

Three-dimensional distribution of GFP-tagged *Pseudomonas putida* during biofilm formation on solid PAHs assessed by confocal laser scanning microscopy

A.C. Rodrigues¹, S. Wuertz², A.G. Brito¹ and L.F. Melo³

¹ University of Minho, Centro de Engenharia Biológica/IBQF, 4710-057 Braga, Portugal

² Department of Civil and Environmental Engineering, University of California, Davis, One Shields Avenue, Davis CA 95616, USA

³ Faculty of Engineering of the University of Porto, Chemical Engineering Department, LEPAE, 4200-465 Porto, Portugal

Abstract Confocal laser scanning microscopy was used to monitor the colonization pattern of the *gfp*-labelled derivative strain of *Pseudomonas putida* ATCC 17514 on fluorene and phenanthrene crystals. The *in situ* experiments showed that *P. putida* tends to grow directly on phenanthrene, forming a biofilm on accessible crystalline surfaces. On the other hand, no significant biofilm formation was observed in the presence of fluorene. The higher biopolymers production in the presence of phenanthrene rather than fluorene may be a strategy to increase substrate accessibility.

Keywords CLSM, EPS, GFP, polycyclic aromatic hydrocarbons, *Pseudomonas putida*

Introduction

Fluorene and phenanthrene are among the most common polycyclic aromatic hydrocarbons (PAHs) found at contaminated industrial sites. Besides their toxicity, such hydrocarbons are recalcitrant to biodegradation due to their low water solubility. Advances in nondestructive methods of microscopic analysis using confocal laser scanning microscopy (CLSM) and cell markers such as the gene encoding the green fluorescent protein (GFP) have led to more detailed information regarding biological processes (Chalfie *et al.*, 1994).

In order to better understand the strategies employed by bacteria to feed on such hydrophobic compounds, the time course of biofilm formation by a GFP-labelled *Pseudomonas putida* on fluorene and phenanthrene crystals as sole carbon and energy source was monitored using CLSM.

Materials and Methods

The experiments with the *gfp*-labelled derivative strain of *P. putida* ATCC 17514 were carried out in a four-channel (5x10x40 mm) flow-cell, limited by two microscope glass coverslips. The glass coverslip at the bottom of the flow-cell was coated with PAH crystals. In channels 1 and 2, a single PAH was used (phenanthrene in channel 1 and fluorene in channel 2). In channel 3, fluorene was placed at the inlet and phenanthrene at the outlet. Each flow channel was inoculated with a culture of *gfp*-labelled *P. putida* cells pregrown on the respective PAH. After 1 hour of incubation, mineral medium was continuously pumped at a flow rate of 5 mL/h. The flow-cell was placed under a microscope AXIOVERT 135 TV with a confocal laser unit, coupled to a Leica QUANTIMET image analysis computer. In each channel, three distinct areas, at the inlet (a), at the middle (b) and at the outlet (c) were monitored. Twenty horizontal optical sections were scanned at 2 µm vertical intervals. The bacteria were detected with an Ar 488 nm laser using a 515 nm long-pass filter, and the PAH crystals with a HeNe 543 nm laser line using a 550 nm long-pass filter. Images were obtained with a 40x/1.3 oil immersion lens. The growth of *P. putida::gfp* cells was monitored during six days and biovolumes were determined by numeric integration of microbial colonization profiles, following the method described in Kuehn *et al.* (1998).

Batch assays were performed in order to assess the production of extracellular polymeric substances (EPS) during *P. putida* growth on fluorene and phenanthrene. Polysaccharides were analysed by the phenol-sulphuric acid method and protein concentration was measured by the Folin-Ciocalteu phenol method.

Results and Discussion

Growth of *P. putida::gfp* cells was detected in all scanned regions in channels 1 (phenanthrene crystals only) and 2 (fluorene crystals only). In both channels, the colonization pattern was random and no external effect on growth was detected. In contrast, in channel 3 (inlet: fluorene, outlet: phenanthrene), *P. putida* cells were mainly concentrated where the phenanthrene crystals were located, with the cell volume at the inlet and at the centre of the channel being relatively low. In all channels, a decrease in the

biovolume in the different stacks was occasionally observed, a phenomenon that was considered to be the result of cell motility or detachment. Figure 1 depicts the growth of *P. putida::gfp* cells in flow channel 3, in the different stacks.

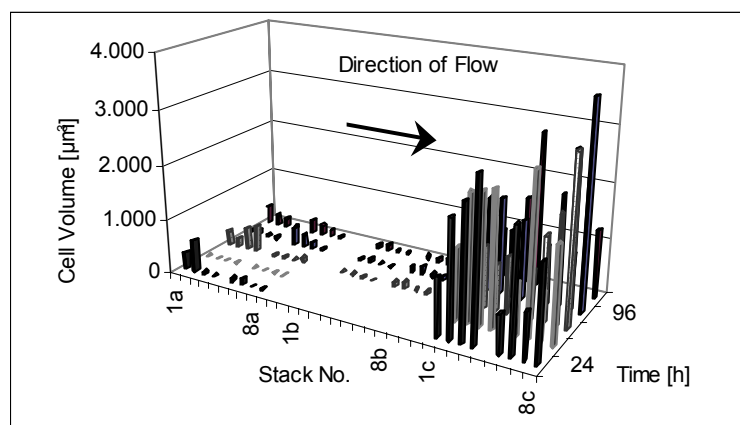


Figure 1 Time profiles of the *gfp*-labelled *P. putida* cell volume during growth on fluorene (inlet) and phenanthrene (outlet), in different stacks, for three areas (a, b and c) of flow channel 3.

The *in situ* experiments indicated that substrate characteristics affected bacterial strategy regarding uptake. *P. putida* cells attach and grow on the phenanthrene crystals throughout the formation of a biofilm on accessible surfaces, despite the fact that they can also feed on dissolved fluorene, more water-soluble than phenanthrene, supplied from upstream.

Active processes, such as polymer production, are required for adhesion of cells to substrate (van Schie and Fletcher, 1999). The production of EPS during bacterial growth on fluorene and phenanthrene is shown in Figure 2.

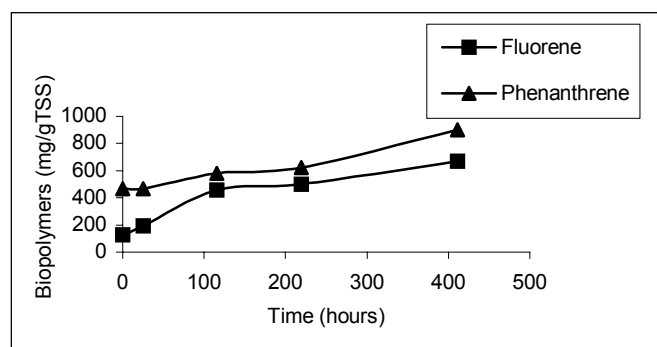


Figure 2 Evolution of EPS during growth on fluorene and phenanthrene.

A higher cell production of EPS was observed in the presence of phenanthrene compared to fluorene. The adhesive properties of extracellular polymers are well documented (Oliveira, 1992). By reducing the radius of cell-substrate interaction through the production of EPS, the repulsive barrier can be bridged. Furthermore, as aromatic compounds adsorb to or interact via hydrogen bonding with EPS (Rattee and Breuer, 1974), PAHs may become more available to the cells in a biofilm. This might be the reason for the confluent growth of *P. putida* cells on phenanthrene crystals (Figure 1).

Conclusions

Confocal laser microscopy showed that *P. putida* seems to overcome the low aqueous phase substrate concentration by colonizing directly phenanthrene crystals. The production of EPS may be a bacterial strategy to adhere to PAHs, thus promoting the availability and uptake of such less water-soluble compounds by bacteria.

Acknowledgements

The authors acknowledge grant PRAXISXXI/BD/15944/98 from the Foundation for Science and Technology/M.C.T., Portugal, awarded to A.C. Rodrigues, and the financial support provided by Instituto de Biotecnologia e Química Fina (IBQF).

The authors are also grateful to L. Hendrickx for assistance in the labelling of *P. putida* and A. Schnell for helping set-up flow-cell experiments.

References

- Chalfie M., Yuan T., Euskirchen G., Ward W.W. and Prasher D.C. (1994). Green fluorescent protein as a marker for gene expression. *Science*, 263, 802-805.
- Kuehn M., Hausner M., Bungartz H.J., Wagner M., Wilderer P., and Wuertz S. (1998). Automated confocal laser scanning microscopy and semiautomated image processing for analysis of biofilms. *Appl. Environ. Microbiol.*, 64(11), 4115-4127.
- Oliveira D.R. (1992). Physico-chemical aspects of adhesion. NATO ASI Ser. Ser. E 223, 45-58.
- van Schie P.M. and Fletcher M. (1999). Adhesion of biodegradative anaerobic bacteria to solid surfaces. *Appl. Environ. Microbiol.*, 65, 5082-5088.
- Rattee I.D. and Breuer M.M. (1974). The physical chemistry of dye adsorption, 323. Academic Press, New York.