that decrease the surface tension and excretion of high-molecular-weight polysaccharide polymers that serve to emulsify the oil and water into small particles that provide increased surface area for enzymatic attack. In several studies, these exopolymers appear along with fibrils and wall appendages (Marin *et al.*, 1996; Macedo *et al.*, 2005), and can include embedded flagella that are used for both motility and attachment of the cells to the oil surface (Marin *et al.*, 1996). Another microscopic study further reports the appearance of cellular aggregates that form over the surface of oil droplets and invade the oil as the biofilm matures (Macedo *et al.*, 2005). Altogether, these studies provide the basis for comparisons of different model systems. On the other hand, there have been few comparative studies examining different microorganism and substrate conditions using the same methods. Moreover, the three-dimensional (3D) structures of the microhabitats that are generated by exocellular polymers have not yet been described using 3D reconstructions of serial sections cut through oil droplets that are colonized by microorganisms. With the current interest in the remediation of oil-polluted marine and freshwater environments, a better description of

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the feeding structures is highly relevant for understanding how biophysical processes and cell wall adaptations influence the rate of oil degradation.

The research described here used a combination of cytochemical stains and microscopy techniques to describe the specific exocellular fibrils, films and internal granules that are generated by yeasts and bacteria during oil droplet colonization. A novel aspect of the present research was the use of serial sections and computer imaging to generate a 3D reconstruction of the habitat that is formed by selected yeast and bacteria on the oil droplet surfaces. These trophic structures appear as pits and cavities that enclose microbial cells along with the polymers and enzymes that are produced by the oil-degrading microorganisms. We further document variations among different oil-degrading yeasts in their exopolymer production and localization of oxidative enzymes on the cell surface. Our results suggest that the formation of these 'trophosomes' provides an effective strategy for concentrating enzymes and surfactants in and on the oil droplets, thereby reducing their loss by diffusion and allowing a more efficient attack on the oil.

Materials and methods

Microbial cultures and cultivation conditions

The bacterial strains *Rhodococcus* sp. S67 and *Pseudomonas putida* BS3701, and yeasts *Schwanniomyces occidentalis* IBPM-Y-395, *Torulopsis candida* IBPM-Y-451, *Candida tropicalis* IBPM-Y-303, *Candida lipolytica* IBPM-Y-155 and *Candida maltosa* IBPM-Y-820 were from the Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences (RAS). The yeast *Candida paralipolytica* No. 739 was a gift from the Institute of Microbiology, RAS. Bacteria were grown at 24 °C in rotary flasks (120 r.p.m.) containing Evans medium amended with crude oil (2%). Yeasts were cultivated at 28 °C in yeast nitrogen base medium (Difco) supplied with a 1% mixture of hydrocarbons (C_{12}–C_{20}) or crude oil as a carbon source.

Electron microscopy

Yeast cell wall fractions were obtained by the differential centrifugation of mechanically disintegrated cells. To obtain ultrathin sections, cell pellets were fixed (1 h, 4 °C) in 0.05 M cacodylate buffer (pH 7.2) containing 1.5% glutaraldehyde and postfixed (3 h, 20 °C) with 1% OsO_4 in 0.05 M cacodylate buffer (pH 7.2). After dehydration, the cells were embedded in Epoxy resin Epon 812. Ultrathin sections were prepared on an ultramicrotome Ultracut E (Austria) using a diamond knife and a 'perfect loop,' and viewed through an electron microscope JEM-100B (JEOL, Japan) at an accelerating voltage of 80 kV. Freeze fracture and the preparation of sputter-coated carbon–platinum replicas were carried out as described by Fikhte *et al.* (1973).

Cytochemical staining

For the detection of polysaccharides, cells were fixed with ruthenium red according to Luft (1966). For electron cytochemical detection of heme-containing oxidative enzymes, cells were stained with oxidized diaminobenzidine according to Hirai (1971). For immune cytochemistry, cells were fixed in a 1.5% glutaraldehyde, and embedded in Lovicryl K4 resin polymerized at − 40 °C. Ultrathin sections were double stained using specific polyclonal antibodies to yeast cytochrome P-450 and complex 'protein A – gold' (15 nm golden particles).

Petroleum degradation

The quantity of residual oil hydrocarbons in the medium following biodegradation was determined using a gravimetric method according to Drugov & Rodin (2007). Residual oil was extracted from 50 mL of culture broth with chloroform (2 : 1), after which the extract was centrifuged for 30 min at 4000 ***g***. The pellet was dried by mixing over anhydrous sodium sulfate. Chloroform was removed by heating at 70–75 °C for 3–4 h and at 35–40 °C overnight. The degree of oil degradation was determined according to the formula:

$$D = (P_c - P_e)/P_c \times 100\%$$

where P_c and P_e are the residual oil in control (crude oil without addition of bacteria) and experimental samples; D is degradation.

Visualization of 3D trophic structures

For the 3D reconstruction of bacterial and yeast colonies associated with aqueous-suspended oil droplets, semi-thin sections (0.5 μm thick) were cut from the entire cross-section of a sample using a diamond knife. Each series contained at least 40 sections. Sections were viewed and analyzed with a light Axio Imager A1 (Carl Zeiss) microscope; images were captured using an AxioCam (Carl Zeiss) digital camera with the software AXIOVISION AC. Trace software IGL TRACE 1.26b (Fiala, 2002) was used to adjust serial sections by their contours. The 3D images were exported to WRML format, with final rendering using 3D Studio Max9 (Autodesk).

Results

Electron microscopy of the cell surfaces of yeasts grown in oil-containing media revealed profound structural alterations in the cell walls as compared with yeasts grown without hydrocarbons. Depending on the species, the yeasts could be

divided into two groups. Group one included several species of *Candida*, *Torulopsis* and *Shwanniomyces*. When grown on either hexadecane, *n*-alkane mixtures (C_{12}–C_{20}) or crude oil, these yeasts formed 'canals' in their cell walls. The canals were numerous, with up to 100 canals per cell, and were especially vivid on the carbon–platinum replicas of the cell surface (Fig. 1a and b). The formation of the canals was substrate-dependent. When cells that had been grown on oil were transferred to a medium with glucose as a sole carbon source, the canal-forming yeasts reverted to a morphotype without canals (Fig. 1c).

Along with canal formation, the yeasts *S. occidentalis*, *T. candida* and *C. maltosa* also secreted copious amounts of fibrilar substances when cultivated in media with hexadecane, a mixture of *n*-alkanes or crude oil. Ultrathin sections and freeze fraction micrographs vividly demonstrated that this exosubstance was anatomically bound with the canal features (Fig. 2a–c).

Cytochemical staining of these cells with diaminobenzidine further revealed the presence of oxidative enzymes that were concentrated in the canals (Fig. 3a and b). The oxidative enzymes could also be observed in canals of partially purified cell wall fractions from these yeasts, suggesting that these enzymes are ionically or covalently bound with these modified sites of the cell wall (Fig. 3c). Immunocytochemical staining (Fig. 3d) further showed that cytochrome P-450 was concentrated in distinct locations within the cell walls. All of these facts confirmed the supposition that primary oxidation of hydrocarbons by yeasts occurs mainly in the canals where degradative enzymes are entrapped in a polymer matrix.

In contrast to the canal-forming yeast, a second group of yeasts including *C. lipolytica* and *C. paralipolytica* did not form canals when grown in hydrocarbon- or crude oil-containing media, but instead secreted large amounts of fibrilar substances. The carbon–platinum replicas of these yeasts were quite smooth (Fig. 3e) and the yeasts appeared to secrete the fibrilar substances over their entire cell surface. The products of the cytochemical staining reaction targeting oxidative enzymes were located both on the cell surfaces and on the exocellular films (Fig. 3f).

The two bacterial species studied here behaved differently during growth in the oil-containing media: *P. putida* BS3701 attacked the oil globules from the outside (Fig. 4a), whereas *Rhodococcus* sp. S67 penetrated inside the oil globules (Fig. 4b). Irrespective of their locations, these bacteria, both in pure and mixed culture, secreted large amounts of polysaccharide materials in the form of films and granules (Fig. 4c), which were assessed by electron microscopic examinations of ultrathin sections stained with ruthenium red (Fig. 4d). Cytochemical staining with diaminobenzidine showed that oxidative enzymes were located in the cell walls of both bacteria as well as in the extracellular films that were evenly distributed over the cell surfaces (Fig. 4e and f). The exocellular substances were most abundant when bacteria were cultivated in a mixed culture. Moreover, the mixed bacterial culture showed a greater efficiency in oil degradation in water medium than the individual bacteria (more than 70% in mixed cultures as compared with 50–60% observed for *P. putida* BS3701 and *Rhodococcus* sp. S67 as pure cultures).

A 3D reconstruction of bacterial consortia grown on oil (Fig. 5a and b) was performed to answer the questions: (1) Is there any expediency in the abundant release of polymer substances by microorganisms grown on oil hydrocarbons? (2) Do cells form any specific structures that facilitate the use of potential growth substrates contained in oil?

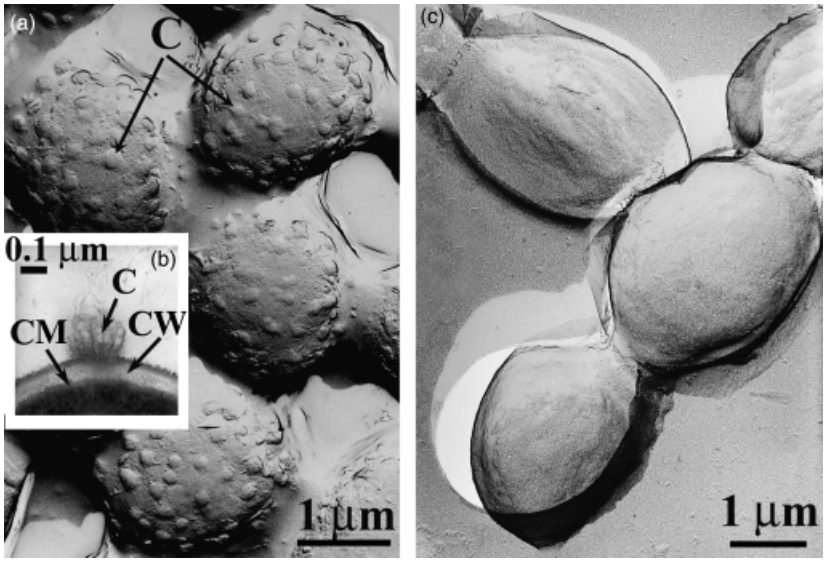

Fig. 1. (a) A carbon–platinum replica of the surface of the yeast *Schwanniomyces occidentalis* grown on *n*-alkanes. (b) Ultrathin section of the yeast *S. occidentalis* grown on *n*-alkanes. (c) A carbon–platinum replica of the surface of the yeast *S. occidentalis* grown on glucose. C, canal; CW, cell wall; CM, cytoplasm membrane.

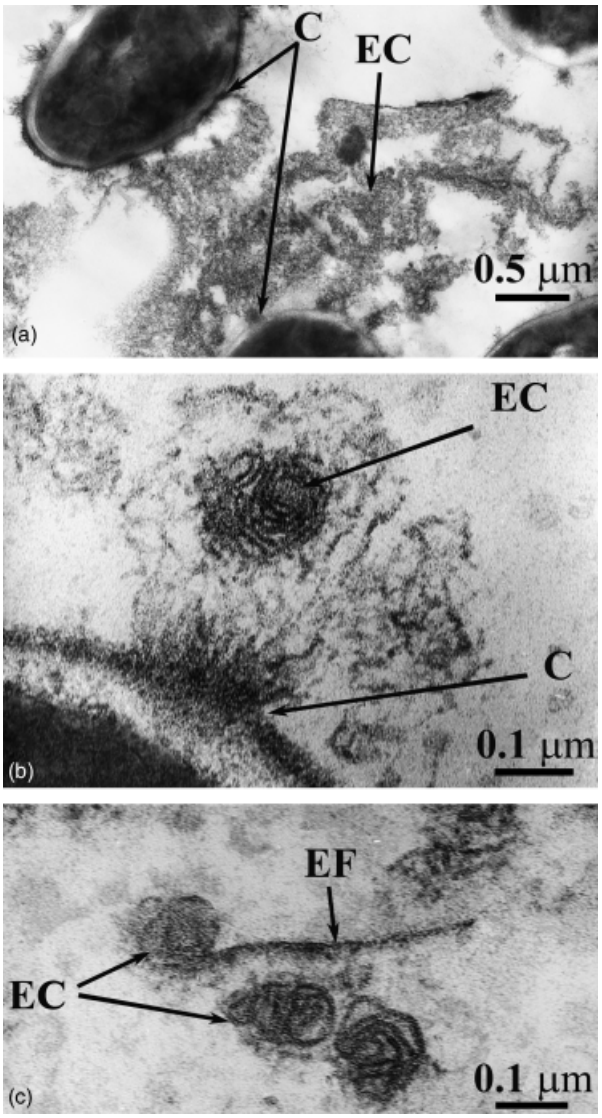

Fig. 2. (a) Ultrathin section of yeasts *Schwanniomyces occidentalis* grown on *n*-alkanes. (b, c) Ultrathin section of exocellular components of *S. occidentalis* yeasts grown on *n*-alkanes. EC, exocellular components; C, canal; EF, exocellular films.

To understand the structural behavior of microorganisms, a bacterial consortium containing cells of *Rhodococcus* sp. S67 and *P. putida* was used as a model. The consortium was grown in shaking flasks with crude oil as a sole carbon source. A visual analysis of the 3D features of bacterial structures formed in the oil demonstrated that the bacteria inhabited discrete cavities in the oil droplets that constituted a kind of 'trophic' vesicle or granule. All of the granules were bound to one another by polymer films and all of the unified structures comprised a well-developed network over the surface of the oil globules. Granules in the globules were either closed or open to the aqueous medium. Open granules probably served as emulsion traps for metabolites generated by oil degradation. The analysis of serial sections showed that after complete utilization of the substrate, the trophic units, or 'trophosomes,' broke down and the entire process of the substrate utilization involved a continuous assembly and decay of functional units in the network of exocellular granule vesicles.

Discussion

The present study reveals a possibly common scenario by which different yeasts and bacteria may colonize and utilize hydrophobic substrates as oil and its components (specifically *n*-alkanes) when suspended as droplets in an aqueous medium. The most notable feature for several of the yeasts studied here was the substrate-induced formation of 'canals' that permeated the cell walls and that were lined with exopolymers and oxidative enzymes. This observation is in agreement with earlier studies reporting the development of modified sites in the cell walls of yeasts grown on oil hydrocarbons. The biochemical aspects of 'canal' formation have been particularly well studied for *S. occidentalis* (Dmitriev *et al.*, 1980), in which the appearance of canals was proposed to occur as the result of enzyme hydrolysis of basic polysaccharides at particular sites in the cell wall; these sites thereafter become hydrophobic and accumulate protein. The presence of reaction products produced by oxidative enzymes in the canals and by cytochrome P-450 at distinct sites in the cell walls indicates that the complexes of enzymes participating in the primary oxidation of hydrocarbons most likely are localized in these structures (Van Beilen *et al.*, 2006). In the other group of microorganisms studied here, namely the non-canal-forming yeasts and bacteria, oxidative enzymes revealed by cytochemical staining occurred primarily on the surface layer of the cell wall and within exocellular polymer structures, suggesting that the primary oxidation of oil hydrocarbons occurs in both locations.

It is highly improbable that the above-described exopolymers are emulsifiers (Van Hamme *et al.*, 2003; Wentzel *et al.*, 2007) that are released by microorganisms during growth on petroleum hydrocarbons. The exocellular polymer constructions, described in the present paper, are sufficiently strong; they remain strongly bound to the cells even during treatment with alcohol and acetone that was used to prepare the samples for electron microscopic examinations. Probably, these exopolymers are similar to the earlier reported 'flocs' produced by *Rhodococcus jostii* RHA1 during growth on hydrocarbons (Perry *et al.*, 2007). The flocs were shown to consist of a high-molecular-mass polymer of a repeating tetrasaccharide unit composed of D-glucuronic acid, D-glucose, D-galactose, L-fucose and *O*-acetyl (1 : 1 : 1 : 1 : 1). In the present work, it was shown that the exocellular polymers yielded a positive cytochemical reaction for

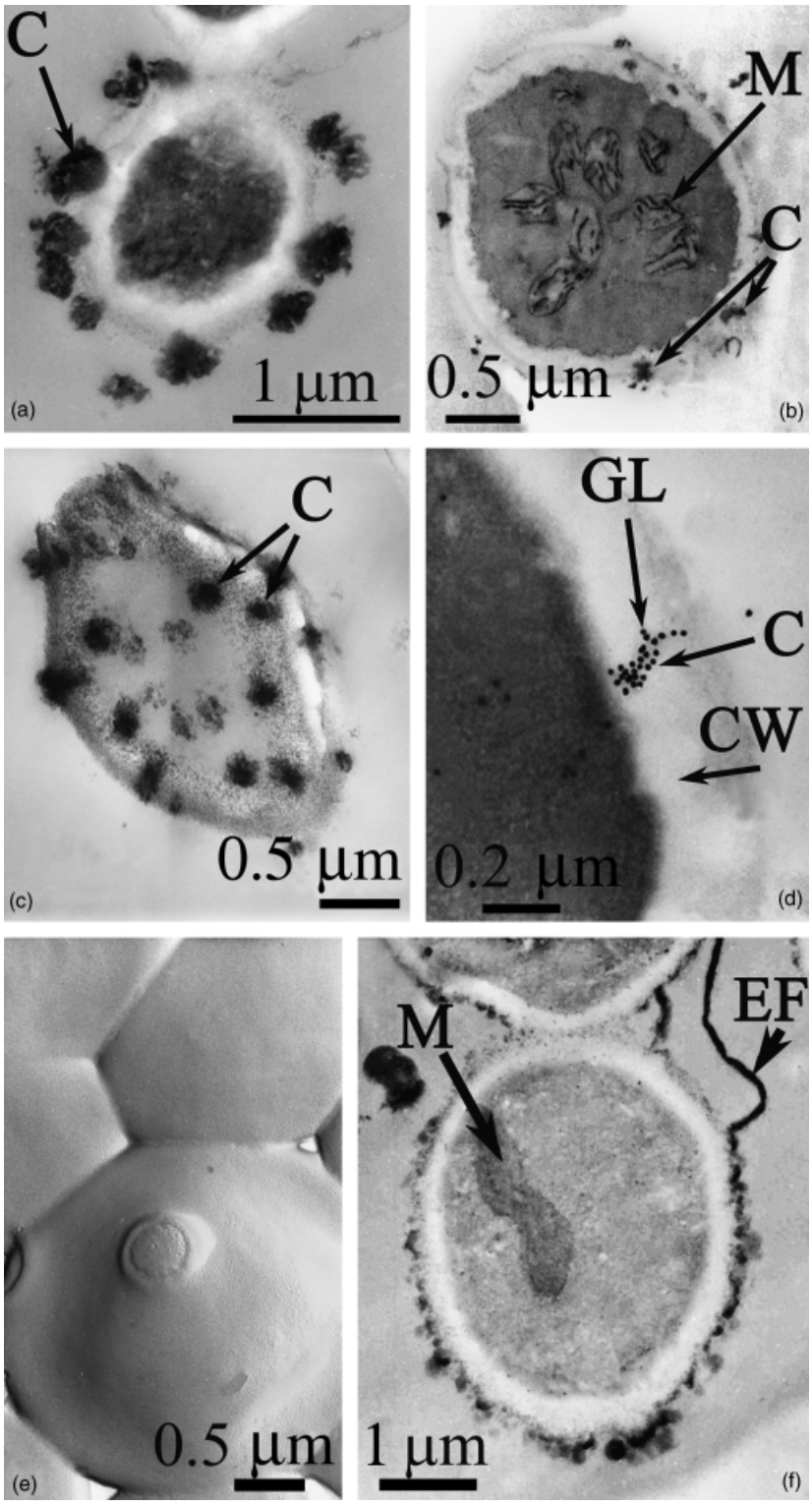

Fig. 3. (a) *Torulopsis candida* grown on *n*-alkanes; electron cytochemical reaction on oxidative enzymes (reaction with 3′3′-diaminobenzidine). (b) *Schwanniomyces occidentalis* grown on *n*-alkanes; electron cytochemical reaction on oxidative enzymes. (c) A fraction of the cell walls of *T. candida* grown on *n*-alkanes; electron cytochemical reaction on oxidative enzymes (reaction with DAB). (d) The yeast *Candida maltosa* grown on *n*-alkanes; immune cytochemical reaction with cytochrome P-450 (protein A – gold label, 15 nm). (e) A carbon–platinum replica of the surface of the yeast *Candida paralipolytica* grown on *n*-alkanes. (f) Ultrathin section of yeasts *C. paralipolytica* grown on *n*-alkanes; electron cytochemical reaction on oxidative enzymes (reaction with DAB). C, canal; M, mitochondria; CW, cell wall; GL, gold label; EF, exocellular film.

oxidative enzymes. It is known that depending on the physiological situation, exopolymers fulfill various functions in microbial associations. They can: (1) retain cells inside a local space, thus establishing a macrostability against outer physical factors, preventing wash-out; (2) maintain a macrostructure of the microbial community providing short diffusion distances for metabolite transfer; (3) bind nutrients; and (4) protect the association against adverse outer factors, for

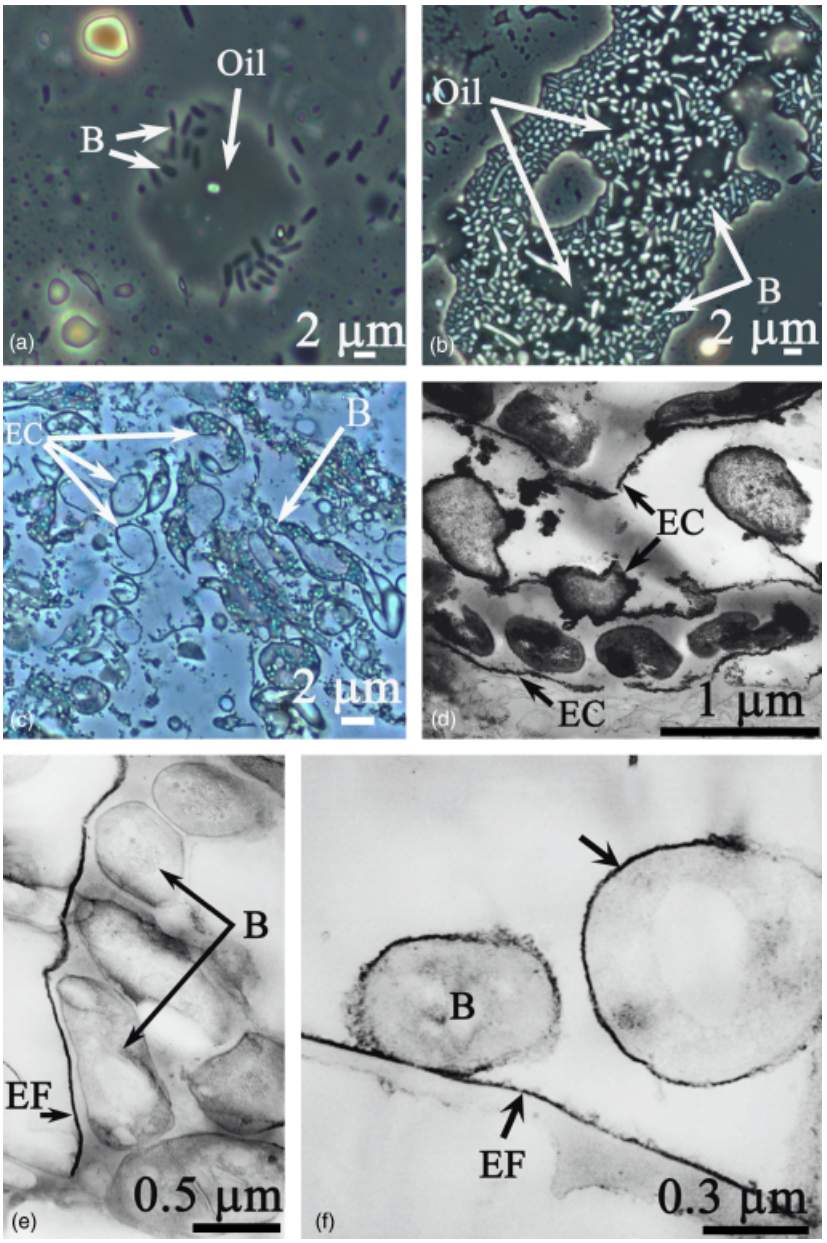

Fig. 4. (a) Phase-contrast microscopy (× 100) showing the interactions of the bacterium *Pseudomonas putida* BS3701 with an oil drop. (b) Phase-contrast microscopy (× 100) showing the interactions of the bacterium *Rhodococcus* sp. S67 with an oil drop. (c) Light microscopy; a semi-thin (0.5 μm) section of a bacterial associate (*P. putida* BS3701 and *Rhodococcus* sp. S67) growth on oil; embedding in Epon 812. (d) Electron microscopy; a bacterial associate (*P. putida* BS3701 and *Rhodococcus* sp. S67); a cytochemical reaction on polysaccharides (staining with ruthenium red); growth on oil. (e, f) Electron cytochemical reaction on oxidative enzymes (with DAB). The bacteria *Rhodococcus* sp. S67 (e) and *P. putida* BS3701 (f) grown on oil (an arrow denotes the reaction product). 'Oil' is an oil drop; B, bacterium; EC, exocellular components; EF, exocellular film.

example such as toxic chemicals or predation by protozoa. The present study has added one more function: participation of exopolymers in the primary utilization of hydrophobic substrates by the formation of trophosomes. The structural investigations carried out in the present work correlate well with the universally adopted idea of a physical structure of a microbial community that allows cooperation and rapid efficient exchange of metabolites and growth factors between its members. A polymer film, such as that described in the present work, isolates a part of the culture medium together with microorganisms and oil. When formed by mixed cultures, this kind of structuring results in the formation of granules containing different, but metabolically related microorganisms, potential growth substrates contained in the oil and a pool of enzymes that is produced by the entire community to carry out the degradation of oil molecules as they are stripped out of the hydrophobic interface by surfactants. These results have important practical applications, and might be used to increase the stability and viability of microbial associates in biopreparations aimed at the destruction of hydrophobic substrates. For example, it may be possible to artificially construct biopreparations of microbial consortia that include specific microorganisms that construct particularly efficient trophosomes. Studies on interactions

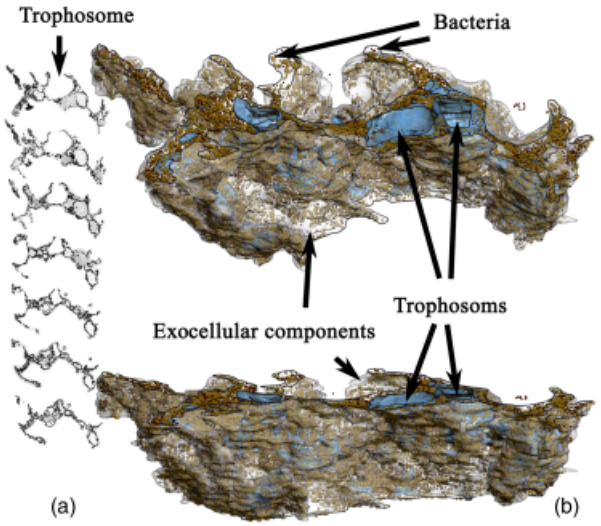

Fig. 5. (a) Serial semi-thin sections of a bacterial associate (*Pseudomonas putida* and *Rhodococcus* sp. S67). (b) 3D reconstruction of the network of vesicles granules based on serial semi-thin sections of the bacterial (*P. putida* and *Rhodococcus* sp.) associate.

between degrader organisms may also consider the compatibility of various degrader organisms with the exopolymers contained in these trophic structures that differentially affect bioavailability to different species. Still another consideration is the effect of dispersants, commonly used in remediation, on the production of trophosomes. In future work, it may be interesting to evaluate the extent to which the rate of oil degradation is influenced not only by the types of enzymes and surfactants that are produced by microorganisms but also by differences in the ability of cells to produce these trophic structures or to coexist with bacteria and yeasts that perform this function. Often, the rate of degradation by mixtures of bacteria is improved over that obtained by pure cultures of single species. Possibly, this may reflect such interactions, involving the creation of microhabitats comprised of mixtures of exopolymers, with different species contributing to the overall features of community-level trophic structures. For example, in a study examining the mechanical properties of the oil–film interface (Kang *et al.*, 2008), it was shown that the bacteria *Acinetobacter venetianus* RAG-1 and *Rhodococcus erythropolis* 20S-E1-c produced substances that created very different surface properties of the oil–water interface: one was soapy and the other was more firm or papery. A comparative analysis of the trophosome habitat generated by different combinations of microorganisms could be a logical follow-up to the research conducted here.

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