



Aerobic Hydrocarbon-Degrading *Gammaproteobacteria: Porticoccus*

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Tony Gutierrez

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Abstract

The class *Gammaproteobacteria* contains the most important genera and largest diversity of obligate and generalist hydrocarbonoclastic bacteria that are found in the marine environment. With the exception of *Planomicrobium alkanoclasticum* (a Gram-positive of the Firmicutes), the class *Gammaproteobacteria* contains all known obligate hydrocarbonoclastic bacteria (OHCB), as represented by the genera *Alcanivorax*, *Cycloclasticus*, *Neptunomonas*, *Oleibacter*, *Oleiphilus*, *Oleispira*, and *Thalassolituus*. Prospecting studies aimed in identifying new taxa of hydrocarbonoclastic bacteria from underexplored biotopes in the ocean have uncovered novel OHCB within the *Gammaproteobacteria*, further increasing the known diversity of these organisms within this physiologically and phylogenetically diverse class. In this respect, one underexploited biotope is the cell surface, or phycosphere, of marine eukaryotic phytoplankton (microalgae) as a source of OHCB. Members of the *Alcanivorax* and *Marinobacter* have been commonly reported living

T. Gutierrez (✉)

Institute of Mechanical, Process and Energy Engineering, School of Engineering and Physical Sciences, Heriot-Watt University, Edinburgh, UK

e-mail: tony.gutierrez@hw.ac.uk

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T. J. McGenity (ed.), *Taxonomy, Genomics and Ecophysiology of Hydrocarbon-Degrading Microbes*, Handbook of Hydrocarbon and Lipid Microbiology,

https://doi.org/10.1007/978-3-030-14796-9_32

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associated with many species of phytoplankton (diatoms, dinoflagellates, coccolithophores), and novel genera and species of OHCB (*Polycyclovorans*, *Algiphilus*, *Porticoccus hydrocarbonoclasticus*) have also been uncovered. This chapter discusses *P. hydrocarbonoclasticus*, which is a recently discovered OHCB that is not commonly represented in sequencing surveys, even from oil-polluted sites, and whose functional role in the water column and as a symbiont of phytoplankton remains to be resolved.

1 Introduction

Members of three major phytoplankton lineages (dinoflagellates, diatoms, coccolithophores) have been found to harbor obligate hydrocarbonoclastic bacteria (OHCB), including novel taxa of these organisms (Green et al. 2006; Gutierrez et al. 2012a, b, 2013, 2014; Mishamandani et al. 2016). Their association with phytoplankton raises important questions with respect to their evolutionary genesis, ecology, and response in the event of an oil spill at sea, and while the underlying basis for this remains unresolved, there is evidence suggesting that the enrichment of hydrocarbons on phytoplankton cell surfaces plays an important role. By nature of their surface chemistry, phytoplankton cell surfaces have been shown to adsorb and accumulate hydrocarbons, such as the polycyclic aromatic hydrocarbons (PAHs) (Mallet and Sardou 1964; Andelman and Suess 1970). Phytoplankton may also be a biogenic source of PAHs by synthesizing these chemicals (Andelman and Suess 1970; Gunnison and Alexander 1975) and translocating them into the algal cell wall (Gunnison and Alexander 1975; Zelibor et al. 1988). Many phytoplankton also produce alkenones (Marlowe et al. 1984), which are long-chain hydrocarbon-like compounds, and almost all produce the volatile hydrocarbon isoprene (Shaw et al. 2010; Exton et al. 2012). Whether through intracellular synthesis or adsorption of hydrocarbons from the surrounding seawater, the cell surface of phytoplankton cells – i.e., phycosphere – may be considered as a “hot spot” to which OHCB are prone to reside and live in symbiosis with their eukaryotic algal hosts.

All known genera of OHCB are *Gammaproteobacteria*, aerobic and, with the exception of *Planomicrobium alkanoclasticum* which belongs to the phylum Firmicutes of Gram-positive bacteria, they include *Alcanivorax*, *Cycloclasticus*, *Oleibacter*, *Oleiphilus*, *Oleispira*, *Neptunomonas*, and *Thalassolituus*. Recent work has uncovered novel OHCB (*Algiphilus aromaticivorans*, *Polycyclovorans algicola*, *Porticoccus hydrocarbonoclasticus*) that comprise novel genera and/or species and that were isolated from eukaryotic phytoplankton. While another chapter in this volume describes *A. aromaticivorans* and *P. algicola*, this chapter provides an overview on *P. hydrocarbonoclasticus* that was originally isolated from *Lingulodinium polyedrum* and found also inhabiting the phycosphere of various other species of dinoflagellates and diatoms.

2 *Porticoccus hydrocarbonoclasticus*

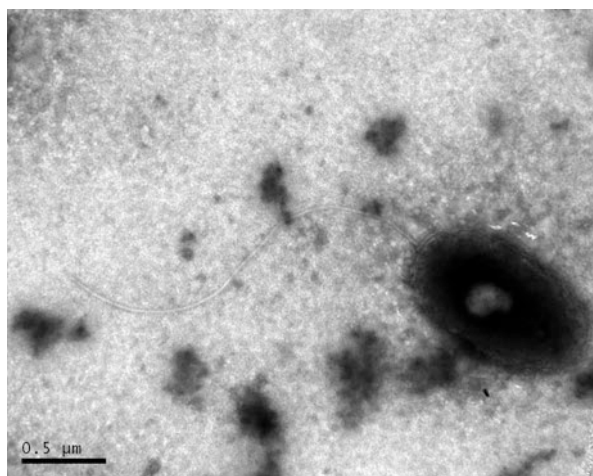
P. hydrocarbonoclasticus MCTG13d^T (=ATCC BAA-2274^T) is a rod-shaped, Gram-stain-negative, halotolerant bacterium. The strain was isolated from a non-axenic laboratory culture of the marine dinoflagellate *Lingulodinium polyedrum* (CCAP 1121/2), which was isolated originally from Loch Creran in Argyll, Scotland (Gutierrez et al. 2012).

On ONR7a agar plates amended with acetate as the sole carbon and energy source, colonies of *P. hydrocarbonoclasticus* develop within 2 weeks of incubation at 28 °C and appear round and nonpigmented with diameters 0.5–2 mm. On ONR7a agar sprayed with a PAH substrate (e.g., phenanthrene, anthracene, fluorene, or pyrene), colonies that developed were larger (4–7 mm in diameter), surrounded by clearing zones (i.e., indicative of PAH degradation) and appear pale yellow-green, slightly raised with rough surfaces and undulate margins. Under the microscope, cells are short to long, non-spore-forming rods (1.0–2.0 × 0.5–0.6 μm in average size) (Fig. 1). They contain intracellular inclusion bodies and surface blebs, and are motile by means of a single polar flagellum.

P. hydrocarbonoclasticus is an obligate aerobe that produces catalase, oxidase, and reduces nitrate to nitrite. The G+C content of the organism's DNA is 54.9 mol% and the predominant isoprenoid quinone is Q-8. The dominant fatty acids are C_{16:0}, C_{16:1}ω7c, and C_{18:1}ω7c.

The bacterium is able to grow at temperatures ranging from 10 °C to 37 °C (optimal, 15 °C), and at pH values ranging from 6.5 to 9.0 (optimal, pH 8.0). The organism is negative for lipase (Tween 80) and the hydrolysis of agar and gelatin, but positive for phosphatase activity. It does not accumulate polyhydroxybutyrate (PHB) granules, although cells contain intracellular granules that fluoresce after staining with Nile Blue. The organism is able to grow well in a medium containing NaCl at concentrations of 0–6%, although growth is markedly reduced in the absence of

Fig. 1 Transmission electron micrograph of a cell with negative staining of strain *Porticoccus hydrocarbonoclasticus* MCTG13d^T. Bar, 0.5 μm (Source: Gutierrez et al. (2012). Reprinted with the permission from Appl. Environ. Microbiol)



NaCl and completely inhibited at 10% NaCl. Hence, *P. hydrocarbonoclasticus* is a slightly halotolerant bacterium.

From a BLAST analysis in 2016, the highest levels ($\geq 95\%$) of sequence similarity for *P. hydrocarbonoclasticus* was to 70 environmental clones. The most closely related type strain is *Porticoccus litoralis* IMCC2115^T (96.5% sequence identity), which originated from coastal surface seawater in the Yellow Sea, South Korea (Oh et al. 2010). The next closest cultivated relatives included members of the *Microbulbifer* (91.4–93.7%) and *Marinimicrobium* (90.4–92.0%) genera. The affiliation of *P. hydrocarbonoclasticus* with the genus *Porticoccus* is supported by a moderate bootstrap value of 85%, and RDP-II Classifier (Wang et al. 2007) set to a confidence threshold of 80% indicates the organism is an unclassified member of the family *Alteromonadaceae*. The organism is distinctly grouped within a clade of mainly uncultivated bacterial clones that lies adjacent to the OM60 clade, represented by strain HTCC2080 (Cho et al. 2007), and the SAR92 clade, represented by strain HTCC2207 (Stingl et al. 2007). Thus, *P. hydrocarbonoclasticus* is a member of a phylogenetic clade that lies adjacent to the OM60 and SAR92 clades, and which except for *P. hydrocarbonoclasticus* and *P. litoralis*, this clade is almost entirely represented by several hundred uncultured clones that includes the representative bacterial clones D53 (Zeng et al. 2005) and ELB16-080 (Glatz et al. 2006). The most closely related clones and type strains originated from pristine or oil-contaminated coastal, polar and open ocean seawater and sediment, soil, hydrocarbon seeps, Mariana Trench sediment, and the bacterial community associated with sponges and phytoplankton blooms. Some of these, together with closest type strains, are represented alongside *P. hydrocarbonoclasticus* in the phylogenetic tree shown in Fig. 2. Using genome wide gene-content analyses, Spring et al. (2015) revealed the existence of two distinct ecological guilds within the lineage of marine *gammaproteobacteria*. Their results revealed a novel order within the class *Gammaproteobacteria*, which is designated *Cellvibrionales* that comprises the family *Porticoccaceae*, and in which comprises the genera *Porticoccus*. Four other novel families were revealed to comprise this order (*Cellvibrionaceae*, *Halieaceae*, *Microbulbiferaceae*, *Spongiibacteraceae*).

The following hydrocarbons are those that have been tested and which are utilized as sole carbon sources for growth and/or are mineralized by *P. hydrocarbonoclasticus*: phenanthrene, anthracene, pyrene, fluorene, and *n*-hexadecane. The strain is able to grow on acetate and indole as sole sources of carbon, but unable to grow on mannitol, fructose, glucose, xylose, arabinose, decane, hexane, pentane, pyruvate, methanol, and methane. The organism does not grow in rich or diluted nutrient marine medium, such as marine agar 2216 medium. Based on its nutritional spectrum, *P. hydrocarbonoclasticus* utilizes hydrocarbons as its preferred source of carbon over other naturally occurring organic substrates, and thus represents a novel OHCB of the genus *Porticoccus*.

Interestingly, of the two type strains that represent the genus *Porticoccus*, only *P. hydrocarbonoclasticus* is an OHCB based on its almost exclusive requirement to use hydrocarbons, preferably PAHs, as a source of carbon and energy. The other type strain of this genus, *P. litoralis* IMCC2115^T, is however not recognized for degrading

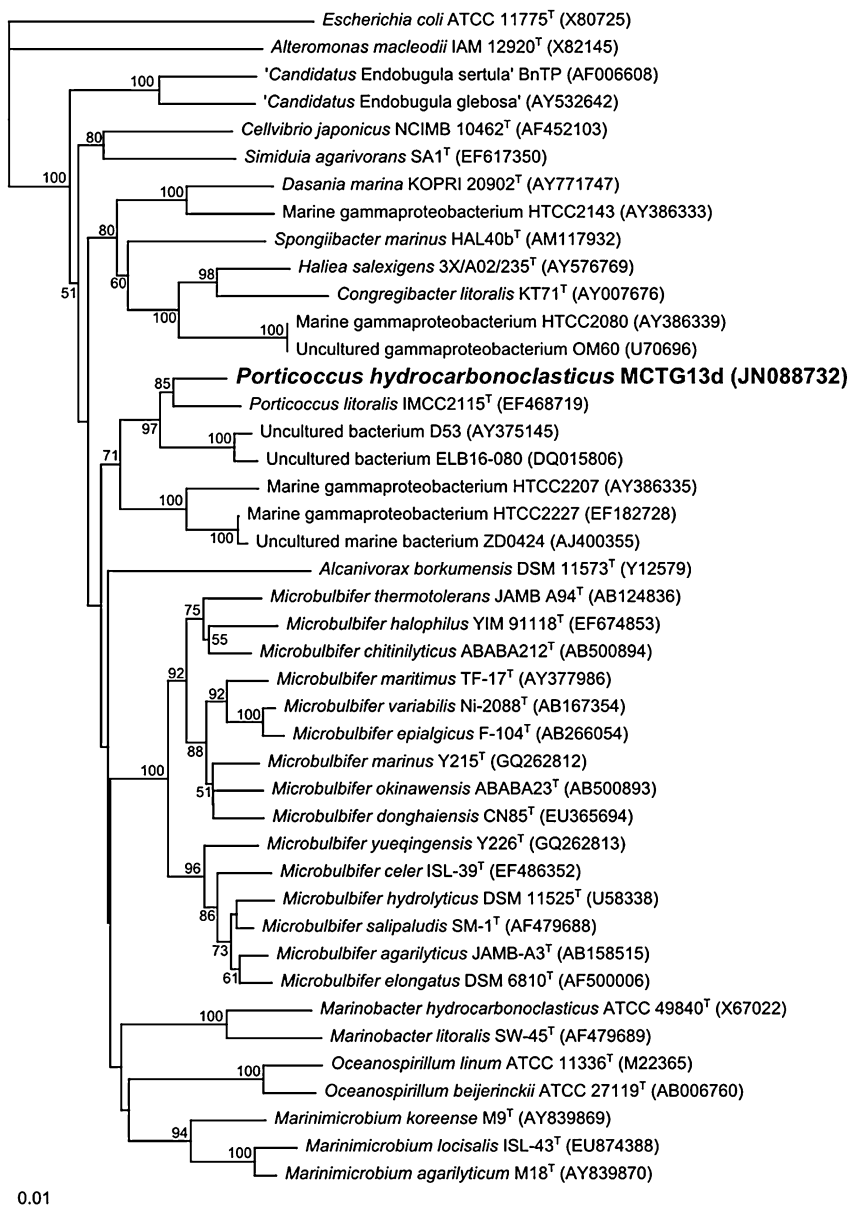


Fig. 2 Neighbour-joining phylogenetic tree, based on 16S rRNA gene sequences (>1200 bp), showing the relationships between *P. hydrocarbonoclasticus* strain MCTG13d^T and representative type strains and environmental clones. *Escherichia coli* ATCC 11775^T and *Alteromonas macleodii* IAM 12920^T were used as the outgroup. Bootstrap values (expressed as percentages of 1000 replications) of >50% are shown at each node. GenBank accession numbers are shown in parentheses. Bar represents 0.01 substitutions per nucleotide position

hydrocarbons, and it is capable of utilizing sugar substrates (e.g., mannitol, fructose, glucose, arabinose) as a source of carbon and energy for growth. This is intriguing from the perspective that when we consider the other well-established OHCB genera (*Alcanivorax*, *Cycloclasticus*, *Neptunomonas*, *Oleiphilus*, *Oleispira*, and *Thalassolituus*), all members within these genera that have been cultivated and characterized are able to degrade hydrocarbons. From this current state of knowledge, we can assume with a good level of confidence that when we identify a member comprising any one of these genera in environmental samples, it would encode the trait to degrade hydrocarbons, even if we do not have the means to directly test for this phenotype – as for example, the organism was identified through a sequencing survey. As a disclaimer, biology does not always follow defined rules; we may someday identify an *Alcanivorax* or other member of a recognized OHCB genus, for example, that is unable to degrade hydrocarbons. The discovery of an OHCB, such as *P. hydrocarbonoclasticus*, within a genus (i.e., *Porticoccus*) that contains members that are nonhydrocarbon degraders suggests that the reciprocal of this may also exist for genera like *Alcanivorax*, *Cycloclasticus*, and any of the other recognized genera of OHCB.

Using qPCR primers targeting the 16S rRNA gene of *P. hydrocarbonoclasticus*, the organism has been shown to be associated with a range of other species of diatoms, dinoflagellates (Gutierrez et al. 2012; Fig. 3) and coccolithophores (unpublished data). Considering the organism is phylogenetically grouped within a clade that comprises several hundred uncultivated clones of which many were derived from living marine surfaces (e.g., sponges, fish) and phytoplankton, the clade may have an evolutionary genesis of having developed symbiotic associations with higher organisms in the marine environment. With increasing technological advances in genome sequencing of environmental samples, as well as techniques to coax cultivation-recalcitrant organisms into cultivation, we may be able to reveal whether this clade contains other OHCB like *P. hydrocarbonoclasticus*.

3 Research Needs

While our knowledge has increased on the diversity and functioning of OHCB in the ocean, for which almost all are represented within the class *Gammaproteobacteria*, the question remains: Have we got them all? Quite often, though, the methods used to isolate or detect these organisms are biased toward those that, respectively, rely on cultivating these organisms in the laboratory or that bloom sufficiently to be easily detected by, for example, sequencing approaches. Hydrocarbonoclastic bacteria that have evaded identification in environmental samples may be because they do not grow to sufficient abundance, or they are simply not amenable to cultivation in the laboratory. Seawater sampling programs aimed to study microbial population diversity and dynamics do not often employ operational fractionation to tease apart the various microbial populations (based on size) that constitute the water sample, hence leading to the misconception that hydrocarbon-degrading bacteria identified in seawater samples were present there in a free-living state. It is not inconceivable that many

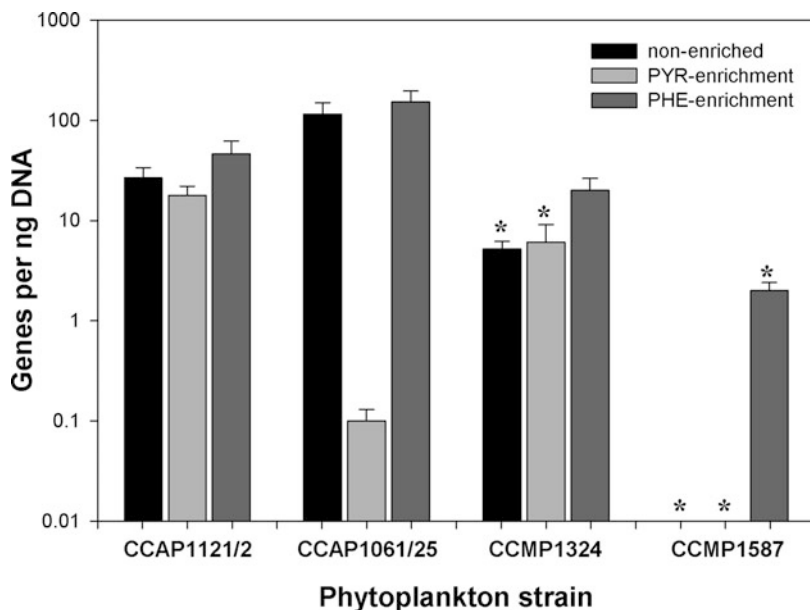


Fig. 3 Abundance of *P. hydrocarbonoclasticus* MCTG13d^T 16S rRNA genes in various marine phytoplankton species using PCR-targeted probes. Phytoplankton cultures were enriched with pyruvate (PYR-enriched), phenanthrene (PHE-enriched), or no added carbon source (unenriched). Bars are the average and standard deviation of duplicate qPCR reactions measuring the abundance of *P. hydrocarbonoclasticus*-specific 16S rRNA genes per ng DNA. Phytoplankton strains used were *Lingulodinium polyedrum* CCAP1121/2, *Pseudo-Nitzschia* CCAP1061/25, *Isochrysis* sp. CCMP1324, and *Thalassiosira weissflogii* CCMP1587. Asterisks represent values that were below the quantification limit (<5 gene copies per reaction) of the assay (Source: Gutierrez et al. (2012). Reprinted with the permission from Appl. Environ. Microbiol)

hydrocarbon-degrading bacteria, including OHCB, that have been reported in the literature describing their isolation, molecular identification and/or dynamics, were likely physically attached to phytoplankton cells at the time of their sampling. For example, *P. hydrocarbonoclasticus* is not well represented in the pool of 16S rRNA sequence data available online, such as in the GenBank and RDP databases. This may be because the occurrence of this species in the marine environment is possibly confined to a life associated with certain species of phytoplankton, and their abundance, even per phytoplankton cell, may be sufficiently low to escape detection by sequencing approaches (e.g., Sanger sequencing of clone libraries, MiSeq, or pyrosequencing). These peculiar OHCB were identified because they were targeted using selective techniques to coax them into cultivation and because they were searched for in the right place, which was in cultures of nonaxenic marine phytoplankton. Many new taxa of hydrocarbonoclastic bacteria are likely to exist in seawater and sediments of the global ocean that await discovery. More work is needed to better understand the association of these fastidious and somewhat elusive OHCB with phytoplankton, as well as their function and ecology in the wider context of oil degradation in the ocean.

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