Handbook of
Hydrocarbon and Lipid Microbiology
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Terry J. McGenity *Editor*

Taxonomy, Genomics and Ecophysiology of Hydrocarbon-Degrading Microbes

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Handbook of Hydrocarbon and Lipid Microbiology

This handbook is the unique and definitive resource of current knowledge on the diverse and multifaceted aspects of microbial interactions with hydrocarbons and lipids, the microbial players, the physiological mechanisms and adaptive strategies underlying microbial life and activities at hydrophobic material:aqueous liquid interfaces, and the multitude of health, environmental and biotechnological consequences of these activities.

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With 36 Figures and 32 Tables

Springer

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I dedicate this book to my father, Jim McGenity, who passed away during its production. He left school aged 14 and returned to formal education much later in his life, which led to his being a passionate and inspirational teacher who shared his love of learning with everyone around him.

Introduction

Taxonomy draws on many disciplines in biology and in turn is used in all aspects of the biosciences. A *robust* taxonomy defines natural relations between organisms and importantly has *predictive* power. This book provides comprehensive, authoritative discussions about cultivated and characterized microbes that degrade hydrocarbons, building on the first edition of the *Handbook of Hydrocarbon and Lipid Microbiology* (Timmis et al. 2010). The motivation for this compilation stems from the increased breadth and depth in our understanding of hydrocarbon-degrading taxa over the last decade, which is partly a consequence of: (1) novel approaches to cultivation of hydrocarbon degraders (see McGenity 2016); (2) insights into yet-to-be-cultivated hydrocarbon degraders from molecular community analysis (see McGenity 2019); (3) taxonomic refinements due to availability of better tools to investigate microbes, such as those described in the complementary 17-volume Hydrocarbon and Lipid Microbiology Protocols series, especially the increased application of genomic data (see Orata et al. 2018; Parks et al. 2019); and (4) a global explosion, post-Macondo, of research into hydrocarbon degradation (see Redmond and Valentine 2018).

The first three chapters provide the context for the rest of the book. The first chapter compiles all the bacterial and archaeal genera known to us at the time of writing that have hydrocarbon-degrading representatives (Prince et al. 2018). The current tally of ~320 genera from across the domain *Bacteria* highlights the breadth of this physiological trait, probably reflecting the diverse array of hydrocarbons that can serve as carbon and energy sources in distinct environments with different redox couplings, as well as the capacity for horizontal transfer of many of the genes involved in their degradation (Diaz 2004). Our understanding of the diversity of hydrocarbon degradation grows rapidly, for example, the candidate phylum Atribacteria was recently shown to have the capacity to ferment short-chain *n*-alkanes based on metagenome-assembled genomes (Liu et al. 2019). Oren (2017) details the aerobic hydrocarbon-degrading *Archaea*, exclusively haloarchaea, highlighting the importance of this trait in extreme halophiles owing to the frequent juxtaposition of salt and oil. Prince (2018a) covers hydrocarbon-degrading *Eukarya*, primarily fungi, but also considers the unclear role of microalgae and protozoa in hydrocarbon degradation.

Kwon et al. (2019) set the scene for the *Bacteroidetes*, a phylum that is well known for degrading biopolymers but not specifically hydrocarbons, by discussing their potential role in oil-contaminated environments determined by metagenomic and stable-isotope probing data, especially from the Deepwater Horizon spill. Then they provide a detailed description of the taxonomy and hydrocarbon-degrading potential of several formally described species and other strains.

Buchan et al. (2019) provide a genome-focused overview of the alphaproteobacterial family *Rhodobacteraceae*, colloquially referred to as Roseobacters, for which there is much evidence of important contributions to aerobic degradation of a wide range of hydrocarbons. The alphaproteobacterial order *Sphingomonadales*, reviewed by Kertesz et al. (2018), has a similar breadth of aerobic hydrocarbon-degrading activities as their cousins, the Roseobacters. Both taxa are primarily known for their aromatic, especially polycyclic aromatic, hydrocarbon-degrading abilities, compared with the biodegradation of alkanes. While Roseobacters are mostly restricted to the sea and coast, the *Sphingomonadales* inhabit a wide range of terrestrial, freshwater, and marine environments.

Tan and Parales (2019) expertly cover the *Betaproteobacteria*, which are often major hydrocarbon degraders in soil and aquifer environments (together with *Gammaproteobacteria*), particularly responsible for aerobic degradation of aromatic and chloro-, nitro-, and amino-aromatic hydrocarbons and, to a lesser extent, alkanes. Moreover, several genera of *Betaproteobacteria* contribute to anaerobic aromatic and alkane biodegradation with nitrate and perchlorate as terminal electron acceptors.

Most known hydrocarbon degraders are from the *Gammaproteobacteria*, which frequently dominate hydrocarbon-contaminated environments, and so the class merits an overview chapter to itself. This is provided by Gutierrez (2017a), who outlines the diversity of both the generalist and specialist (or obligate) hydrocarbonoclastic *Gammaproteobacteria*. Korzhenkov et al. (2019) carried out a meta-analysis of the *Oleiphilaceae*, which is currently represented by a single obligate alkane-degrading marine species. They discuss evidence from genomic and phylogenetic analysis of strains isolated by Sosa et al. (2017), suggesting that there is another genus in the family with several new species. Yakimov et al. (2019) review the latest developments in the taxonomy, genomics, and physiology of the family *Alcanivoraceae*, which has become the archetypal aerobic marine obligate hydrocarbonoclastic family, primarily responsible for alkane biodegradation in saline waters and sediments. They focus on the dozen species of the genus *Alcanivorax* and also introduce a species from the related genus *Ketobacter*. Intriguingly, the distribution of yet-to-be-cultivated *Alcanivorax* species hints at associations with a wide range of marine organisms from cyanobacteria to sponges. The next chapter focuses on a single marine species, *Porticoccus hydrocarbonoclasticus*, which was isolated from a dinoflagellate and can grow on both PAHs and hexadecane (Gutierrez 2017b). The final chapter on the *Gammaproteobacteria* provides an overview of hydrocarbon-degrading *Xanthomonadales* and then focuses on two marine species

Polycyclovorans algicola and *Algiphilus aromaticivorans*, both of which were isolated from marine algae and degrade aliphatic, polycyclic, and monocyclic aromatic hydrocarbons (Gutierrez 2017c).

Davidova et al. (2018) give a thorough exposition of the anaerobic hydrocarbon-degrading *Deltaproteobacteria*, mostly marine sulfate reducers, but also iron reducers and fermentative microbes that usually exist syntrophically with methanogens. They discuss the mechanisms by which hydrocarbons are activated in the absence of oxygen, notably: fumarate addition, carboxylation, hydroxylation, and hydration of alkynes.

Kalyuzhnaya et al. (2019) provide in-depth analysis of the aerobic methane-oxidizing bacteria (methanotrophs), which can be found in the *Alphaproteobacteria* and *Betaproteobacteria* as well as *Verrucomicrobia* and Phylum NC10, which houses the candidate species *Methylomirabilis oxyfera.* The chapter concludes by highlighting how developments in molecular genetic systems and whole-genome metabolic modelling can enhance fundamental understanding of methanotrophy as well as their biotechnological potential. Dedysh and Dunfield (2018) focus on the facultative methanotrophs, with emphasis on the most-studied genus *Methylocella*, in which the soluble methane monoxygenase is repressed when alternative multi-carbon substrates are present.

Fungal hydrocarbon degraders were discussed in a book in the same series by Prenafeta-Boldú et al. (2018). In this book Rafin and Veignie (2018) provide an in-depth appraisal of one ascomycete, infamous for biodeterioration of fuels, known variously as *Amorphotheca resinae* or *Hormoconis resinae*, or the "kerosene fungus."

The final three chapters are broader in scope, highlighting some global consequences of the activities of hydrocarbon-degrading bacteria. Prince (2018b) considers the broad distribution and diversity of hydrocarbon degraders and how they impinge on our lives for better or worse. Gutierrez (2018) discusses how obligate hydrocarbonoclastic bacteria survive in the apparent absence of oil spills or seeps, revealing that hydrocarbons are often present in low abundance in the environment and also produced by many organisms, resulting in cryptic short-term hydrocarbon cycles between producer and consumer. Coscolín et al. (2018) conclude the book by compiling information about the hydroxylase enzymes from aerobic marine obligate hydrocarbon degraders, which have great potential for industrial biocatalysis.

I would like to finish by expressing my gratitude to the authors, who have provided such wide-ranging and fascinating insights into hydrocarbon-degrading microbes. In particular, I thank Roger Prince and Tony Gutierrez for valuable inputs into designing the structure and content of this book.

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About the Series Editor-in-Chief

Kenneth N. Timmis
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Kenneth Timmis studied microbiology and obtained his Ph.D. at Bristol University. He undertook postdoctoral training at the Ruhr-University Bochum, Yale, and Stanford, at the latter two as a Fellow of the Helen Hay Whitney Foundation. He was then appointed Head of an independent research group at the Max Planck Institute for Molecular Genetics in Berlin and subsequently Professor of Biochemistry in the University of Geneva, Faculty of Medicine. Thereafter, for almost 20 years, he was Director of the Division of Microbiology at the National Research Centre for Biotechnology (GBF)/now the Helmholtz Centre for Infection Research (HZI), and concomitantly Professor of Microbiology in the Institute of Microbiology of the Technical University Braunschweig. He is currently Emeritus Professor in this institute.

The Editor-in-Chief has worked for more than 30 years in the area of environmental microbiology and biotechnology, has published over 400 papers in international journals, and is an ISI Highly Cited Microbiology-100 researcher. His group has worked for many years, inter alia, on the biodegradation of oil hydrocarbons, especially the genetics and regulation of toluene degradation, and on the ecology of hydrocarbon-degrading microbial communities, discovered the new group of marine oil-degrading hydrocarbonoclastic bacteria, initiated genome sequencing

projects on bacteria that are paradigms of microbes that degrade organic compounds (*Pseudomonas putida* and *Alcanivorax borkumensis*), and pioneered the topic of experimental evolution of novel catabolic activities.

He is Fellow of the Royal Society, Member of the European Molecular Biology Organisation, Fellow of the American Academy of Microbiology, Member of the European Academy of Microbiology, and Recipient of the Erwin Schrödinger Prize. He is the founder and Editor-in-Chief of the journals *Environmental Microbiology*, *Environmental Microbiology Reports*, and *Microbial Biotechnology*.

About the Volume Editor

Terry J. McGenity is a Professor at the University of Essex, UK. His Ph.D., investigating the microbial ecology of ancient salt deposits (University of Leicester), was followed by postdoctoral positions at the Japan Marine Science and Technology Centre (JAMSTEC, Yokosuka) and the Postgraduate Research Institute for Sedimentology (University of Reading). Terry's overarching research interest is to understand how microbial communities function and interact to influence major biogeochemical processes. He worked as a postdoc with Ken Timmis at the University of Essex, where he was inspired to investigate microbial interactions with hydrocarbons at multiple scales, from communities to cells, and as both a source of food and stress. Terry has broad interests in microbial ecology and diversity, particularly with respect to carbon cycling (especially the second most abundantly produced hydrocarbon in the atmosphere, isoprene), and is driven to better understand how microbes cope with, or flourish, in hypersaline and poly-extreme environments.

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Prokaryotic Hydrocarbon Degraders

1

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Contents

Abstract

Hydrocarbons have been part of the biosphere for millions of years, and a diverse group of prokaryotes has evolved to use them as a source of carbon and energy. To date, the vast majority of formally defined genera are eubacterial, in 7 of the 24 major phyla currently formally recognized by taxonomists (Tree of Life, http://tolweb.org/Eubacteria. Accessed 1 Sept 2017, 2017), principally in the

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Actinobacteria, the Bacteroidetes, the Firmicutes, and the Proteobacteria. Some Cyanobacteria have been shown to degrade hydrocarbons on a limited scale, but whether this is of any ecological significance remains to be seen – it is likely that all aerobic organisms show some basal metabolism of hydrocarbons by non-specific oxygenases, and similar "universal" metabolism may occur in anaerobes. This chapter focuses on the now quite large number of named microbial genera where there is reasonably convincing evidence for hydrocarbon metabolism. We have found more than 320 genera of Eubacteria and 12 genera of Archaea. Molecular methods are revealing a vastly greater diversity of currently uncultured organisms – Hug et al. (Nat Microbiol 1:16048, 2016) claim 92 named bacterial phyla, many with almost totally unknown physiology – and it seems reasonable to believe that the catalog of genera reported here will be substantially expanded in the future.

1 Introduction

Hydrocarbons have always been part of earth's biosphere. They are found in interstellar space (Kaiser et al. 2015), are produced by living organisms (Valentine and Reddy 2015), and are produced by geological processes (Vandenbroucke and Largeau 2007) and during incomplete combustion (Wang et al. 2014c). Hydrocarbons are relatively stable molecules, but can provide a source of carbon and energy to any organism able to activate them. It is this activation that distinguishes hydrocarbon-degrading organisms from their brethren, for once activated by the aerobic insertion of oxygen or anaerobic addition of fumarate (Rabus et al. 2016), for example, hydrocarbons enter the standard biochemistry of living organisms and are readily oxidized to carbon dioxide and water.

All aerobic organisms seem to have some ability to metabolize hydrocarbons (e.g., Ortiz de Montellano 2009), and it would not be surprising if similar basal metabolism occurred in anaerobes. But this article focuses on those organisms able to grow in pure culture using hydrocarbons as sole, or major source of carbon for growth. Only an isolated organism can be given a valid taxonomic name, and the limitation that an organism must be available in pure culture must substantially underestimate the diversity of organisms that productively metabolize hydrocarbons, because it is becoming clear that organisms often grow in mutually beneficial consortia (e.g., Allen and Banfield 2005; Brenner et al. 2008; McGenity et al. 2012; Hays et al. 2015). Co-metabolism may be essential for some compounds (see Kanaly and Harayama 2000; Hazen et al. 2016), and may require interspecies "cooperation." The phenomenon of syntrophy, where organisms cooperate to metabolize substrates with benefits for all, is also beginning to be understood (e.g., Gray et al. 2011; Gieg et al. 2014). Indeed the need for interspecies interactions may well be a principal reason for the oft-quoted statement that only a tiny fraction of microbes can be "cultivated." Nevertheless some species thrive in pure culture in the laboratory, and these have allowed a detailed understanding of their biochemistry.

Here, we provide context for the other chapters in this volume by listing all genera (known to us) with species that can degrade hydrocarbons, and outline their taxonomy.

2 Bacterial Taxonomy

Despite its origins before the Darwin-Wallace identification of Natural Selection as the driving force for speciation (Darwin and Wallace 1858), Linnaean taxonomy still plays the central role in describing organisms, giving each organism a binomial name consisting of a generic and a specific identifier (e.g., Gest 1999). Genera are then grouped in families, families in orders, orders in phyla, and phyla into domains. In theory this classification ought to fit neatly into an evolutionary classification – the taxonomy should reflect phylogeny. Indeed it is fairly effective for multicellular sexual organisms, where species can be defined as organisms that can interbreed, genera can be distinguished on morphological grounds, and there has been little gene transfer between otherwise unrelated organisms. Even then, species are only a transient category as evolution continues (Dobzhansky 1937).

Microbial taxonomy is rather more complicated, and widely discussed (Gest 1999; Federhen 2014; Hug et al. 2016; Rosselló-Mora and Amann 2001; Thompson et al. 2015). As can be seen in Table 1, some genera were defined in the early nineteenth century, but an explosion in their number has occurred since the advent of molecular taxonomy based on ribosomal RNA (Woese 1987; Cole et al. 2009). As this has developed, it has become clear that microbial diversity is only poorly understood – Hug et al. (2016) suggest that <10% of putative phyla within the bacterial domain contain cultivated representatives, and there may be a similar disparity in the Archaea (Spang et al. 2017; Adam et al. 2017). The uncultivated phyla, which have largely unknown metabolic pathways/capabilities, are not necessarily minor components of the biosphere – for example, SAR11 was known to be the most abundant organism in the world's oceans before it was cultivated and formally described as *Pelagibacter ubique* (Rappé et al. 2002). We may thus confidently expect that many more organisms will be cultured and described when more emphasis is placed on classical microbial cultivation (Gest 2008; McGenity 2016). It is noteworthy that some of what are currently believed to be the most important and widespread oil-consuming genera, such as *Alcanivorax* (Yakimov et al. 1998) and *Cycloclasticus* (Dyksterhouse et al. 1995) were discovered only rather recently.

Modern molecular taxonomy uses ribosomal RNA genes as the preeminent molecular signature for understanding the relatedness of different organisms (Cole et al. 2009). This is certainly reasonable in that it is hard to imagine an organism replacing such an essential part of its metabolism. But lateral gene transfer is now very well established (Popa et al. 2017; Soucy et al. 2015), using the "mobilome" – plasmids, bacteriophages, and transposons (Frost et al. 2005). Phenotypic traits such as hydrocarbon degradation are frequently carried on plasmids (e.g., Oct, Nah7, dox, TOL, etc., see Abbasian et al. 2016), and might be expected to pass readily between even quite unrelated organisms, at least on an evolutionary timescale. Such transfer means that different traits will exhibit different phylogenies from that of the ribosomal RNA molecules, and may well be dispersed over a broad taxonomic space. The fact that hydrocarbon degradation is not yet found in all bacterial phyla may indicate some barriers to such transmission (Popa et al. 2017), or merely that such organisms have not yet been found.

Table 1 Currently published genera of bacteria able to grow using hydrocarbons as sole or major carbon source

Genus[a]	Typical substrate	References[b]
Phylum Actinobacteria		
Order Actinomycetales		
Actinomyces Harz (1877)	Crude oil	ZoBell (1946)
Order Actinopolysporales		
Actinopolyspora Gochnauer et al. (1975)	Alkanes	Al-Mueini et al. (2007)
Order Corynebacteriales		
Amycolicicoccus Wang et al. (2010)	Crude oil	Nie et al. (2013)
Corynebacterium Lehmann and Neumann (1896)	Fuel oil	Chaineau et al. (1999)
Dietzia Rainey et al. (1995)	Alkanes	Yumoto et al. (2002)
Gordonia Stackebrandt et al. (1989)	Alkanes	Kummer et al. (1999)
Mycobacterium Lehmann and Neumann (1896)	Phenanthrene	Willumsen et al. (2001)
Nocardia Trevisan (1889)	Alkanes	Mikolasch et al. (2003)
Rhodococcus Zopf (1891)	Phenanthrene	Meyer et al. (1999)
Smaragdicoccus Adachi et al. (2007)	Crude oil	Adachi et al. (2007)
Tsukamurella Collins et al. (1988)	Hexadecane	Hassanshahian et al. (2013)
Williamsia Kampfer et al. (1999)	Crude oil	Obuekwe et al. (2009)
Order Micrococcales		
Arthrobacter Conn & Dimmick (1947)	Gas oil	Le Petit et al. (1975)
Brachybacterium Collins et al. (1988)	Diesel	Wang et al. (2010)
Brevibacterium Breed (1953)	Alkanes	Pirnik et al. (1974)
Cellulomonas Bergey et al. (1923)	Biphenyl	Plotnikova et al. (2006)
Cellulosimicrobium Schumann et al. (2001)	Crude oil	Dashti et al. (2009)
Citricoccus Altenburger et al. (2002)	Crude oil	Al-Awadhi et al. (2007)
Clavibacter Davis et al. (1984)	Naphthalene	Dore et al. (2003)
Cryobacterium Suzuki et al. (1997)	Alkanes	Lo Giudice et al. (2010)
Georgenia Altenburger et al. (2002)	Crude oil	Al-Awadhi et al. (2007)
Herbiconiux Behrendt et al. (2011)	Benzo[*a*]pyrene	Okai et al. (2015a)
Isoptericola Stackebrandt et al. (2004)	Crude oil	Al-Awadhi et al. (2007)
Janibacter Martin et al. (1997)	Phenanthrene	Yamazoe et al. (2004)
Kocuria Stackebrandt et al. (1995)	Crude oil	Dashti et al. (2009)
Kytococcus Stackebrandt et al. (1995)	Crude oil	Al-Saleh et al. (2009)
Leifsonia Evtushenko et al. (2000)	Crude oil	Dashti et al. (2009)
Microbacterium Orla-Jensen (1919)	Pyrene	Gauthier et al. (2003)
Micrococcus Cohn (1872)	Hexadecane	Ilori et al. (2000)
Plantibacter Behrendt et al. (2002)	Toluene	Lo Giudice et al. (2010)
Sanguibacter Fernández-Garayzábal et al. (2008)	Alkanes	Lo Giudice et al. (2010)
Terrabacter Collins et al. (1989)	Fluorene	Iida et al. (2003)
Order Propionibacteriales		
Aeromicrobium Miller et al. (1991)	Crude oil	Chaillan et al. (2004)

(*continued*)

Table 1 (continued)

Genus[a]	Typical substrate	References[b]
Nocardioides Prauser (1976)	Alkanes	Hamamura and Arp (2000)
Order Pseudonocardiales		
Amycolatopsis Lechevalier et al. (1986)	Phenanthrene	Bourguignon et al. (2014)
Prauserella Kim and Goodfellow (1999)	Crude oil	Almutairi (2015)
Pseudonocardia Henssen (1957)	Toluene	Juteau et al. (1999)
Saccharothrix Labeda et al. (1984)	Pyrene	Hu et al. (2004)
Order Streptomycetales		
Streptomyces Waksman and Henrici (1943)	Alkanes	Barabas et al. (2001)
Order Streptosporangiales		
Nocardiopsis Brocq-Rousseau (1904)	Crude oil	Dixit and Pant (2000)
Order Thermoleophilales		
Thermoleophilum Zarilla and Perry (1984)	Alkanes	Zarilla and Perry (1984)
Phylum Bacteroidetes		
Order Cytophagales		
Algoriphagus Bowman et al. (2003)	Crude oil	Wang et al. (2014b)
Cytophaga Winogradsky (1929)	Crude oil	Bossert and Bartha (1984)
Echinicola Nedashkovskaya et al. (2005)	Crude oil	Al-Awadhi et al. (2012)
Fabibacter Lau et al. (2006)	Crude oil	Wang et al. (2014b)
Roseivirga Nedashkovskaya et al. (2005)	Alkanes	Selvaratnam et al. (2016)
Order Flavobacteriales		
Arenibacter Ivanova et al. (2001)	Phenanthrene	Gutierrez et al. (2014)
Chryseobacterium Vandamme et al. (1994)	Kerosene	Szoboszlay et al. (2008)
Cloacibacterium Allen et al. (2006)	Crude oil	Jurelevicius et al. (2013)
Flavobacterium Bergey et al. (1923)	Diesel oil	Stucki and Alexander (1987)
Mesoflavibacter Asker et al. (2008)	Benzo[*a*]pyrene	Okai et al. (2015a)
Myroides Vancanneyt et al. (1996)	Crude oil	Maneerat et al. (2006)
Olleya Mancuso Nichols et al. (2005)	Benzo[*a*]pyrene	Okai et al. (2015b)
Polaribacter Gosink et al. (1998)	Crude oil	Wang et al. (2014b)
Tenacibaculum Suzuki et al. (2001)	Crude oil	Wang et al. (2014b)
Weeksella Holmes et al. (1987)	Crude oil	Yuste et al. (2000)
Yeosuana Kwon et al. (2006)	Benzo[*a*]pyrene	Kwon et al. (2006)
Zobellia Barbeyron et al. (2001)	Crude oil	Wang et al. (2014b)
Zunongwangia Qin et al. (2007)	Crude oil	Shao et al. (2014)
Order Sphingobacteriales		
Parapedobacter Kim et al. (2007)	Pyrene	Zhao et al. (2013)
Pedobacter Steyn et al. (1998)	Diesel	Margesin et al. (2003)
Olivibacter Ntougias et al. (2007)	Diesel	Szabó et al. (2011)
Terrimonas Xie and Yokota (2006)	Benzo[*a*]pyrene	Song et al. (2015)
Phylum Verrucomicrobia		
Methylacidimicrobium van Teeseling et al. (2014)	Methane	van Teeseling et al. (2014)
'Methylacidiphilum'	Methane	Hou et al. (2008)

(*continued*)

Table 1 (continued)

Genus[a]	Typical substrate	References[b]
Phylum Cyanobacteria		
Order Chroococcales		
Agmenellum De Brebisson (1839)	Phenanthrene	Narro et al. (1992)
Aphanocapsa Nägeli (1849)	Crude oil	Raghukumar et al. (2001)
Aphanothece Nägeli (1849)	Alkanes	Abed and Köster (2005)
Coccochloris Sprengel (1807)	Naphthalene	Cerniglia et al. (1979)
Order Nostocales		
Anabaena St. Vincent (1886)	Alkanes	Gamila et al. (2003)
Nostoc Vaucher (1888)	Naphthalene	Cerniglia (1992)
Order Oscillatoriales		
Dactylococcopsis Hansgirg (1893)	Alkanes	Abed and Köster (2005)
Halothece Margheri et al. (2008)	Alkanes	Abed and Köster (2005)
Microcoleus Desmaziéres (1892)	Alkanes	Al-Hasan et al. (1998)
Oscillatoria Vaucher (1892)	Alkanes	Gamila et al. (2003)
Phormidium Kutzing (1892)	Alkanes	Al-Hasan et al. (1998)
Plectonema Thuret (1892)	Crude oil	Raghukumar et al. (2001)
Phylum Deinococcus-Thermus		
Order Thermales		
Thermus Brock and Freeze (1969)	Pyrene	Feitkenhauer et al. (2003)
Phylum Firmicutes		
Order Bacillales		
Aeribacillus Minana-Galbis et al. (2010)	Alkanes	Tourova et al. (2016)
Aneurinibacillus Shida et al. (1996)	Toluene	Godheja et al. (2017)
Bacillus Cohn (1872)	Toluene	Li et al. (2008)
Brevibacillus Shida et al. (1996)	Crude oil	Dashti et al. (2009)
Exiguobacterium Collins et al. (1984)	Diesel oil	Mohanty and Mukherji (2008)
Geobacillus Nazina et al. (2001)	Crude oil	Nazina et al. (2001)
Kurthia Trevisan (1885)	Anthracene	Bisht et al. (2010)
Lysinobacillus Ahmed et al. (2007)	Asphaltenes	Jahromi et al. (2014)
Oceanobacillus Lu et al. (2002)	Crude oil	Al-Awadhi et al. (2007)
Paenibacillus Ash et al. (1994)	Phenanthrene	Meyer et al. (1999)
Planococcus Migula (1894)	Benzene	Li et al. (2006)
Planomicrobium Yoon et al. (2001)	Alkanes	Engelhardt et al. (2001)
Staphylococcus Rosenbach (1884)	Diesel	Saadoun et al. (1999)
Order Clostridiales		
Abyssivirga Schouw et al. (2016)[c]	Alkanes	Schouw et al. (2016)
Desulfitobacterium Utkin et al. (1994)	Toluene	Kunapuli et al. (2010)
Desulfotomaculum Campbell and Postgate (1965)	*o*-Xylene	Morasch et al. (2004)
Desulfosporosinus Stackebrandt et al. (1997)	Gasoline	Robertson et al. (2001)
Garciella MirandoTello et al. (2003)	Asphalt	Lavania et al. (2012)
Peptococcus Kluyver and van Niel (1936)	Crude oil	Floodgate (1984)
Sarcina Goodsir (1842)	Crude oil	Bossert and Bartha (1984)

(*continued*)

Table 1 (continued)

Genus[a]	Typical substrate	References[b]
Order Lactobacillales		
Lactobacillus Beijerinck (1901)	Crude oil	Floodgate (1984)
Phylum Proteobacteria		
Class Alphaproteobacteria		
Order Caulobacterales		
Brevundimonas Segers et al. (1994)	Fuel oil	Chaineau et al. (1999)
Order Kordiimonadales[d]		
Kordiimonas Kwon et al. (2005)	Benzo[*a*]pyrene	Kwon et al. (2005)
Order Rhizobiales		
Afipia Brenner et al. (1992)	Phenanthrene	Bodour et al. (2003)
Agrobacterium Conn (1942)	Gasoline	Prantera et al. (2002)
Aquamicrobium Kämpfer et al. (2009)	Hexadecane	Tzintzun-Camacho et al. (2012)
Beijerinckia Derx (1950)	Phenanthrene	Surovtseva et al. (1999)
Blastochloris Hiraishi (1997)	Toluene	Zengler et al. (1999)
Chelatococcus Auling et al. (1993)	Cyclohexane	Salamanca and Engesser (2014)
Ensifer Casida (1982)	Phenanthrene	Muratova et al. (2014)
Hyphomicrobium Stutzer and Hartleb (1898)	Crude oil	Ozaki et al. (2006)
Kaistia Im et al. (2005)	Hexadecane	Al-Mailem et al. (2014b)
Martelella Rivas et al. (2005)	Phenanthrene	Feng et al. (2012)
Mesorhizobium Jarvis et al. (1997)	*o*-Xylene	Khomenkov et al. (2005)
Methylobacterium Patt et al. (1976)	Phenanthrene	Bodour et al. (2003)
Methylocapsa Dedysh et al. (2002)	Methane	Dedysh et al. (2002)
Methylocella Dedysh et al. (2000)	Methane	Dedysh et al. (2000)
Methylocystis Bowman et al. (1993)	Methane	Bowman et al. (1993)
Methylosinus Bowman et al. (1993)	Methane	Bowman et al. (1993)
Microvirga Kanso and Patel (2003)	Creosote	Simarro et al. (2013)
Nitratireductor Labbe et al. (2004)	Crude oil	Dashti et al. (2015)
Ochrobactrum Holmes et al. (1988)	Crude oil	Peressutti et al. (2003)
Oligotropha Meyer et al. (1994)	Anthracene	Ntougias et al. (2015)
Parvibaculum Schleheck et al. (2004)	Linear alkanes	Schleheck et al. (2004)
Rhizobium Frank (1889)	Phenanthrene	Bodour et al. (2003)
Shinella An et al. (2006)	Anthracene	Ntougias et al. (2015)
Sinorhizobium Chen et al. (1988)	Phenanthrene	Bodour et al. (2003)
Sneathiella (Jordan et al., 2007)	Phenanthrene	Sauret et al. (2014)
Xanthobacter Wiegel et al. (1978)	Dibenzothiophene	Padden et al. (1997)
Order Rhodobacterales		
Catellibacterium Tanaka et al. (2004)	Alkanes	Harwati et al. (2007)
Celeribacter Ivanova et al. (2010)	Phenanthrene	Lai et al. (2014)
Citreicella Sorokin et al. (2006)	Crude oil	Wang et al. (2014b)
Confluentimicrobium Park et al. (2014)	Naphthalene	Jeong et al. (2015)

(*continued*)

Table 1 (continued)

Genus[a]	Typical substrate	References[b]
Defluviimonas Foesal et al. (2013)	Pyrene	Zhang et al. (2018)
Jannaschia Wagner-Dobler et al. (2003)	Alkanes	Harwati et al. (2007)
Labrenzia Biebl et al. (2007)	Crude oil	Kostka et al. (2011)
Loktanella Van Trappen et al. (2004)	Alkanes	Harwati et al. (2007)
Maribius Choi et al. (2007)	Alkanes	Harwati et al. (2007)
Maritimibacter Lee et al. (2007)	Hexadecane	Green et al. (2015)
Nesiotobacter Donachie et al. (2006)	Crude oil	Dashti et al. (2015)
Pelagibaca Cho and Giovannoni (2006)	Crude oil	Wang et al. (2014b)
Polymorphum Cai et al. (2011)	Crude oil	Nie et al. (2012)
Roseivivax Suzuki et al. (1999)	Alkanes	Harwati et al. (2007)
Roseovarius Labrenz et al. (1999)	Alkanes	Harwati et al. (2007)
Salipiger Martínez-Cánovas et al. (2004)	Alkanes	Harwati et al. (2007)
Stappia Uchino et al. (1999)	Crude oil	Al-Awadhi et al. (2007)
Sulfitobacter Sorokin (1996)	Phenanthrene	Crisafi et al. (2016)
Thalassobius Arahal et al. (2005)	Polycyclic aromatic hydrocarbons	Rodrigo-Torres et al. (2017)
Tranquillimonas Harwati et al. (2008)	Alkanes	Harwati et al. (2008)
Tropicibacter Harwati et al. (2009b)	Naphthalene	Harwati et al. (2009b)
Tropicimonas Harwati et al. (2009a)	Alkanes	Harwati et al. (2009a)
Order Rhodospirillales		
Acidocella Kishimoto et al. (1995)	Naphthalene	Dore et al. (2003)
Azospirillum Tarrand et al. (1979)	Alkane	Roy et al. (1988)
Magnetospirillum Schleifer et al. (1992)	Toluene	Shinoda et al. (2005)
Oceanibaculum Lai et al. (2009)	Phenanthrene	Lai et al. (2009)
'Oleomonas' Kanamori et al. (2002)	Toluene, alkanes	Kanamori et al. (2002)
Thalassospira Lopez-Lopez et al. (2002)	Pyrene	Kodama et al. (2008)
Tistrella Shi et al. (2002)	Diesel	Wang et al. (2010)
Zavarzinia Meyer et al. (1994)	Benzene	Rochman et al. (2017)
Order Sphingomonadales[e]		
Altererythrobacter Kwon et al. (2007)	Alkanes	Harwati et al. (2007)
Croceicoccus Xu et al. (2009)	Phenanthrene	Huang et al. (2015)
Erythrobacter Shiba and Simidu (1982)	Alkanes	Harwati et al. (2007)
'Lutibacterium' Chung and King (2001)[f]	Phenanthrene	Chung and King (2001)
Novosphingobium Takeuchi et al. (2001)	Phenanthrene	Shi et al. (2001)
Porphyrobacter Fuerst et al. (1993)	Phenanthrene	Gauthier et al. (2003)
Sphingobium Takeuchi et al. (2001)	Phenanthrene	Prakash and Lal (2006)
Sphingomonas Yabuuchi et al. (1990)	Dibenzofuran	Halden et al. (1999)
Sphingopyxis Takeuchi et al. (2001)	Phenanthrene	LaRoe et al. (2010)
Sphingorhabdus Jogler et al. (2013)	Crude oil	Jeong et al. (2016)
Class Betaproteobacteria		
Order Burkholderiales		
Achromobacter Yabuuchi and Yano (1981)	Gas oil	Le Petit et al. (1975)
Acidovorax Willems et al. (1990)	Phenanthrene	Meyer et al. (1999)

(*continued*)

Table 1 (continued)

Genus[a]	Typical substrate	References[b]
Advenella Coenye et al. (2005)	Pyrene	Wang et al. (2014a)
Alcaligenes Castellani and Chalmers (1919)	Gas oil	Le Petit et al. (1975)
Alicycliphilus Mechichi et al. (2003)	Benzene	Weelink et al. (2008)
Bordetella Moreno-López (1952)	Phenanthrene	Pinyakong et al. (2012)
Brachymonas Hiraishi et al. (1995)	Cyclohexane	Brzostowicz et al. (2005)
Burkholderia Yabuuchi et al. (1993)	Toluene	Parales et al. (2000)
Castellaniella Kämpfer et al. (2006)	α-Pinene	Kämpfer et al. (2006)
Comamonas De Vos et al. (1985)	Phenanthrene	Meyer et al. (1999)
Cupriavidus Makkar and Casida (1987)	Biphenyl	Wittich and Wolff (2007)
Delftia Wen et al. (1999)	Phenanthrene	Shetty et al. (2015)
Diaphorobacter Khan and Hiraishi (2002)	Pyrene	Klankeo et al. (2009)
Herbaspirillum Baldani et al. (1986)	Fluoranthene	Xu et al. (2011)
Herminiimonas Fernandes et al. (2005)	Toluene	Kim et al. (2014b)
Hydrogenophaga Willems et al. (1989)	Benzene	Fahy et al. (2008)
Janthinobacterium De Ley et al. (1978)	Phenanthrene	Bodour et al. (2003)
Leptothrix Kutzing (1843)	Phenanthrene	Bodour et al. (2003)
Massilia La Scola et al. (2000)	Phenanthrene	Bodour et al. (2003)
Methylibium Nakatsu et al. (2006)	Toluene	Nakatsu et al. (2006)
Pandoraea Coenye et al. (2000)	Crude oil	Ozaki et al. (2006)
Paraburkholderia Sawana et al. (2015)	Gasoline	Lee and Jeon (2018)
Polaromonas Irgens et al. (1996)	Naphthalene	Jeon et al. (2004)
Pusillimonas Stolz et al. (2005)	Diesel	Cao et al. (2011)
Ralstonia Yabuuchi et al. (1996)	Toluene	Parales et al. (2000)
Ramlibacter Heulin et al. (2003)	*m*-xylene	Xie et al. (2010)
Rhodoferax Hiraishi et al. (1992)	Propylbenzene	Eriksson et al. (2005)
Sphaerotilus Kutzing (1833)	Crude oil	Austin et al. (1977)
Variovorax De Ley et al. (1991)	Benzene	Posman et al. (2017)
Order Hydrogenophilales		
Thiobacillus Beijerinck (1904)	Phenanthrene	Bodour et al. (2003)
Order Nitrosomonadales		
Spirillum Ehrenberg (1832)	Crude oil	Bossert and Bartha (1984)
Order Rhodocyclales		
'Aromatoleum'	Ethylbenzene	Trautwein et al. (2008)
Azoarcus Reinhold-Hurek et al. (1993)	Toluene	Hess et al. (1997)
Dechloromonas Achenbach et al. (2001)	Benzene	Coates et al. (2001b)
Georgfuchsia Weelink et al. (2009)	Toluene	Weelink et al. (2009)
Methyloversatalis Kalyuzhnaya et al. (2006)	Toluene	Kalyuzhnaya et al. (2006)
Rugosibacter Corteselli et al. (2016)	Phenanthrene	Corteselli et al. (2017a)
Thauera Macy et al. (1993)	Toluene	Shinoda et al. (2004)
Zooglea Itzigsohn (1868)	Crude oil	Farkas et al. (2015)
Class Gammaproteobacteria		

(*continued*)

Table 1 (continued)

Genus[a]	Typical substrate	References[b]
Order Aeromonadales		
Aeromonas Stanier (1943)	Diesel	Odokumal and Dickson (2003)
Oceanisphaera Romanenko et al. (2003)	Crude oil	Buzoleva et al. (2017)
Zobellella Lin and Shieh (2006)	Phenanthrene	Lee et al. (2018)
Order Alteromonadales		
Aestuariibacter Yi et al. (2004)	Crude oil	Wang et al. (2014b)
Aestuariicella Lo et al. (2015)	*n*-Alkanes	Lo et al. (2015)
Aliiglaciecola Jean et al. (2013)	*n*-Alkanes	Jin et al. (2015)
Alteromonas Baumann et al. (1972)	Crude oil	Iwabuchi et al. (2002)
Colwellia Deming (1988)	Crude oil	Baelum et al. (2012)
Glaciecola Bowman et al. (1998)	Phenanthrene	Sauret et al. (2014)
Halioxenophilus Iwaki et al. (2018)	Xylene	Iwaki et al. (2018)
Idiomarina Ivanova et al. (2000)	Crude oil	Wang et al. (2010)
Marinobacter Gauthier et al. (1992)	Crude oil	Gauthier et al. (1992)
Marinobacterium González et al. (1997)	Crude oil	Pham et al. (2009)
Microbulbifer Gonzalez et al. (1997)	Crude oil	Brito et al. (2006)
Pseudidiomarina Jean et al. (2006)	Crude oil	Kostka et al. (2011)
Pseudoalteromonas Gauthier et al. (1995)	Crude oil	Kostka et al. (2011)
Shewanella MacDonell and Colwell (1986)	Crude oil	Gentile et al. (2003)
Order Cellvibrionales[g]		
Porticoccus Oh et al. (2010)	Phenanthrene	Gutierrez et al. (2012b)
Zhongshania Li et al. (2011)	Alkanes	Naysim et al. (2014)
Order Enterobacteriales		
Edwardsiella Ewing and McWhorter (1965)	Lubricating oil	Okpokwasili and Okorie (1988)
Enterobacter Hormaeche and Edwards (1960)	Alkanes	Saadoun et al. (1999)
Erwinia Winslow et al. (1920)	Alkanes	Saadoun et al. (1999)
Franconibacter Stephan et al. (2014)	Crude oil	Pal et al. (2017)
Gallaecimonas Rodríguez-Blanco et al. (2010)	Pyrene	Rodríguez-Blanco et al. (2010)
Klebsiella Trevisan (1885)	Crude oil	Odokumal and Dickson (2003)
Leclercia Tamura et al. (1987)	Pyrene	Sarma et al. (2004)
Pantoea Gavini et al. (1989)	Crude oil	Obuekwe et al. (2009)
Proteus Hauser (1885)	Crude oil	Odokumal and Dickson (2003)
Rahnella Izard et al. (1981)	Naphthalene	Ma et al. (2006)
Serratia Bizio (1823)	Crude oil	Odokumal and Dickson (2003)
Trabulsiella McWhorter et al. (1992)	Pyrene	Kuppusamy et al. (2016)
Order Immundisolibacterales		
Immundisolibacter Corteselli et al. (2017b)	Pyrene	Corteselli et al. (2017b)

(*continued*)

Table 1 (continued)

Genus[a]	Typical substrate	References[b]
Order Legionellales		
Legionella Brenner et al. (1979)	Anthracene	Zhang et al. (2012)
Order Methylococcales		
Clonothrix Roze (1896)	Methane	Vigliotta et al. (2007)
Crenothrix Cohn (1870)[h]	Methane	Stoecker et al. (2006)
Methylobacter Bowman et al. (1993)	Methane	Bowman et al. (1993)
Methylocaldum Bodrossy et al. (1998)	Methane	Bodrossy et al. (1997)
Methylococcus Foster and Davis (1966)	Methane	Bowman et al. (1993)
Methylogaea Geymonat et al. (2011)	Methane	Geymonat et al. (2011)
Methyloglobulus Deutzmann et al. (2014)	Methane	Deutzmann et al. (2014)
Methylohalobius Heyer et al. (2005)	Methane	Heyer et al. (2005)
Methylomagnum Khalifa et al. (2015)	Methane	Khalifa et al. (2015)
Methylomarinovorum Hirayama et al. (2014)	Methane	Hirayama et al. (2014)
Methylomarinum Hirayama et al. (2013)	Methane	Hirayama et al. (2013)
Methylomonas Leadbetter (1974)	Methane	Bowman et al. (1993)
Methyloparacoccus Hoefman et al. (2014)	Methane	Hoefman et al. (2014)
Methyloprofundus Tavormina et al. (2015)	Methane	Tavormina et al. (2015)
Methyloterricola Frindte et al. (2017)	Methane	Frindte et al. (2017)
Methylothermus Tsubota et al. (2005)	Methane	Tsubota et al. (2005)
Methylomicrobium Bowman et al. (1995)	Methane	Kalyuzhnaya et al. (2008)
Methylosarcina Wise et al. (2001)	Methane	Wise et al. (2001)
Methylosoma Rahalkar et al. (2007)	Methane	Rahalkar et al. (2007)
Methylosphaera Bowman et al. (1997)	Methane	Bowman et al. (1997)
Methylovulum Iguchi et al. (2011)	Methane	Iguchi et al. (2011)
Order Oceanospirillales		
Alcanivorax Yakimov et al. (1998)	Alkanes	Yakimov et al. (1998)
Bermanella Pinhassi et al. (2009)	Crude oil	Hu et al. (2017)
Chromohalobacter Ventosa et al. (1989)	Crude oil	Al-Mailem et al. (2014a)
Cobetia Arahal (2002)	Dibenzothiophene	Ibacache-Quiroga et al. (2013)
Halomonas Vreeland et al. (1980)	Crude oil	Wang et al. (2007)
Ketobacter Kim and Rhee (2017)	Alkanes	Kim et al. (2018)
Marinomonas van Landschoot and De Ley (1984)	Phenanthrene	Melcher et al. (2002)
Neptuniibacter Arahal et al. (2007)	Carbazole	Nagashima et al. (2010)
Neptunomonas Hedlund et al. (1999)	Naphthalenes	Hedlund et al. (1999)
Oleibacter Teramoto et al. (2011)	Alkanes	Teramoto et al. (2011)
Oleiphilus Golyshin et al. (2002)	Alkanes	Golyshin et al. (2002)
Oleispira Yakimov et al. (2003)	Alkanes	Yakimov et al. (2003)
Profundimonas Cao et al. (2014)	Crude oil	Cao et al. (2014)
Salinicola Anan'ina et al. (2007)	Naphthalene	Anan'ina et al. (2007)
Thalassolituus Yakimov et al. (2004)	Alkanes	Yakimov et al. (2004)
Order Pasteurellales		

(*continued*)

Table 1 (continued)

Genus[a]	Typical substrate	References[b]
Pasteurella Trevisan (1887)	Fluoranthene	Sepic et al. (1997)
Order Pseudomonadales		
Acinetobacter Brisou & Prévot (1954)	Gas oil	Le Petit et al. (1975)
Alkanindiges Bogan et al. (2003)	Alkanes	Bogan et al. (2003)
Azotobacter Beijerinck (1901)	Paraffins	Gradova et al. (2003)
Moraxella Lwoff (1939)	Biphenyl	Stucki and Alexander (1987)
Perlucidibaca Song et al. (2008)	Crude oil	Viggor et al. (2015)
Pseudomonas Migula (1894)	Gas oil	Le Petit et al. (1975)
Psychrobacter Juni (1986)	Phenanthrene	Harwati et al. (2007)
Order Thiotrichales		
Cycloclasticus Dyksterhouse et al. (1995)	Biphenyl	Dyksterhouse et al. (1995)
Leucothrix Oersted (1844)	Crude oil	Floodgate (1984)
Order Vibrionales		
Salinivibrio Mellado et al. (1996)	Crude oil	Al-Mailem et al. (2014a)
Vibrio Pacini (1854)	Phenanthrene	Hedlund and Staley (2001)
Order Xanthomonadales[i]		
Algiphilus Gutierrez et al. (2012)	Phenanthrene	Gutierrez et al. (2012a)
Alkanibacter Friedrich and Lipski (2008)	Hexane	Friedrich and Lipski (2008)
Dokdonella Yoon et al. (2006)	Phenanthrene	Bacosa and Inoue (2015)
Dyella Xie and Yokota (2005)	Biphenyl	Ang et al. (2009)
Hydrocarboniphaga Palleroni et al. (2004)	Alkanes	Palleroni et al. (2004)
Luteibacter Johansen et al. (2005)	Phenanthrene	Muangchinda et al. (2013)
Lysobacter Christensen and Cook (1978)	Phenanthrene	Maeda et al. (2009)
Nevskia Famintzin (1892)	Toluene	Juteau et al. (1999)
Oleiagrimonas Fang et al. (2015)	Polycyclic aromatic hydrocarbons	Fang et al. (2015)
Polycyclovorans Gutierrez et al. (2013)	Phenanthrene	Gutierrez et al. (2013)
Pseudoxanthomonas Finkmann et al. (2000)	Toluene	Kim et al. (2008)
Rhodanobacter Nalin et al. (1999)	Benzo[*a*]pyrene	Kanaly et al. (2002)
Salinisphaera Antunes et al. (2003)	Diesel	Wang et al. (2010)
Singularimonas Friedrich and Lipski (2008)	Hexane	Friedrich and Lipski (2008)
Solimonas Kim et al. (2007)	Diesel	Wang et al. (2010)
Stenotrophomonas Palleroni and Bradbury (1993)	Pyrene	Juhasz et al. (2000)
Xanthomonas Dowson (1939)	Phenanthrene	Hamann et al. (1999)
Xylella Wells et al. (1987)	Alkanes	Yuste et al. (2000)
Class Deltaproteobacteria		
Order Desulfobacterales		
Desulfatibacillum Cravo-Laureau et al. (2004)	Alkanes	Cravo-Laureau et al. (2004)
Desulfatiferula Cravo-Laureau et al. (2007)	Alkenes	Cravo-Laureau et al. (2007)

(*continued*)

Table 1 (continued)

Genus[a]	Typical substrate	References[b]
Desulfatitalea Higashioka et al. (2013)	Toluene	Higashioka et al. (2013)
Desulfobacterium Bak and Widdel (1988)	Xylene	Harms et al. (1999)
Desulfobacula Rabus et al. (2000)	Toluene	Rabus et al. (1993)
Desulfococcus Widdel (1981)	Alkanes	Kleindienst et al. (2014)
Desulfosarcina Widdel (1981)	Xylene	Harms et al. (1999)
Desulfotignum Kuever et al. (2001)	Toluene	Ommedal and Torsvik (2007)
Order Desulfovibrionales		
Desulfothermus Kuever et al. (2005)	Alkanes	Kuever et al. (2005)
Desulfovibrio Kluyver and van Niel (1936)	Alkanes	Davis and Yarbrough (1966)
Order Desulfuromonales		
Geobacter Lovley et al. (1995)	Toluene	Coates et al. (2001a)
Pelobacter Schink and Pfennig (1983)	Acetylene	Schink (1985)
Order Syntrophobacterales		
Desulfoglaeba Davidova et al. (2006)	Alkanes	Davidova et al. (2006)
Smithella Liu et al. (1999)	Alkanes	Wawrik et al. (2016)
Candidate Phylum NC10		
Candidatus Methylomirabilis Ettwig et al. (2010)	Methane	Ettwig et al. (2010)

[a]The primary taxonomic guide for assigning genera to orders, and orders to phyla, was Bergey's Manual of Systematics of Archaea and Bacteria (2017), last updated on 27th March 2017 and outlined at https://wol-prod-cdn.literatumonline.com/pb-assets/assets/9781118960608/Taxonomic_Outline_October_2017-1507044705000.pdf. Cyanobacterial taxonomy is in a state of flux. In Bergey's Manual of Systematics of Archaea and Bacteria (2017) the phylogenetic groups equivalent to orders have been given numbers. Therefore, the NCBI taxonomy browser (https://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi) was used to sort Cyanobacteria into recognisable orders. Certain genera were not present in Bergey's Manual of Systematics of Archaea and Bacteria (2017), but their names had been validly published. Their assignment to an order is based on taxonomic papers, primarily those from the International Journal of Systematic and Evolutionary Microbiology.

[b]References are to a 'typical' reference, not necessarily the first, last or best.

[c]Postec et al. (2017) object to the genus assignation of *Abyssivirga*, and suggest that the strain should be assigned to *Vallitalea*.

[d]Kordiimonadales was not identified as an order in Bergey's Manual of Systematics of Archaea and Bacteria (2017), but appears to have standing in nomenclature (Kwon et al. 2005).

[e]Early work ascribed several hydrocarbon degraders to *Sphingomonas*, but this genus has undergone taxonomic scrutiny, and most have been moved to *Sphingobium* or *Novosphingobium*. 'Lutibacterium' is closely allied (Leys et al. 2004; Pal et al. 2006; Kim et al. 2007; Kertesz and Kawasaki 2009).

[f]The genus name 'Lutibacterium' is not italicised because, along with 'Aromatoleum', 'Oleomonas' and 'Methylacidiphilum', it does not seem to have been validly published.

[g]Although the order Cellvibrionales is not yet formally recognised, there is a strong case for including the genera *Porticoccus* and *Zhongshania* in this order (Spring et al. 2015).

[h]*Crenothrix* was originally thought to be an alga (Cohn 1873).

[i]Naushad et al. (2015) have proposed the transfer of *Algiphilus, Alkanibacter, Hydrocarboniphaga, Nevskia, Salinisphaera, Singularimonas* and *Solimonas* from the Xanthomonadales to the new order Nevskiales. But here we retain these genera in the Xanthomonadales.

3 Currently Described Hydrocarbon-Degrading Bacteria

Bacterial taxonomy is curated at several sites; The German Collection of Microorganisms and Cell Cultures (DSMZ 2017), The National Center for Biological Information (NCBI 2017), Bergey (2012), RDP (2016), and Euzéby (2017) for examples. Table 1 lists the almost 300 currently published genera with representatives that are able to grow using hydrocarbons as sole or major carbon source. Figure 1 illustrates the phylogenetic distribution of known hydrocarbon-degrading Bacteria, and Fig. 2 focuses on the Proteobacteria.

4 Phylum Actinobacteria

These Gram-positive bacteria are colloquially known as Actinomycetes because some grow as branching filaments that resemble fungal mycelia. They are common in soil environments, and play a central role in the decomposition of organic matter and recycling of soil nutrients. Some produce potent antibiotics.

Eight orders within the Actinobacteria have known hydrocarbon-degrading genera, the Actinomycetales, Corynebacteriales, Micrococcales, Propionibacteriales, Pseudonocardiales, Streptomycetales, Streptosporangiales and Thermoleophilales. Most characterized actinobacterial oil-degraders are in the Corynebacteriales and Micrococcales, (Table 1), and are typically mesophiles. The one characterized genus in the Thermoleophilales, *Thermoleophilum* (Zarilla and Perry 1984), is obligately thermophilic, with an optimum growth temperature of 60 °C. All are aerobes.

Rhodococcus from the order Corynebacteriales seems able to grow on polyethylene as sole carbon sources (Santo et al. 2013; Eyheraguibel et al. 2017) – even on polystyrene 96-well plates in carbon free mineral medium! There is remarkable versatility within this group, with strains of *Gordonia*, *Mycobacterium*, and *Rhodococcus* able to remove sulfur from dibenzothiophene to yield hydroxybiphenyl (see Martínez et al. 2017) and grow on isoprene as sole source of carbon and energy (Acuña Alvarez et al. 2009).

5 Phylum Bacteroidetes

Species of Bacteroidetes are well known for the degradation of complex polymers (Fernández-Gómez et al. 2013). Three out of four phyla within the Bacteroidetes, the Cytophagales, Flavobacteriales, and Sphingobacteriales, currently contain hydrocarbon-degraders (Table 1). All the known Bacteroidetes hydrocarbon-degraders are aerobes.

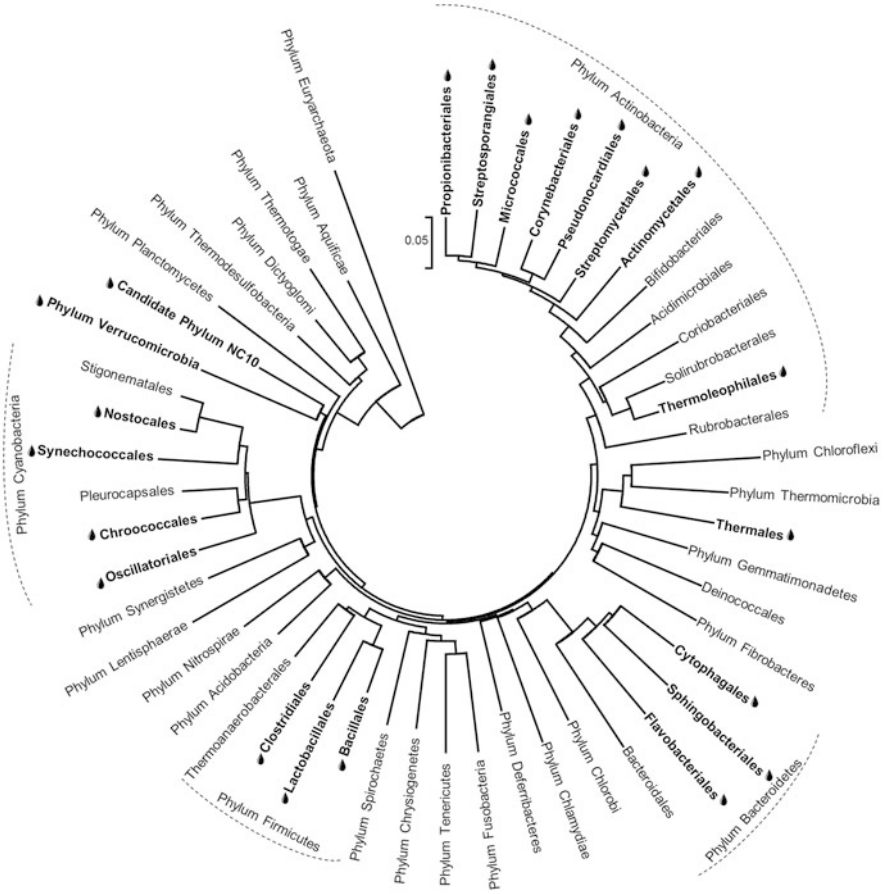

Fig. 1 Phylogenetic distribution of the capacity to degrade hydrocarbons in Bacteria (except Proteobacteria). For those phyla that have hydrocarbon-degrading species, the presence of that capacity in different orders is shown (bold font and oil droplet). The sequence of one representative species from each order of each phylum (when possible from the type species) was selected according to the current bacterial taxonomy available from the online taxonomic resources: Bergey (2017), NCBI (2017) and RDP (2016). Orders are not shown for those phyla that, to date, contain no hydrocarbon-degrading species; instead, one organism was selected as a representative of these phyla. Although the orders Deinococcales and Thermales belong to the phylum Thermus-Deinococcus according to Bergey (2017), they did not form a monophyletic grouping in this tree. The tree is based on 16S rRNA gene sequence comparisons. Evolutionary distances were computed using the Jukes-Cantor method (Jukes and Cantor 1969). The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei 1987). All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA7 (Kumar et al. 2016). *Halosarcina limi*, belonging to the phylum Euryarchaeota of the Archaea, served as the outgroup. The bar represents 0.05 nucleotide substitutions per site. See Fig. 2 for details of the proteobacterial orders with species able to grow with hydrocarbons as sole or main carbon source.

Fig. 2 Phylogenetic distribution of the capacity to degrade hydrocarbons in phylum Proteobacteria. For those classes that have hydrocarbon-degrading species, the presence of that capacity in different orders is shown (bold font and oil droplet). The sequence of one representative species from each order (when possible from the type species) was selected according to the current bacterial taxonomy available from the online taxonomic resources: Bergey (2017), NCBI (2017) and RDP (2016). The tree is based on 16S rRNA gene sequence comparisons. Evolutionary distances were computed as described in Fig. 1. The tree is not rooted with an outgroup. The bar represents 0.02 nucleotide substitutions per site. The order Campylobacterales is included as a representative of the Epsilonproteobacteria (ε). Although the order Desulfurellales is currently in the class Deltaproteobacteria, there is phylogenomic evidence (Waite et al. 2017) that Desulfurellales should be ascribed to a new phylum, Epsilonbacteraeota, along with the rest of the current Epsilonproteobacteria

6 Phylum Cyanobacteria

The Cyanobacteria are typically considered obligate photoautotrophs, but in fact some can grow heterotrophically (Francisco et al. 2014), and many take advantage of reduced carbon compounds in their environment. Their ability to degrade hydrocarbons has been debated for some time, and while there are credible reports using labeled substrates (Table 1), there is some discussion as to how significant this is, and indeed whether the metabolism is actually carried out by the phototrophs, or by commensal bacteria (Abed and Köster 2005; Abed et al. 2014; Al-Wahaib et al. 2016; Radwan et al. 2005). *Microcoleus chthonoplastes*, for example, forms a consortium with oil-degrading bacteria within the cyanobacterial sheath (Sánchez et al. 2005). Axenic cultures of Cyanobacteria may well be a laboratory artifact – Morris et al. (2008) demonstrate that *Prochlorococcus* (not reported to degrade hydrocarbons) grows poorly in the absence of "helper" heterotrophs, at least in dilute cultures.

Agmenellum quadruplicatum (Narro et al. 1992) is currently assigned to *Synechococcus* (e.g., NCBI 2009). Cyanobacterial taxonomy remains in flux, and current convention is to eschew specific names for new isolates, and to use numbers.

In summary, there is currently no evidence that any Cyanobacterium can grow with a hydrocarbon as sole carbon source, but it does seem clear that Cyanobacteria can provide at least some of the oxygen and nitrogen required by heterotrophic oil-degraders in oiled sediments (e.g., Musat et al. 2006). Microbial mats containing Cyanobacteria may be important sinks for hydrocarbon in the environment (e.g., Abed and Köster 2005; Abed et al. 2014; Bordenave et al. 2008; Chronopoulou et al. 2013; Cohen 2002; Hernandez-Raquet et al. 2006; Radwan et al. 2005), and a potential treatment option for saline wastewaters such as those produced in hydraulic fracturing (Akyon et al. 2015).

7 Phylum Deinococcus-Thermus

This phylum currently contains only a single species known to grow on hydrocarbons as sole source of carbon; *Thermus brockii* (Feitkenhauer et al. 2003). This aerobic organism degrades hexadecane and a broad spectrum of polycyclic aromatic hydrocarbons at an optimum temperature of 70 °C, and retains some activity at 80 °C.

8 Phylum Firmicutes

Most Firmicutes ("strong-skinned") are spore-forming gram-positive organisms, and three orders, the Bacillales, the Lactobacillales and the Clostridiales, contain hydrocarbon-degrading genera (Table 1). Strains of *Bacillus subtilis* and *Paenibacillus polymyxa* are able to remove sulfur from dibenzothiophene to yield hydroxybiphenyl

(see Mohebali and Ball 2008). *Brevibacillus borstelensis*, a thermophilic aerobe, can even degrade polyethylene (Hadad et al. 2005), as can a *Bacillus* (Yang et al. 2014).

The organism isolated by Engelhardt et al. (2001) as a *Planococcus* has been reassigned to *Planomicrobium* by Dai et al. (2005).

Two genera of the Clostridiales, *Desulfotomaculum* and *Desulfosporosinus*, are sulfate-reducers, and *Desulfitobacterium* is an iron-reducer; all degrade hydrocarbons under anaerobic conditions.

The identification of *Sarcina* is curious – these organisms have a very characteristic morphology, occurring in packets of eight or more cells, but all the formally described species are fermentative anaerobes or microaerophiles. So are the formally described species of *Peptococcus* and *Lactobacillus*, and there have been few subsequent reports of such organisms as hydrocarbon-degraders. They were identified in aerobic incubations in the references cited in Table 1.

Members of the order Halanaerobiales, consisting of fermentative, obligately anaerobic halophiles, are frequently associated with oil reservoirs and produced waters. Although they have not been shown to use hydrocarbons as a source of carbon and energy, they likely contribute to the production of acid, gas and potentially sulfide using intermediates released by hydrocarbon degraders (Lipus et al. 2017).

9 Phylum Proteobacteria

The majority of the formally described genera of hydrocarbon-degrading bacteria are in this very large phylum of gram-negative organisms. The phylum is divided into five large classes, and all but the smallest (epsilon) contain known hydrocarbon-degrading genera. Most methanotrophic bacteria are in this phylum (Bowman 2006; Knief 2015), which also contains the recently discovered obligate hydrocarbon-degraders (Yakimov et al. 2007), including some strains of *Alcanivorax, Cycloclasticus, Oleiphilus, Oleispira* and *Thalassolituus,* and many organisms able to degrade hydrocarbons under anaerobic conditions. For example, *Alicycliphilus denitrificans* degrades benzene under chlorate-reducing conditions (Weelink et al. 2008), and *Georgfuchsia toluolica* can grow on toluene and ethylbenzene at the expense of reducing nitrate, MnO_2, or ferric ions (Weelink et al. 2009). *Blastochloris sulfoviridis* is a heterotrophic anaerobic phototroph that can degrade toluene (Zengler et al. 1999), although closely related strains cannot. *Rhodoferax ferrireducens* oxidizes propylbenzene under iron-reducing conditions (Eriksson et al. 2005); other members of this genus are phototrophs. *Thauera* is able to degrade toluene under both aerobic and nitrate-reducing anaerobic conditions (Shinoda et al. 2004). A psychrophilic isolate of *Shewanella* is predicted to have a minimal growth temperature of −13 °C (Gentile et al. 2003).

Strains of *Novosphingobium* and *Acidovorax* are able to remove sulfur from dibenzothiophene to yield hydroxybiphenyl (see Mohebali and Ball 2008, although in that review they were identified as *Sphingomonas* and *Pseudomonas*).

Methylibium petroleiphilum, a member of the Betaproteobacteria, is able to degrade the gasoline oxygenate methyl-*t*-butylether (MTBE) as well as aromatic hydrocarbons such as toluene (Nakatsu et al. 2006).

Most known deltaproteobacterial hydrocarbon-degraders are sulfate-reducers and obligate anaerobes. One exception is *Geobacter*, which has catholic tastes and can use oxygen while "waiting" for its preferred anaerobic iron-reducing mode of growth (Lin et al. 2004). Another is *Pelobacter acetylenicus*, which ferments acetylene by disproportionation to acetate and ethanol (Schink 1985).

The epsilon class of Proteobacteria is not known to contain hydrocarbon degraders, but it has recently been recognized as globally ubiquitous in marine and terrestrial ecosystems, and its members likely play a significant role in biogeochemical cycles (Campbell et al. 2006). They are found in substantial numbers in produced waters from biodegraded oil reservoirs (Hubert et al. 2012), and may remove the sulfur from dibenzothiophenes. Recent taxonomic scrutiny suggests that these organisms ought to be assigned to their own phylum – the Epsilonbacteraeota (Waite et al. 2017).

10 Verrucomicrobia

The Verrucomicrobia – the warty microbes - are a relatively recently recognized phylum of Gram-negative organisms that contains some remarkably acidophilic methanotrophs; although preferring slightly less acidic conditions, *Methylacidimicrobium tartarophylax* grew at pH 0.5 (van Teeseling et al. 2014).

11 Candidate Phyla

Taxonomic rules mandate that an organism must be cultivated in order to be formally allotted to a genus and phylum, but modern molecular techniques are revealing that there is an enormous diversity of uncultured microbes on this planet (Hedlund et al. 2014; Hug et al. 2016), and techniques such as stable isotope probing (Kim et al. 2014a; Uhlik et al. 2013) are allowing the taxonomic identification of organisms involved in the biodegradation of labeled substrates such as ^{13}C-benzene and ^{13}C-toluene, even if other aspects of their physiology remain obscure. This approach has identified methane oxidation in Candidate phylum NC10 (Ettwig et al. 2010) and benzene and toluene degradation in Candidate phylum TM7 (Luo et al. 2009; Xie et al. 2011).

12 Archaeal Hydrocarbon Degradation

For many years the contribution of Archaea to hydrocarbon degradation in the environment seemed limited. For example, Röling et al. (2004) found that archaeal species disappeared when oil was applied to mesocosms, even as bacteria thrived.

More recently there has been renewed interest in the Archaeal kingdom, and their enormous diversity has begun to be appreciated (Spang et al. 2017). Several oil-degrading Archaea have now been characterized (Table 2). *Archaeoglobus* is a sulfate-reducing organism, while the Halobacteria, described in detail by Oren (2017), typically live aerobically in several molar salt.

13 Evolution in Real Time?

As mentioned in the introduction, hydrocarbons have always been part of the biosphere, but some molecular structures have only been present in recent years. We have already mentioned the biodegradation of polyethylene (Hadad et al. 2005; Yang et al. 2014) and polystyrene (Mor and Sivan 2008), which have been present in the biosphere for less than 100 years. *Iso*-octane, 2,2,4-trimethylpentane, is another example; it is not found in crude oils, but only in gasolines containing alkylates (Ritter 2005). These were first available in the 1930s. Nevertheless, *iso*-octane is readily aerobically degraded by microbes in apparently pristine waters (Prince et al. 2007), and Solano-Serena et al. (2000) have isolated a *Mycobacterium* able to grow on it as sole carbon and energy source. The organism seems to use a modified alkane monooxygenase to attack the *iso*-octane at the *iso*-propyl end (Solano-Serena et al. 2004).

Table 2 Currently published genera of Archaea able to grow using hydrocarbons as sole or major carbon source

Genus	Typical substrate	Reference
Class Archaeoglobi		
Order Archaeoglobales		
Archaeoglobus Stetter (1988)	Hexadecane	Khelifi et al. (2010)
Class Halobacteria		
Order Halobacteriales		
Haloarcula Torreblanca et al. (1986)	Diesel	Kebbouche-Gana et al. (2009)
Halobacterium Elazari-Volcani (1957)	Octadecane	Al-Mailem et al. (2010)
Halococcus Schoop (1935)	Octadecane	Al-Mailem et al. (2010)
Halorientalis Cui et al. (2011)	Hexadecane	Zhao et al. (2017)
Natronomonas Kamekura et al. (1997)	Crude oil	Selim et al. (2012)
Order Haloferacales		
Haloferax Torreblanca et al. (1986)	Octadecane	Al-Mailem et al. (2010)
Halorubrum McGenity and Grant (1996)	Phenanthrene	Erdoğmuş et al. (2013)
Order Natrialbales		
Halovivax Castillo et al. (2006)	Diesel	Kebbouche-Gana et al. (2009)
Natrialba Kamekura and Dyall-Smith (1996)	Naphthalene	Khemili-Talbi et al. (2015)
Natronococcus Tindall et al. (1984)	Crude oil	Selim et al. (2012)
Natronolimnobius Itoh et al. (2005)	Crude oil	Selim et al. (2012)

Methyl-*t*-butyl ether (MTBE) is another recent addition to the biosphere – it has only been in commerce since the 1980s. For some time it was thought to be very recalcitrant to biodegradation, but it is now recognized that the ability to degrade MTBE is quite widespread (Bradley et al. 2001; Prince 2000), and bioreactors for its destruction have been developed (Maciel et al. 2008). Hristova et al. (2007) have found that important enzymes in the MTBE degradation pathway of *Methylibrium petroleiphilum* are plasmid borne, and suggest that the MTBE monooxygenase is related to alkane monooxygenases, albeit with a broader substrate acceptance. One can imagine that such plasmids would have a significant selective advantage, and be passed to other organisms.

Are these recently acquired activities? No anaerobic degradation has yet been reported, but now that these compounds are in the biosphere there would probably be an advantage to any organism able to use them under anaerobic conditions.

14 Research Needs

It remains a challenge to identify the most active hydrocarbon-degrading microbes in various environments. Approaches such as stable-isotope probing (SIP) provide a link between phylogeny and function and have demonstrated that there are novel hydrocarbon degraders to be found, and some are in currently obscure phyla. Microbial taxonomy proceeds very rapidly, and many strains will be described in the near future. Understanding their taxonomic position will suggest new approaches to discovering novel pathways and indicate areas of the taxonomic landscape that warrant closer attention.

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Aerobic Hydrocarbon-Degrading Archaea

2

Aharon Oren

Contents

Abstract

Enrichment cultures for microorganisms able to degrade aliphatic and aromatic hydrocarbons at high salt concentrations have resulted in the isolation of a number of strains of hydrocarbon-utilizing aerobic extremely halophilic Archaea of the class *Halobacteria*. Such isolates have been obtained from salt marshes and saltern ponds in the south of France, coastal sabkhas in Kuwait, a solar saltern in Turkey, Lake Urmia in Iran, an oil field in Tatarstan, and other hypersaline environments. Phylogenetically these isolates are affiliated with the genera *Halobacterium*, *Haloarcula*, *Halococcus* (order *Halobacteriales*), *Haloferax* and *Halorubrum* (order *Haloferacales*), and *Natrialba* (order *Natrialbales*). They grow at near-neutral pH at salt concentrations from >10% up to saturation. Compounds reported to be used as growth substrates include crude oil, C_{10}-C_{34} *n*-alkanes, pristane, and aromatic hydrocarbons such as benzene, toluene, naphthalene, acenaphthene, phenanthrene, anthracene, and pyrene. Unfortunately none

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of the strains have been well documented taxonomically, biochemically, and genomically, and none of the isolates is currently available from publicly accessible culture collections.

1 Introduction

The aerobic halophilic Archaea of the class *Halobacteria* are not widely known as hydrocarbon degraders. None of the descriptions of the 215 species belonging to 52 genera (numbers of named taxa with standing in the nomenclature as of April 2016) contained any information on the potential of the type of strains to grow on aliphatic or aromatic hydrocarbons. This is undoubtedly due to the fact that such substrates were never tested as growth substrates. But no genes commonly associated with oil degradation have been annotated in the many published genomes of *Halobacteria*, so that the ability of the halophilic Archaea to grow on hydrocarbons must be limited.

Early attempts to demonstrate biodegradation of aliphatic hydrocarbons at the highest salt concentrations that are optimal for growth of the *Halobacteria* confirmed this apparent inability of natural communities of halophilic archaea to degrade oil components. Thus, incubation of water samples of varying salinity (3.3–27.4% salt) collected from the salt evaporation ponds near the south end of Great Salt Lake, Utah, and also from the lake itself, for periods up to two and half weeks in the presence of [1-^{14}C]-hexadecane, failed to yield significant amounts of $^{14}CO_2$ at salinities over 20%, in spite of the fact that oxygen and inorganic nutrients (ammonium, phosphate) were provided. No increase in oxygen uptake was found at salinities above 20% following addition of mineral oil (Ward and Brock 1978).

However, in the early 1990s the first reports were published of isolates of halophilic Archaea growing on aliphatic and aromatic hydrocarbons, even as the sole carbon and energy source (Bertrand et al. 1990; Kulichevskaya et al. 1991). The existence of such strains is not surprising in view of the wide occurrence of hypersaline brines associated with oil fields. But although more strains were isolated later, our information on the physiological, biochemical, and genomic basis of hydrocarbon degradation by members of the *Halobacteria* is still extremely limited (for earlier reviews see Oren et al. 1992; Margesin and Schinner 2001; Oren 2002; Peyton et al. 2004; Patzelt 2005; Le Borgne et al. 2008; McGenity 2010; Andrei et al. 2012; Martins and Peixoto 2012; Fathepure 2014; Edbeib et al. 2016).

2 Diversity of Hydrocarbon-Degrading Extremely Halophilic Archaea

The data presented in Table 1 show that a wide range of aliphatic and aromatic hydrocarbons were reported to be degraded by different representatives of the class *Halobacteria*, orders *Halobacteriales* (genera *Halobacterium*, *Haloarcula*,

Table 1 Aliphatic and aromatic hydrocarbons degraded by halophilic Archaea of the class *Halobacteria*. The table is based in part on data presented by Fathepure (2014) and references cited therein

Hydrocarbon	Chemical structure	Degrading organisms	Salinity (%)	References
Aliphatic hydrocarbons				
n-Alkanes	C_{10}-C_{30}	*Halobacterium* (*Halorubrum*) *distributum* VKM B-1916	29	Kulichevskaya et al. 1991
n-Alkanes	C_{10}-C_{34}	*Haloferax* sp., *Halobacterium* sp., *Halococcus* sp.		Al-Mailem et al. 2010
Tetradecane	$C_{14}H_{30}$	*Haloarcula* EH4	>20	Bertrand et al. 1990
Hexadecane	$C_{16}H_{34}$	*Haloarcula* EH4	>20	Bertrand et al. 1990
Heptadecane	$C_{17}H_{36}$	*Haloarcula* sp., *Haloferax* sp.	>22	Tapilatu et al. 2010
Octadecane	$C_{18}H_{38}$	*Haloferax* sp., *Halobacterium* sp., *Halococcus* sp.	>26	Al-Mailem et al. 2010
Pristane	$C_{19}H_{40}$	*Haloarcula* EH4	>20	Bertrand et al. 1990
Eicosane	$C_{20}H_{42}$	*Haloarcula* EH4, *Haloarcula* sp., *Haloferax* sp.	>20–22	Bertrand et al. 1990; Tapilatu et al. 2010
Heneicosane	$C_{21}H_{44}$	*Haloarcula* EH4	>20	Bertrand et al. 1990
Aromatic hydrocarbons				
Benzene	C_6H_6	*Haloferax* sp., *Halobacterium* sp., *Halococcus* sp.	>26	Al-Mailem et al. 2010
Toluene	C_7H_8	*Haloferax* sp., *Halobacterium* sp., *Halococcus* sp.	>26	Al-Mailem et al. 2010
Naphthalene	$C_{10}H_8$	*Haloferax* spp., *Halobacterium* spp., *Halococcus* sp., *Halorubrum* sp., *Haloarcula* spp., *Natrialba* C21	20 or >26	Al-Mailem et al. 2010; Bonfá et al. 2011; Erdoğmuş et al. 2013; Khemili-Talbi et al. 2015
Acenaphthene	$C_{12}H_{10}$	*Haloarcula* EH4	>20	Bertrand et al. 1990
Biphenyl	$C_{12}H_{10}$	*Haloferax* sp., *Halobacterium* sp., *Halococcus* sp.	>26	Al-Mailem et al. 2010
Anthracene	$C_{14}H_{10}$	*Haloarcula* EH4, *Haloferax* sp.	>20	Bertrand et al. 1990; Bonfá et al. 2011
Phenanthrene	$C_{14}H_{10}$	*Haloarcula* EH4, *Haloferax* spp., *Halobacterium* spp., *Halococcus* sp.,	>20–>26	Bertrand et al. 1990; Al-Mailem et al. 2010; Tapilatu et al. 2010; Bonfá et al. 2011; Erdoğmuş et al. 2013

(*continued*)

Table 1 (continued)

Hydrocarbon	Chemical structure	Degrading organisms	Salinity (%)	References
		Halorubrum spp., *Haloarcula* spp.		
Pyrene	$C_{16}H_{10}$	*Haloferax* spp., *Halobacterium* spp., *Halorubrum* spp., *Haloarcula* spp., *Natrialba* C21	20	Bonfá et al. 2011; Erdoğmuş et al. 2013; Khemili-Talbi et al. 2015

Halococcus), *Haloferacales* (genera *Haloferax*, *Halorubrum*), and *Natrialbales* (genus *Natrialba*). Strains were obtained from a salt marsh and salt production facility in the south of France (Bertrand et al. 1990; Tapilatu et al. 2010), from oil-contaminated saline water in Algeria (Khemili-Talbi et al. 2015), from coastal sabkhas in Kuwait (Al-Mailem et al. 2010, 2012, 2015), from solar saltern ponds in Turkey (Erdoğmuş et al. 2013), from Lake Urmia in Iran (Taran 2011), and from oil fields in Tatarstan and Kazakhstan (Kulichevskaya et al. 1991; Zvyagintseva et al. 1995).

The phylogenetic affiliation of the isolates with the genera *Halobacterium*, *Haloarcula*, *Halococcus*, *Haloferax*, *Halorubrum* was ascertained on the basis of (often partial) sequencing of 16S rRNA genes (Table 2). Unfortunately, none of the isolates has been characterized in-depth.

3 Ecology

Some isolates of hydrocarbon-degrading Archaea have been isolated from hypersaline environments where oil hydrocarbons are abundantly found: brines associated with oil fields in Kazakhstan (Zvyagintseva et al. 1995) and Tatarstan (Kulichevskaya et al. 1991), coastal sabkhas on the shores of the Arabian Gulf (Al-Mailem et al. 2010, 2015), and oil contaminated saline water in Algeria (Khemili-Talbi et al. 2015). But other strains were obtained from high-salt environments without a history of oil contamination such as salt marshes and salt production facilities in the Camargue, France (Bertrand et al. 1990; Tapilatu et al. 2010), and Turkey (Erdoğmuş et al. 2013). Another example is the (not further documented) *Halococcus salifodinae* strain Br3 isolated from solution-mined brine in northern England that could grow with straight-chain and branched alkanes as carbon source (Greedy RS and Grant WD, unpublished work cited by McGenity et al. (2000)).

It is not clear how abundant such hydrocarbon-degrading Archaea are in hypersaline lakes. The early studies by Ward and Brock (1978) of samples of increasing salinity (3.3–28.4% salt) from evaporation ponds near the southern end of Great Salt Lake, Utah, and from different sites from the lake itself did not show significant mineralization of [1-^{14}C]-hexadecane to $^{14}CO_2$ following two and a half weeks of incubation at salt concentrations above 20% which are optimal for most members of

Table 2 16S rRNA gene sequences of isolates of hydrocarbon-degrading members of the *Halobacteria*[a]

GenBank accession number	Length (bp)	Strain designation	Phylogenetic affiliation (% identity)	Reference
FJ868731	1415	MSNC 2	*Haloarcula argentinensis* (99)	Tapilatu et al. 2010
FJ868736	1472	EH4	*Haloarcula marismortui* (99)	Tapilatu et al. 2010
GU550442	365	HA-3	*Halobacterium salinarum* (100)	Al-Mailem et al. 2010
KJ922542	496	M5	*Halobacterium salinarum* (100)	Al-Mailem et al. 2015
JX067388	1463	C37	*Halobacterium salinarum* (*piscisalsi*) (100)	Erdoğmuş et al. 2013
GU550443	367	HA-4	*Halococcus salifodinae* (92)	Al-Mailem et al. 2010
HQ438272	891	BO3	*Haloferax alexandrinus* (99)	Bonfá et al. 2011
HQ438273	897	BO6	*Haloferax alexandrinus* (99)	Bonfá et al. 2011
HQ438278	895	AA31	*Haloferax alexandrinus* (99)	Bonfá et al. 2011
FJ868732	1467	MSNC 4	*Haloferax alexandrinus* (99)	Tapilatu et al. 2010
FJ868734	1468	MSNC 13	*Haloferax alexandrinus* (98)	Tapilatu et al. 2010
FJ868735	1485	MSNC 16	*Haloferax alexandrinus* (99)	Tapilatu et al. 2010
KJ922541	492	M4	*Haloferax elongans* (100%)	Al-Mailem et al. 2015
GU573917	512	HA-2	*Haloferax gibbonsii* (99)	Al-Mailem et al. 2010
GU550441	405	HA-1	*Haloferax lucentense* (96)	Al-Mailem et al. 2010
JX861380	530	HOA1	*Haloferax lucentense* (100)	Al-Mailem et al. 2014
HQ438274	879	BO7	*Haloferax lucentense* (99)	Bonfá et al. 2011
HQ438275	925	PR13	*Haloferax lucentense* (99)	Bonfá et al. 2011
HQ438276	888	MM17	*Haloferax lucentense* (99)	Bonfá et al. 2011
HQ438277	912	MM27	*Haloferax lucentense* (99)	Bonfá et al. 2011
HQ438280	889	AA41	*Haloferax lucentense* (99)	Bonfá et al. 2011
JX067385	771	C24	*Haloferax lucentense* (99)	Erdoğmuş et al. 2013

(*continued*)

Table 2 (continued)

GenBank accession number	Length (bp)	Strain designation	Phylogenetic affiliation (% identity)	Reference
JX067386	1435	C27	*Haloferax lucentense* (97)	Erdoğmuş et al. 2013
JX861381	530	HOA4	*Haloferax mucosum* (100)	Al-Mailem et al. 2014
HQ438279	870	AA35	*Haloferax prahovense* (99)	Bonfá et al. 2011
HQ438281	899	CL47	*Haloferax sulfurifontis* (98)	Bonfá et al. 2011
JX067390	811	C43	*Halorubrum chaoviator* (98)	Erdoğmuş et al. 2013
JX067389	1471	C41	*Halorubrum ezzemoulense* (100)	Erdoğmuş et al. 2013
JX067391	1470	C46	*Halorubrum ezzemoulense* (100)	Erdoğmuş et al. 2013
HG423210	885	C21	*Natrialba aegyptia* (99)	Khemili-Talbi et al. 2015

[a]Sequences JQ350849, JX861384, JX861385, and JX861383, presented as belonging to Archaea by Al-Mailem et al. (2012, 2014), are not archaeal, and are therefore not listed

the *Halobacteria*. Attempts to isolate halophilic hydrocarbon degraders from the oil seeps at Rozel Point in the northern arm of the lake by enrichment with benzene as carbon source yielded mainly of *Gammaproteobacteria* and *Bacteroidetes* (Sei and Fathepure 2009). Archaea affiliated with the genera *Halopenitus*, *Halosarcina*, and others grew in enrichment cultures of Rozel Point sediment with benzoate as carbon source, but their ability to also degrade hydrocarbons was not tested (Dalvi et al. 2016).

Enrichment cultures set up in which hypersaline soil with white salt crusts and red brine samples from a supertidal coastal sabkha area in Kuwait were mixed with 3% crude oil showed enhanced oil removal following fertilization with organic nitrogen and illumination. After 4–6 weeks of incubation at 40 °C, 63–66% of the oil was consumed by the soil and the brine. Samples treated also with 3% casamino acids lost 86–89% of the oil. Under continuous illumination oil degradation by soil-water mixtures was twice as rapid as in the dark. No degradation occurred in autoclaved control experiments (Al-Mailem et al. 2012). Cultivation-dependent and cultivation-independent analyses of hydrocarbonoclastic Archaea were reported for soil and salt pond samples from two Kuwait sabkha areas. Isolates were affiliated with the genera *Haloferax* (*Hfx. lucentense*, *Hfx. mucosum*, *Hfx. sulfurifontis*) and *Halobacterium* (*Hbt. salinarum* and *Hbt. piscisalsi*, reported to be a later synonym of *Hbt. salinarum*), as based on partial (~500 bp) 16S rRNA gene sequences. Cultivation-independent analysis of the Archaea in the samples yielded sequences affiliated with the genera *Halorussus*, *Halomicrobium*, and *Halorientalis*. To what extent representatives of these genera may degrade hydrocarbons is unknown (Al-Mailem et al. 2014).

Development of different groups of Archaea in microcosms from the hypersaline Vermelha Lagoon (Massambaba Environmental Protection Area, Rio de Janeiro, Brazil) was monitored using molecular methods targeting DNA and RNA following contamination with heptadecane, naphthalene, or crude oil. After 4, 12, and 32 days of incubation, sequences affiliated with the *Halobacteria* corresponded to 36%, 14%, and 36% of the sequenced bands from the RNA-based analysis of the heptadecane-, naphthalene- and crude oil-enriched microcosms, respectively. Identical sequences related to the genus *Halorhabdus* were retrieved from heptadecane-and naphthalene-contaminated microcosms by RNA-based analysis (Jurelevicius et al. 2014).

A study of microcosms of near-salt-saturated brines (~31% salt) from the Engrenier pond, a hypersaline lake on the Mediterranean coast of France, incubated at 40 °C under a 12-h photoperiod and enriched with 0.02% light petroleum showed a 24% decrease in the amount of petroleum hydrocarbons after 4 weeks. This decrease could not be attributed to biological activity as a similar decrease was observed in sterilized controls. Addition of 0.2% casamino acids and 0.1% trisodium citrate led to an additional 12.8% decrease in hydrocarbons in the nonsterilized systems. Monitoring the abundant groups of prokaryotes by16S rRNA gene amplification and denaturing gradient gel electrophoresis (DGGE) showed that phylotypes related to *Haloarcula*, *Haloterrigena*, and *Halorhabdus* increased in abundance, as did *Salinibacter* (bacteria). However, no specific difference in the archaeal DGGE fingerprints was observed between the contaminated and uncontaminated microcosms (Corsellis et al. 2016).

4 Cultivation

All isolates discussed above grow well on complex carbon sources (yeast extract, casamino acids) commonly used for the cultivation of members of the *Halobacteria*, without added hydrocarbons. All are aerobic; hydrocarbon degradation in the presence of alternative electron acceptors such as nitrate has not been tested. Salt concentrations above 10–15% and up to saturation are required for the growth of halophilic Archaea of the class *Halobacteria*. Representatives of the genus *Haloferax*, a genus that includes isolates found to degrade hydrocarbons, prefer the lower salt concentration range. Improved degradation at the lower salinities may also be correlated with the limited solubility of oxygen at the highest salt concentrations (Al-Mailem et al. 2010). All strains of hydrocarbon utilizing Archaea grow in the neutral pH range, and the salt composition of standard media used for the cultivation of neutrophilic members of the *Halobacteria* (Oren 2006 and references therein) may be satisfactory for growth of hydrocarbon degraders. For example, the mineral constituents of the medium used by Al-Mailem et al. (2014) were (g/l): NaCl, 240; $MgCl_2 \cdot 6H_2O$, 30; $MgSO_4 \cdot 7H_2O$, 35; and KCl, 7.0. The basal medium used by Bonfá et al. (2011) contained (g/l): NaCl, 200; KCl, 3.75; NH_4Cl, 0.267; K_2HPO_4, 0.174; $MgSO_4$, 37; and $CaCl_2$, 0.5, supplemented with trace elements and vitamins. McGenity and Gramain (2010) provided general information about methods for the cultivation of halophilic hydrocarbon degraders, not specifically directed to the Archaea.

When hydrocarbons are used as the sole organic carbon source in the medium, appropriate sources of inorganic nitrogen (ammonium, nitrate) and phosphate must be supplied. In some cases the addition of complex nitrogenous compounds was found to stimulate hydrocarbon consumption of halophilic archaeal isolates. Thus, following addition of casamino acids (0.2%), some *Haloferax*, *Halobacterium*, and *Halococcus* isolates from Kuwait degraded twice as much crude oil as in the presence of 0.2% NH_4NO_3 as the sole nitrogen source. Illumination (81 μmol quanta m^{-2} s^{-1}) further stimulated degradation, but the mechanism of the effect was not elucidated (Al-Mailem et al. 2012). Further understanding of these results could be helpful to develop strategies to enhance hydrocarbon degradation in high salinity environments. Yeast extract at a concentration of 0.05% was reported to stimulate growth of a number of halophilic archaea, affiliated with different genera, in the presence of naphthalene, anthracene, phenanthrene, pyrene, and benzanthracene (0.3 mM each), especially after longer incubation times (Bonfá et al. 2011). However, it was not reported whether degradation of the aromatic compounds was also improved. Yeast extract at a concentration of 0.04% was also included in the medium used to grow *Haloarcula* sp. IRU1 from Lake Urmia in Iran for the production of poly (3-hydroxyalkanoate) (PHA) from oil, added at a concentration of 2% (Taran 2011).

5 Biochemistry

Very little information is available about the biochemistry of hydrocarbon degradation by halophilic Archaea.

Aldehydes and carboxylic acids as well as long-chain esters were found in cultures of an orange archaeon obtained from a former petroleum produced water pit in Oman and growing at 20% salt in the presence of pentadecane and eicosane. This was interpreted as an indication of a split pathway for hydrocarbon degradation, in which α-oxidation and subterminal routes proceed in parallel (Patzelt 2005). Unfortunately this isolate was not further identified and documented.

When grown in the presence of naphthalene, phenanthrene, and pyrene, strains *Haloferax* C-24 and C-27, *Halobacterium piscisalsi* (*salinarum*) C-37, *Halobacterium salinarum* C-51, *Halorubrum ezzemoulense* C-41 and C-46, and *Halorubrum* C-43, all isolated from a Turkish saltern, showed activity of catechol 1,2-dioxygenase, an enzyme of the *ortho* cleavage pathway. This pathway is also used by *Natrialba* C21 during degradation of naphthalene and pyrene (Khemili-Talbi et al. 2015). Activity of the protocatechuate 3,4-dioxygenase enzyme of the *ortho* cleavage pathway could be detected in cells of *Haloarcula* sp. C-52 grown on the same substrates. This suggests that aromatic hydrocarbons are degraded by the *ortho* cleavage of the β-ketoadipate pathway. Gene fragments of halophilic catechol 1,2-dioxygenase (414 bp) were amplified from all these strains, and fragments (330 bp) of protocatechuate 3,4-dioxygenase genes were obtained from three out of the seven isolates, but unfortunately no sequence information was reported (Erdoğmuş et al. 2013).

Phenanthrene degradation by *Haloferax* strain MSNC 14 from a hypersaline pond in the Camargue, France, was accompanied by the formation of 2,2′-diphenic acid in

the culture medium. This suggests that phenanthrene degradation starts from 9,10-dioxygenation and *ortho*-cleavage to yield phenanthrene *cis*-9,10-dihydrodiol that is further catabolized to 2,2′-diphenic acid via 9,10-dihydroxyphenanthrene (Tapilatu et al. 2010); however, no confirmation was supplied on the level of enzymatic activities or genes.

Some halophilic Archaea that degrade hydrocarbons were reported to produce surfactants that emulsify oil. Oil emulsification was observed in part of the hydrocarbon-degrading strains isolated from oil field brines in Tatarstan. Additional strains were isolated that did not degrade hydrocarbons but caused oil emulsification (Kulichevskaya et al. 1991).

Haloferax sp. MSNC14, a heptadecane, pristane, and phenanthrene degrader isolated from the salterns of Salins de Giraud, Camargue, France (Tapilatu et al. 2010), produces surfactants that increase hydrocarbon bioavailability by emulsification of oil and decrease in surface tension (Djeridi et al. 2013).

Hydrocarbon utilizing isolates affiliated with the genera *Haloferax*, *Halobacterium*, and *Halococcus* from a hypersaline super-tidal sabkha of the coast of Kuwait showed a high level of tolerance to mercury: up to100–200 ppm $HgCl_2$ when grown in complex hydrocarbon-free media, up to 10–30 ppm in mineral medium containing 0.5% crude oil as the sole carbon and energy source. Volatilization of mercury was also observed (Al-Mailem et al. 2011).

6 Genomics

No genomic studies were reported on any of the hydrocarbon degrading isolates of halophilic Archaea mentioned above, and no further relevant information was obtained from metagenomic studies of oil-polluted hypersaline environments. Analyses of the genomes of members of the *Halobacteria* not associated with oil degradation (e.g., Falb et al. 2008) did not yield any indication of a potential for hydrocarbon degradation.

7 Research Needs

Unfortunately none of the many strains mentioned in this chapter appears to be available from publicly accessible culture collections. The *Halobacterium* (*Halorubrum*) *distributum* isolate from the Bondyuzhskoe oil field described to degrade C_{10}-C_{30} *n*-alkanes (Kulichevskaya et al. 1991) was deposited in the All-Russian Collection of Microorganisms as VKM B-1916 (Zvyagintseva et al. 1995), but it is a patent strain and access is restricted. Strain MSNC 16, an *n*-alkane-degrading isolate from a saltern crystallizer pond in the south of France and related to *Haloferax alexandrinus* and *Hfx. volcanii* (Tapilatu et al. 2010), was deposited in the Culture Collection, University of Göteborg, Sweden, in July 2009 as CCUG 58481. However, this strain, as well as a new culture of the same isolate deposited in July 2010 as CCUG 59981, could not be revived after lyophilization, so that no viable cultures are available (Moore ERB, personal communication, July 2016).

The lack of availability of any of the hydrocarbon-degrading strains of halophilic Archaea described in the literature is a major problem that currently prevents any progress of the research on this topic. None of the strains have been well documented taxonomically, biochemically, and genomically. Further studies will therefore depend on the isolation of new strains from natural environments or the release of strains that may still exist in the laboratories where the cultures were originally isolated.

Further research on hydrocarbon degradation by halophilic Archaea is relevant in view of their potential role for bioremediation of hypersaline petroleum produced water and oil-polluted hypersaline environments, especially in hot regions (Tapilatu et al. 2010). Oil degrading Archaea may find additional biotechnological applications, one of them being the production of poly(3-hydroxyalkanoate) biodegradable plastics. A *Haloarcula* isolate from Lake Urmia, Iran, strain IRU1, was reported to grow on crude oil as sole carbon and energy source and to produce PHA up to 41% of its dry weight when grown at 47 °C in the presence of 2% crude oil, 0.04% yeast extract, and 0.016% NaH_2PO_4 as carbon, nitrogen, and phosphorus sources, respectively (Taran 2011).

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Eukaryotic Hydrocarbon Degraders

3

Roger C. Prince

Contents

Abstract

Hydrocarbons have been part of the biosphere for millions of years, and a diverse group of eukaryotes has evolved to degrade them. Most of the cultures "in captivity" are fungi, but there are also examples from several algal phyla, and there are reports that some protozoa can degrade hydrocarbons. To date, all hydrocarbon degradation by eukaryotes seems to be aerobic. Only a few fungi and a single achlorophyllous green alga are known to be able to grow on hydrocarbons as their sole source of carbon and energy, but several are economically important, either in "spoiling" fuels or in biofilters. Many more fungi are able to degrade polycyclic aromatic hydrocarbons at a fast enough rate to be useful in the remediation of contaminated soil, and they may play an important role in the attenuation of the perennial natural production of these pyrogenic pollutants.

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1 Introduction

Hydrocarbons have always been part of earth's biosphere. They are found in interstellar space (Parker and Kaiser 2017), are produced by living organisms (Valentine and Reddy 2015), and are produced by geological processes (Vandenbroucke and Largeau 2007) and during incomplete combustion (Lima et al. 2005; Wang et al. 2014b). Hydrocarbons are relatively stable molecules but can provide a source of carbon and energy to any organism able to activate them. It is this activation that distinguishes hydrocarbon-degrading organisms from their brethren, for once activated by the aerobic insertion of oxygen (or the anaerobic addition of fumarate, Prince and Walters 2016), they enter the standard biochemistry of living organisms and are readily oxidized to carbon dioxide and water.

Although all aerobic organisms seem to have some ability to metabolize hydrocarbons (Nelson 2017), only a small fraction relies on this metabolism to support their growth. Almost 300 prokaryotic genera contain species able to grow with hydrocarbons as sole source of carbon and energy (Prince et al. 2018), but rather fewer eukaryotes are known to carry out this feat – a few fungi and a single achlorophyllous alga. Nevertheless, many fungi may be important contributors to hydrocarbon degradation in the biosphere, particularly of polycyclic aromatic hydrocarbons while consuming other substrates. This chapter discusses their taxonomic diversity. Only an isolated organism can be given a valid taxonomic name, and the limitation that an organism must be available in pure culture must substantially underestimate the diversity of organisms that productively metabolize hydrocarbons, because it is becoming clear that organisms often grow in mutually beneficial consortia (e.g., Boonchan et al. 2000; Cavaliere et al. 2017). Nevertheless some species thrive in pure culture in the laboratory, and these have allowed a detailed understanding of their biochemistry.

2 Fungal Taxonomy

Despite its origins before the Darwin-Wallace identification of natural selection as the driving force for speciation (Darwin and Wallace 1858), Linnaean taxonomy continues to be a useful paradigm for describing organisms, giving each organism a binomial name consisting of a generic and a specific identifier (e.g., Gest 1999). Genera are then grouped in families, families in orders, orders in phyla, and phyla into domains. In theory this classification ought to fit neatly into an evolutionary classification – the taxonomy should reflect phylogeny. Indeed it is fairly effective for multicellular sexual organisms, where species can be defined as organisms that can interbreed, genera can be distinguished on morphological grounds, and there has been little gene transfer between otherwise unrelated organisms.

Fungal, algal, and protist taxonomy is considerably more complicated and is undergoing a revolution as molecular techniques are brought to bear on the problem (Hawksworth et al. 2011; Hibbett et al. 2007; James et al. 2006; Kohlmeyer et al.

2006; Lutzoni et al. 2004; Shenoy et al. 2007; Spatafora et al. 2017). These studies confirm that the fungi are monophyletic, but some earlier classifications of potential phyla are paraphyletic and will need revision. There is enormous diversity in the fungi, with estimates of more than several million species (Hawksworth 2012). They share a life cycle with both sexual and asexual reproduction, and a major problem in fungal taxonomy is that sexual and asexual forms of the same organism have often been assigned to different species (when this is discovered, they are known as anamorphs). This chapter follows traditional use and lists anamorphs as separate entities, although this is now considered anachronistic. Yet more complications arise with the *fungi incertae sedis* (Latin for "of uncertain placement"), where sexual reproduction is as yet uncharacterized – their taxonomic placement is often a "best guess." Despite these complications, however, giving an organism a name is an essential prerequisite to disseminating information about it, and this chapter is offered in the spirit of placing current information into an organized framework.

Most of the described fungi fall into the *Ascomycota* (some 75% of total named species) and *Basidiomycota* (some 25%) – together forming the *Dikarya* – fungi that in some stages of growth have two distinct nuclei, from two parental strains, in the same cell. These are the familiar fungi of everyday life. Other phyla are more obscure, but members of some of them are also participants in the global cycle of hydrocarbon metabolism.

2.1 Currently Described Hydrocarbon-Degrading Fungi

Fungal taxonomy is curated on the web at Index Fungorum (2018), Mycobank (2018), and NCBI (2018). As can be seen in Table 1, some genera were defined by Linnaeus (L.) in the eighteenth century, and many were defined by the end of the nineteenth century, when obviously the taxonomic attributes were all visual. There has not (yet?) been the explosion of new genera seen in the prokaryotes with the advent of ribosomal RNA phylogeny (Cole et al. 2009; Woese 1987), attesting to the observational skills of early mycologists, but we can certainly expect an explosion of new species (Lücking et al. 2014).

2.1.1 Phylum Ascomycota

The *Ascomycota* are commonly known as the sac fungi – they do their meiotic reproduction in an ascus to form ascospores. The phylum contains bakers' and brewers' yeasts (*Saccharomyces* sp. in a genus that contains hydrocarbon degraders; see Table 1) and several important producers of antibiotics, such as *Penicillium* sp., again a genus that contains hydrocarbon degraders. As can be seen in Table 1, most research has focused on polycyclic aromatic hydrocarbons; *Yarrowia* is one example of an ascomycete known to degrade alkanes (Mauersberger et al. 2001) and so is *Amorphotheca resinae* (Seifert et al. 2007), which grows in jet fuel, cosmetics, and wood preserved with creosote or coal tar. This fungus is also known by the anamorph name *Hormoconis resinae* or its antique and erroneous synonym *Cladosporium*

Table 1 Fungal genera reported to degrade hydrocarbons

Genus	Typical substrate	Reference[a]
Phylum Ascomycota		
Subphylum Pezizomycotina		
Class Ascomycetes		
Oidiodendron Robak 1932	Crude oil	Floodgate (1984)
Pseudeurotium Beyma 1937	Toluene	Prenafeta-Boldú et al. (2001)
Class Dothideomycetes		
Alternaria Nees 1816	Fluorene	Garon et al. (2004)
Arthrographis Cochet 1939	Fluoranthene	Giraud et al. (2001)
Aureobasidium Viala and Boyer 1891	Crude oil	Bossert and Bartha (1984)
Bipolaris Shoemaker 1959	Anthracene	Giraud et al. (2001)
Cenococcum Mougeot and Fries 1829	Phenanthrene	Braun-Lüllemann et al. (1999)
Cercosporidium Earle 1901	Fuel oil	Snellman et al. (1988)
Cicinobolus Ehrenberg 1853	Anthracene	Krivobok et al. (1998)
Cladosporium Link 1816	Diesel	Chaîneau et al. (1999)
Cochliobolus Drechsler 1934	Crude oil	Bossert and Bartha (1984)
Coniothyrium Corda 1840	Pyrene	Ravelet et al. (1999)
Curvularia Boedijn 1933	Fluorene	Juckpech et al. (2012)
Cyclothyrium Petrak 1923	Pyrene	Da Silva et al. (2003)
Davidiella Braun et al. 2003	Anthracene	Aranda et al. (2017)
Drechslera Ito 1930	Anthracene	Krivobok et al. (1998)
Embellisia Simmons 1971	Anthracene	Krivobok et al. (1998)
Epicoccum Link 1815	Naphthalene	Mueller et al. (1996)
Helminthosporium Link 1809	Crude oil	Floodgate (1984)
Lasiodiplodia Clendenin 1896	Benzo[*a*]pyrene	Wang et al. (2014a)
Phoma Saccardo 1880	Crude oil	Bossert and Bartha (1984)
Pithomyces Berkeley and Broom 1875	Anthracene	Giraud et al. (2001)
Pullularia Berkhout 1923	Alkanes	Markovetz et al. (1968)
Sphaeropsis Léveillé 1842	Coal tar oil	Potin et al. (2004)
Sporormiella Ellis and Everhart 1892	Anthracene	Krivobok et al. (1998)
Ulocladium Preuss 1851	Anthracene	Giraud et al. (2001)
Westerdykella Stolk 1955	Fluorene	Garon et al. (2004)
Class Eurotiomycetes		
Allescheria Hartig 1899	Alkanes	Markovetz et al. (1968)
Amorphotheca Parbery 1969	Crude oil	Chaillan et al. (2004)
Aspergillus Micheli 1729	Diesel	Chaîneau et al. (1999)
Capronia Saccardo 1883	Heavy fuel oil	Garzoli et al. (2015)
Cladophialophora Borelli 1980	Toluene	Prenafeta-Boldú et al. (2001)
Dichotomomyces Saito ex Scott 1970	Anthracene	Krivobok et al. (1998)

(*continued*)

Table 1 (continued)

Genus	Typical substrate	Reference[a]
Emericella Berkeley 1857	Crude oil	Oudot et al. (1993)
Eupenicillium Ludwig 1892	Crude oil	Oudot et al. (1993)
Exophiala Carmichael 1966	Toluene	Prenafeta-Boldú et al. (2001)
Hormoconis Arx and de Vries 1973	Diesel	Gaylarde et al. (1999)
Keratinomyces Vanbreuseghem 1952	Alkanes	Markovetz et al. (1968)
Microsporon Gruby 1842	Alkanes	Markovetz et al. (1968)
Neosartorya Malloch and Cain 1973	Fluoranthene	Garon et al. (2004)
Paecilomyces Bainier 1907	Biphenyl	Gesell et al. (2001)
Penicillium Link 1809	Pyrene	Da Silva et al. (2003)
Phialophora Medlar 1915	Pyrene	Ravelet et al. (1999)
Rhinocladiella Nannfeldt 1934	Diesel	Gaylarde et al. (1999)
Talaromyces Benjamin 1955	Crude oil	Oudot et al. (1993)
Class Leotiomycetes		
Botrytis Micheli 1729	Crude oil	Bossert and Bartha (1984)
Cadophora Lagerberg et al. 1927	Anthracene	Aranda et al. (2017)
Gliomastix Guéguen 1905	Crude oil	Fedorak et al. (1984)
Hyphozyma de Hoog and Smith 1981	Anthracene	Giraud et al. (2001)
Leptodontium de Hoog 1977	Toluene	Prenafeta-Boldú et al. (2001)
Microascus Zukal 1885	Fuel oil	Snellman et al. (1988)
Monilia Bonorden 1851	Crude oil	Bossert and Bartha (1984)
Ophiostoma Sydow and Sydow, 1919	α-Pinene	Jin et al. (2006)
Phomopsis Bubák, 1905	Phenanthrene	Tian et al. (2007)
Pseudogymnoascus Raillo 1929	Phenanthrene	Batista-García et al. (2017)
Class Pezizomycetes		
Morchella Dillenius 1794	Anthracene	Gramss et al. (1999)
Class Sordariomycetes		
Acremonium Link 1809	Diesel	Chaîneau et al. (1999)
Beauveria Vuillemin 1912	Diesel	Chaîneau et al. (1999)
Botryotrichum Saccardo and Marchal 1885	Fluorene	Garon et al. (2004)
Calcarisporium Preuss 1851	Asphalt	Yanto and Tachibana (2013)
Cephalosporium Corda 1839	Crude oil	Floodgate (1984)
Chaetomium Kunze 1817	Anthracene	Giraud et al. (2001)
Claviceps Tulasne 1853	Naphthalene	Mueller et al. (1996)
Clonostachys Corda 1839	Crude oil	Bossert and Bartha (1984)
Colletotrichum Corda 1832	Anthracene	Krivobok et al. (1998)
Corollospora Wedermann 1922	Heavy fuel oil	Garzoli et al. (2015)
Cryphonectria Saccardo 1905	Anthracene	Krivobok et al. (1998)

(*continued*)

Table 1 (continued)

Genus	Typical substrate	Reference[a]
Cylindrocarpon Wollenweber 1913	Crude oil	Bossert and Bartha (1984)
Doratomyces Corda 1829	Coal tar oil	Potin et al. (2004)
Emericellopsis Beyma 1940	Naphthalene	Cerniglia et al. (1978)
Fusarium Link 1809	Diesel	Chaîneau et al. (1999)
Gibberella Saccardo 1877	Heavy fuel oil	Garzoli et al. (2015)
Gibellulopsis Batista 1959	Heavy fuel oil	Garzoli et al. (2015)
Gliocladium Corda 1840	Pyrene	Ravelet et al. (1999)
Gonytrichum Nees and Nees 1818	Crude oil	Floodgate (1984)
Graphium Corda 1837	Crude oil	Bossert and Bartha (1984)
Haematonectria Rossman et al. 1999	Heavy fuel oil	Garzoli et al. (2015)
Humicola Traaen 1914	Crude oil	Bossert and Bartha (1984)
Hypocrea Fries 1825	Pyrene	Hong et al. 2010
Ilyonectria Chaverri et al. 2011	Heavy fuel oil	Garzoli et al. (2015)
Lophotrichus Benjamin 1949	Fuel oil	Snellman et al. (1988)
Memnoniella Höhnel 1923	Crude oil	Embar et al. (2006)
Myceliophthora Costantin 1892	Anthracene	Giraud et al. (2001)
Neurospora Shear and Dodge 1927	Naphthalene	Cerniglia et al. (1978)
Niesslia Auerswald 1869	Heavy fuel oil	Garzoli et al. (2015)
Nigrospora Zimmermann 1902	Phenanthrene	Kannangara et al. (2016)
Pestalotia De Notaris 1841	Naphthalene	Cerniglia et al. (1978)
Pestalotiopsis Steyaert 1949	Alkanes	Yanto and Tachibana (2013)
Purpureocillium Luangsa-ard et al. 2011	Heavy fuel oil	Garzoli et al. (2015)
Pseudallescheria Negrutskii and Fischer 1944	Crude oil	April et al. (1998)
Scedosporium Saccardo ex Castellani and Chalmers 1919	Alkanes	April et al. (1998)
Scopulariopsis Bainier 1907	Pyrene	Ravelet et al. (1999)
Seimatosporium Corda 1833	Fluorene	Garon et al. (2004)
Sepedonium Link 1809	Alkanes	Markovetz et al. (1968)
Sordaria Cesati and De Notaris 1863	Naphthalene	Cerniglia et al. (1978)
Sporothrix Hektoen and Perkins 1901	Anthracene	Krivobok et al. (1998)
Trichocladium Marz 1871	Phenanthrene	Silva et al. (2009)
Trichoderma Persoon 1794	Diesel	Chaîneau et al. (1999)
Verticillium Nees 1816	Crude oil	Bossert and Bartha (1984)
Ascomycota incertae sedis		
Monocillium Saksena 1955	Pyrene	Ravelet et al. (1999)
Pezizomycotina incertae sedis		
Gilmaniella Morinaga, Minoura, and Udagawa, 1978	Phenanthrene	Kannangara et al. (2016)
Tetracoccosporium Szabó 1905	Fuel oil	Snellman et al. (1988)

(*continued*)

Table 1 (continued)

Genus	Typical substrate	Reference[a]
Subphylum *Saccharomycotina*		
Class Saccharomycetes		
Candida Berkhout 1923	Crude oil	Zinjarde and Pant (2002)
Debaryomyces Lodder and Kreger-van Rij 1984	Biphenyl	Lange et al. (1998)
Geotrichum Link 1809	Crude oil	Bossert and Bartha (1984)
Hansenula Sydow and Sydow 1919	Crude oil	Floodgate (1984)
Issatchenkia Kudryavtsev 1960	Crude oil	Katemai et al. (2008)
Kluyveromyces Van der Walt 1956	Benzo[*a*]pyrene	Engler et al. (2000)
Pichia Hansen 1904	Phenanthrene	Pan et al. (2004)
Saccharomyces Meyen ex Hansen 1838	Naphthalene	Cerniglia et al. (1978)
Saccharomycopsis Schiønning 1903	Crude oil	Floodgate (1984)
Torulopsis Spegazzini 1918	Crude oil	Bossert and Bartha (1984)
Yarrowia Van der Walt and Arx 1981	Alkanes	Mauersberger et al. (2001)
Subphylum Taphrinomycotina		
Class Schizosaccharomycetes		
Schizosaccharomyces Lindner 1893	Kerosene	Amanchukwu et al. (1989)
Phylum Basidiomycota		
Subphylum Agaricomycotina		
Class Agaricomycetes		
Agaricus L. 1753	Pyrene	Gramss et al. (1999)
Agrocybe Fayod 1889	Benzo[*a*]pyrene	Steffen et al. (2002)
Allescheriella Apinis 1963	Crude oil	Floodgate (1984)
Armillaria Staude 1857	Benzo[*a*]pyrene	Hadibarata and Kristanti (2012)
Amanita Persoon, 1794	Benzo[*a*]pyrene	Braun-Lüllemann et al. (1999)
Anthracophyllum Cesati 1879	Pyrene	Acevedo et al. (2011)
Bjerkandera Karsten 1879	Benzo[*a*]pyrene	Kotterman et al. (1998)
Boletus L. 1753	Chrysene	Braun-Lüllemann et al. (1999)
Ceratobasidium Rogers 1935	Phenanthrene	Dai et al. (2010)
Ceriporia Donk 1933	Pyrene	Lee et al. (2014)
Ceriporiopsis Domański 1963	Anthracene	Krivobok et al. (1998)
Cerrena Gray 1821	Asphalt	Yanto and Tachibana (2014)
Coniophora de Candolle 1815	Anthracene	Krivobok et al. (1998)
Coprinellus Karsten 1879	Dibenzothiophene	Aranda et al. (2009)
Coprinus Persoon 1797	Anthracene	Gramss et al. (1999)
Coriolopsis Murrill 1905	Pyrene	Pickard et al. (1999)

(*continued*)

Table 1 (continued)

Genus	Typical substrate	Reference[a]
Crinipellis Patouillard 1889	Pyrene	Lambert et al. (1994)
Daedalea Persoon 1801	Benz[*a*] anthracene	Martens and Zadrazil (1998)
Daedaleopsis Schröter 1888	Anthracene	Field et al. (1992)
Dentipellis Donk 1962	Pyrene	Lee et al. (2014)
Dichomitus Reid 1965	Benz[*a*] anthracene	Martens and Zadrazil (1998)
Ganoderma Karsten 1881	Benz[*a*] anthracene	Martens and Zadrazil (1998)
Gymnopilus Karsten 1879	Lignite tar	Gramss et al. (1999)
Gyrodon Opatowski 1836	Phenanthrene	Braun-Lüllemann et al. (1999)
Hebeloma Kummer 1871	Phenanthrene	Braun-Lüllemann et al. (1999)
Heterobasidion Brefeld 1888	Pyrene	Lee et al. (2014)
Hypholoma Kumm 1871	Lignite tar	Gramss et al. (1999)
Irpex Fries 1828	Phenanthrene	Novotny et al. (2000)
Kuehneromyces Singer and Smith 1946	Phenanthrene	Sack et al. (1997)
Laccaria Berkeley and Broom 1883	Pyrene	Braun-Lüllemann et al. (1999)
Lactarius Persoon 1797	Phenanthrene	Braun-Lüllemann et al. (1999)
Laetiporus Murrill 1904	Phenanthrene	Sack et al. (1997)
Leccinum Gray 1821	Benzo[*a*]pyrene	Braun-Lüllemann et al. (1999)
Lentinus Fries 1825	Phenanthrene	Valentín et al. (2006)
Lenzites Fries 1836	Anthracene	Gramss et al. (1999)
Marasmiellus Murrill 1915	Benzo[*a*]pyrene	Wunch et al. (1997)
Merulius Fries 1821	Pyrene	Lee et al. (2013)
Minimedusa Weresub and LeClair 1971	Fluorene	Garon et al. (2004)
Mycoaciella Eriksson, Hjortstam, and Ryvarden 1978	Phenanthrene	Lee et al. (2015)
Nematoloma Karsten 1879	Pyrene	Hofrichter et al. (1998)
Oospora Wallroth 1833	Alkanes	Markovetz et al. (1968)
Oxyporus Donk 1933[b]	Anthracene	Krivobok et al. (1998)
Panaeolus Quélet 1872	Naphthalene	Cerniglia et al. (1978)
Paxillus Fries 1836	Phenanthrene	Braun-Lüllemann et al. (1999)
Peniophora Cooke 1879	Phenanthrene	Lee et al. (2015)
Phanerochaete Karsten 1889	Benzo[*a*]pyrene	Haemmerli et al. (1986)
Phlebia Fries 1821	Phenanthrene	Valentín et al. (2006)
Phlebiella Karsten 1890	Phenanthrene	Lee et al. (2014)
Phyllotopsis Singer 1936	Pyrene	Lee et al. (2014)

(*continued*)

Table 1 (continued)

Genus	Typical substrate	Reference[a]
Pisolithus Albertini and Schwein 1805	Phenanthrene	Braun-Lüllemann et al. (1999)
Pleurotus Kummer 1871	Pyrene	Pickard et al. (1999)
Polyporus Micheli ex Adanson 1763	Phenanthrene	Valentín et al. (2006)
Pseudochaete Wagner and Fischer 2002	Pyrene	Lee et al. (2014)
Psilocybe Kummer 1871	Pyrene	Da Silva et al. (2003)
Pycnoporus Karsten 1898	Benz[*a*] anthracene	Martens and Zadrazil (1998)
Ramaria Fries ex Bonorden 1851	Anthracene	Field et al. (1992)
Rhizochaete Greslebin, Nakasone, and Rajchenberg 2004	Pyrene	Lee et al. (2014)
Rhizoctonia De Candolle 1805	Anthracene	Cerniglia (1992)
Rigidoporus Murrill 1905	Anthracene	Cambria et al. (2008)
Sporotrichum Link 1809	Crude oil	Bossert and Bartha (1984)
Stereum Hill ex Persoon 1794	Phenanthrene	Valentín et al. (2006)
Stropharia Quélet 1872	Benzo[a]pyrene	Steffen et al. (2002)
Suillus Gray 1821	Benzo[a]pyrene	Braun-Lüllemann et al. (1999)
Trametes Fries 1836	Pyrene	Pickard et al. (1999)
Trichaptum Murrill 1904	Pyrene	Lee et al. (2014)
Class Tremellomycetes		
Cryptococcus Vuillemin 1901	Anthracene	Krivobok et al. (1998)
Trichosporon Behrend 1890	Alkanes	Middelhoven et al. (2000)
Subphylum Pucciniomycotina		
Class Microbotryomycetes		
Rhodosporidium Banno 1967	Dibenzothiophene	Baldi et al. (2003)
Rhodotorula Harrison 1927	Crude oil	Bossert and Bartha (1984)
Sporobolomyces Kluyver and Niel 1924	Crude oil	Bossert and Bartha (1984)
Subphylum Ustilaginomycotina		
Class Ustilaginomycetes		
Pseudozyma Boekhout 1985	*n*-Alkanes	Kitamoto et al. (2001)
Phylum Chytridiomycota		
Metarhizium Sorokin 1879	*n*-Alkanes	Lin et al. (2011)
Phlyctochytrium Schröter 1892	Naphthalene	Cerniglia et al. (1978)
Rhizophlyctis Fischer 1892	Naphthalene	Cerniglia et al. (1978)
Phylum Mucoromycota		
Absidia van Tieghem 1878	Fluorene	Garon et al. (2004)
Choanephora Currey 1873	Naphthalene	Cerniglia et al. (1978)
Circinella Tieghem and Le Monnier 1873	Naphthalene	Cerniglia et al. (1978)
Cokeromyces Shanor 1950	Naphthalene	Cerniglia (1992)

(*continued*)

Table 1 (continued)

Genus	Typical substrate	Reference[a]
Cunninghamella Matruchot 1903	Naphthalene	Cerniglia et al. (1978)
Gilbertella Hesseltine 1960	Biphenyl	Dodge et al. (1979)
Gongronella Ribaldi 1952	Pyrene	Ravelet et al. (2000)
Helicostylum Corda 1842	Naphthalene	Mueller et al. (1996)
Lichtheimia Vuillemin 1903	Crude oil	Moustafa (2016)
Mortierella Coemans 1863	Benzo[*a*]pyrene	Mueller et al. (1996)
Mucor Fresenius 1850	Pyrene	Ravelet et al. (1999)
Phycomyces Kunze 1823	Naphthalene	Cerniglia et al. (1978)
Rhizomucor Lucet and Costantin 1900	Anthracene	Giraud et al. (2001)
Rhizopus Ehrenberg 1821	Naphthalene	Cerniglia et al. (1978)
Syncephalastrum Schröter 1886	Biphenyl	Dodge et al. (1979)
Thamnidium Link 1809	Naphthalene	Cerniglia et al. (1978)
Umbelopsis Amos and Barnett 1966	Benzoquinoline	Sutherland et al. (2005)
Zygorhynchus Vuillemin 1903	Naphthalene	Cerniglia et al. (1978)
Phylum Zoopagomycota		
Basidiobolus Eidam 1846	Naphthalene	Cerniglia et al. (1978)
Conidiobolus Brefeld 1884	Naphthalene	Cerniglia (1992)
Linderina Raper and Fennell 1952	Naphthalene	Cerniglia et al. (1978)
Smittium Poisson 1937	Naphthalene	Cerniglia et al. (1978)

[a]Note that the references are to a "typical" reference not necessarily the first, last, or best
[b]Krivobok et al. (1998) called this *Oxysporus*, which is not known in current taxonomies

resinae [note that bona fide members of the latter genus are currently assigned to a different class!] and is known colloquially as the "creosote fungus," the "kerosene fungus," or the "jet fuel fungus" (Rafin and Veignie 2018) in different industries. It is a major problem if it grows in naval or jet fuel contaminated with small amounts of water, because the mycelium can clog fuel lines and corrode metal parts (Neihof 1988; Swift 1988; Yemashova et al. 2007). Fortunately an immunoassay for its rapid detection is available (Kelley 2002). The taxonomic confusion with this species is reminiscent of that described by Darwin (1862) of the three orchid species, in very distinct genera, that turned out to be the male, female, and hermaphrodite forms of a single species, *Catasetum tridentatum*, which itself has been reclassified as *Catasetum macrocarpum* (Davis 2005).

2.1.2 Phylum Basidiomycota

The *Basidiomycota* include the classic mushrooms and toadstools but also the rusts and smuts and the white rot fungi that can degrade lignin. Most work has examined their ability to degrade polycyclic aromatic hydrocarbons – and the enzymology involved has been well studied. Prenafeta-Boldú et al. (2018) discuss the internal cytochrome P450 oxidation and the extracellular peroxidases and laccases that have been implicated in hydrocarbon biodegradation. Less is known about the enzymology of alkane biodegradation.

Hildén et al. (2008) provide an example of taxonomic reevaluation in their work with *Nematoloma frowardii* b19, which they demonstrate should be moved to the genus *Phlebia*. It is likely that many more changes will be suggested as molecular taxonomy is more broadly applied.

2.1.3 Phylum Chytridiomycota

The chytrids were once characterized as members of the protozoa, but they are now firmly established in the fungi. They are typically aquatic species, and some are parasites on algae, but others are terrestrial, including the potato black scab disease, *Synchytrium endobioticum.*

2.1.4 Phylum Mucoromycota

Members of this phylum are typically associated with plants, although some are parasites of other organisms including immunocompromised humans. The organisms that makes tempeh, *Rhizopus oligosporus* and *R. oryzae*, are members of this phylum.

2.1.5 Phylum Zoopagomycota

This phylum is essentially the animal-associated sister phylum of the *Mucoromycot*a and includes organisms that are parasites on flies, nematodes, and amoebae.

3 Algal Taxonomy

Algae are a large and diverse group of simple, typically photoautotrophic organisms, ranging from unicellular to multicellular forms such as the seaweeds. Their taxonomy is complex; they are both paraphyletic and polyphyletic (Brodie and Lewis 2007), even if their plastids are monophyletic (Rodriguez-Ezpeleta et al. 2005*;* Moreira and López-García 2017). Algal taxonomy is curated at the World Register of Marine Species (2017), Algaebase (2017), and the NCBI Taxonomy Homepage (2018). Molecular taxonomy is revealing many unexpected relationships in the algae, but perhaps the most remarkable is the case of the heterokonts or stramenopiles. Many indeed look like algae, ranging from the giant multicellular kelp to the unicellular diatoms, but the group includes *Phytophthora*, the dreaded potato blight, and was perhaps the origin of the apicoplast of the malaria parasite *Plasmodium* (Waller and McFadden 2005). Several diatoms have been shown to degrade aromatic hydrocarbons, but it is not yet known how widespread this ability is within the group. Gamila and Ibrahim (2004) present evidence that *Scenedesmus obliquus* and *Nitzschia linearis*, isolated from the River Nile, were able to degrade a broad range of *n*-alkanes and polycyclic aromatic hydrocarbons in crude oil, but whether the strains were axenic or not was not documented. Similar results were reported for *S. obliquus* and *Chlorella vulgaris* by El-Sheekh et al. (2013), but again it is not clear that the cultures were axenic, and the biodegradation might have been carried out by bacteria (Prince et al. 2018).

The achlorophyllous green alga *Prototheca* (Walker et al. 1975) degrades both saturated and aromatic hydrocarbons in crude oils, but the other algae that have been shown to degrade hydrocarbons (Table 2) do this is in addition to their photoautotrophic way of life, and its importance in the biosphere is unclear. Intriguingly, there are indications of synergistic interactions with hydrocarbon-degrading bacteria (Luo et al. 2014; Thompson et al. 2017; Warshawsky et al. 2007) that may imply a greater importance than is often imagined.

One red alga and several diatoms have been shown to degrade hydrocarbons (Table 2), although again the environmental significance of this is unclear – it may

Table 2 Algae and their relatives reported to degrade hydrocarbons

Genus	Typical substrate	Reference[a]
***Phylum Chlorophyta* (*green algae*)**		
Ankistrodesmus Corda 1838	Benzo[*a*]pyrene	Warshawsky et al. (1995)
Chlorella Beijerinck 1890	Naphthalene	Todd et al. (2002)
Chlamydomonas Agassiz 1846	Phenanthrene	Liebe and Fock (1992)
Dunaliella Teodoresco 1904	Naphthalene	Mueller et al. (1996)
Prototheca[b] Krüger 1894	Crude oil	Walker et al. (1975)
Scenedesmus Meyen 1829	Benzo[*a*]pyrene	Warshawsky et al. (1995)
Selenastrum Reinsch 1867	Benzo[*a*]pyrene	Warshawsky et al. (1995)
Ulva L 1753	Naphthalene	Cerniglia et al. (1980)
***Phylum Rhodophyta* (*red algae*)**		
Porphyridium Nageli 1849	Naphthalene	Cerniglia et al. (1980)
Phylum Heterokontophyta		
Class *Bacillariophyta* (*diatoms*)		
Achnanthes Bory de Saint-Vincent 1822	Alkanes	Antić et al. (2006)
Amphora Ehrenberg ex Kützing 1844	Naphthalene	Cerniglia et al. (1980)
Cyclotella Kützing 1833	Fluoranthene	Liu et al. (2006)
Cylindrotheca Rabenhorst 1859	Naphthalene	Cerniglia et al. (1980)
Navicula Bory 1822	Naphthalene	Cerniglia et al. (1982)
Nitzschia Hassall 1845	Naphthalene	Cerniglia et al. (1982)
Skeletonema Greville 1865	Phenanthrene	Hong et al. (2008)
Synedra Ehrenberg 1830	Naphthalene	Cerniglia et al. (1982)
Class *Phaeophyceae* (*brown algae*)		
Petalonia Derbès and Solier 1850	Naphthalene	Cerniglia et al. (1980)
Class *Oomycetes* (*water molds*)		
Phytophthora de Bary 1876	Naphthalene	Cerniglia et al. (1978)
Saprolegnia Nees 1823	Naphthalene	Cerniglia et al. (1982)
Class *Labyrinthulomycetes* (*slime nets*)		
Thraustochytrium Sparrow 1936	Naphthalene	Cerniglia et al. (1982)
Class Hyphochytriomycetes		
Hyphochytrium Zopf 1884	Naphthalene	Cerniglia et al. (1982)

[a]Note that the references are to a "typical" reference not necessarily the first, last, or best
[b]*Prototheca* is generally considered an achlorophyllous alga (e.g., Wolff et al. 1994), but Mycobank (2018) considers it a relative of the slime molds

merely reflect a particularly active form of some of the basal metabolism common to all living things (Nelson 2017).

4 Hydrocarbon Biodegradation by Protozoans?

Protozoans are not typically considered to be hydrocarbon-degrading organisms, so their complex taxonomy (Adl et al. 2005) need not concern us here. Nevertheless, a recent paper by Kachieng'a and Momba (2017) reports that a consortium of two ciliates (*Aspidisca* sp. and *Trachelophyllum* sp.) with a euglenoid (*Peranema* sp.) was able to degrade hydrocarbons in water from a petroleum-contaminated aquifer. While it is difficult to assess the analytical procedures used, and the efforts to ensure that the biodegradation was indeed due to the protists rather than commensal bacteria, the paper alerts us to a previously understudied research area.

5 Research Needs

Fungal, algal, and protist taxonomy is at the threshold of a molecular revolution. As this proceeds, a clearer view of an individual strain's taxonomic position will suggest new approaches to discovering novel pathways and indicate areas of the taxonomic landscape that warrant closer attention. There are undoubtedly important opportunities for improving bioremediation protocols in understanding the interactions between these eukaryotes and the prokaryotic hydrocarbon degraders, and this will probably be made easier when their taxonomic positions are clearer.

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Aerobic Hydrocarbon-Degrading *Bacteroidetes*

4

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Abstract

Bacteroidetes (*Bacteroidaeota*) is composed of six classes and is widely distributed in natural environments. The contribution of this phylum to hydrocarbon degradation in the Gulf of Mexico during the Deepwater Horizon oil spill was estimated by DNA stable-isotope probing (SIP) and metagenomic analysis. An approximation across different studies suggests that about 3% of hydrocarbon-degrading bacteria were from the phylum *Bacteroidetes*. The number of isolates from the *Bacteroidetes* that can degrade hydrocarbons has been increasingly reported during the last decade. In this chapter, the characteristics of *Arenibacter algicola*, *Bergeyella* sp. RR7, *Carboxylicivirga flava*, *Chryseobacterium hungaricum*, *Echinicola* sp. SWSAL15, *Mesoflavibacter* sp. ITB11, *Myroides pelagicus*, *Olivibacter oleidegradans*, *Olleya* sp. ITB9, *Parapedobacter pyrenivorans*, *Pedobacter cryoconitis*, *Yeosuana aromativorans*, and an unidentified *Flavobacterium* sp. are described.

1 Introduction

The phylum *Bacteroidetes*, previously known as the *Cytophaga-Flavobacterium-Bacteroides* group, is currently composed of six classes: *Bacteroidia*, *Chitinophagia*, *Cytophagia*, *Flavobacteriia*, *Saprospiria*, and *Sphingobacteriia* (Munoz et al. 2016). Recently, the name *Bacteroidaeota* has been proposed for this phylum by including the rank of phylum in the International Code of Nomenclature of Prokaryotes (Oren et al. 2015), and *Balneolaeota* and *Rhodothermaeota* are reclassified from *Bacteroidetes* as novel phyla based on multilocus sequence analysis or phylogenomic analysis (Hahnke et al. 2016; Munoz et al. 2016). However, the proportion of *Balneolaeota* and *Rhodothermaeota* in natural environments is very low compared with *Bacteroidaeota*; therefore, not considering these two phyla has a negligible effect on understanding the distribution of *Bacteroidetes*. In this chapter, *Bacteroidetes* refers to the members formally associated with the six classes in this phylum (Fig. 1).

Members of *Bacteroidetes* comprise 11–22% of the total bacterial community in tidal mudflat or nearshore sediments (Kim et al. 2004) and around 2–5% in cold-seep sediments (Patra et al. 2016). However, in seawater, 6–30% of the total bacterial community are generally classified as *Flavobacteriaceae* (Eilers et al. 2000), one of the major phylogenetic lineages in the phylum *Bacteroidetes*. In the case of terrestrial environments, approximately half of intestinal microbes, 2–12% of freshwater microbes, and 1.7–17% of soil microbes are *Bacteroidetes* (Thomas et al. 2011). In aquatic environments, members of this phylum are thought to be primarily associated with particles and in having a role in initiating the mineralization of

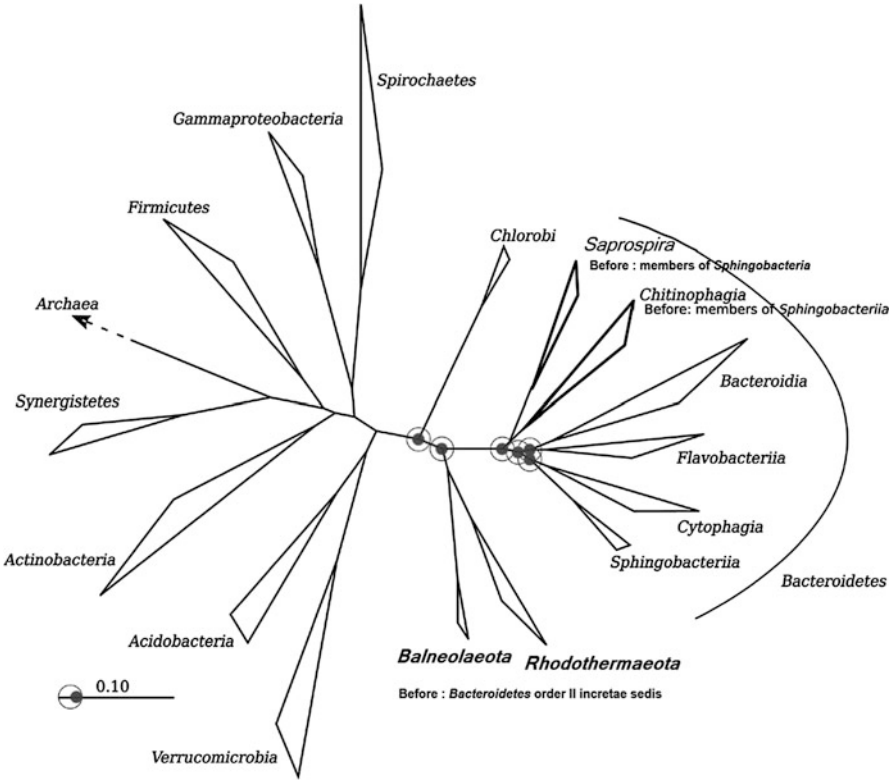

Fig. 1 Consensus tree based on concatenation of the 16S-23S rRNA sequences for *Bacteroidetes* and other bacteria. (Source: Munoz et al. (2016). Reprinted with kind permission from Elsevier)

high-molecular-weight organic matter (Kirchman 2002), a trait that is supported by the presence of several genes encoding hydrolytic enzymes involved in the degradation of high-molecular-weight organic matter (Bauer et al. 2006). Although many *Bacteroidetes* clones and isolates from hydrocarbon-contaminated environments have been reported, only several isolates have been confirmed as hydrocarbon degraders. In this chapter, the distribution of *Bacteroidetes* in hydrocarbon-contaminated environments and the characteristics of 13 reported hydrocarbon-degrading isolates in the phylum *Bacteroidetes*, including eight type species, will be described.

2 Diversity of *Bacteroidetes* in Hydrocarbon-Contaminated Environments

After the "Nakhodka" oil spill, scientists reported a sharp decrease in the number of bacteria in the phylum *Bacteroidetes* and enrichment of hydrocarbon-degrading bacteria such as *Alcanivorax* and *Sphingomonas* (Harayama et al. 2004). Similar

results were observed in mesocosm experiments with soil or seawater/sediment amended with oil (Castle et al. 2006; Saul et al. 2005) and at a sandy beach in Spain where an oil spill from the vessel "Prestige" occurred (Alonso-Gutiérrez et al. 2008). These studies demonstrated that members of the phylum *Bacteroidetes* are sensitive to hydrocarbons. On the other hand, the involvement of *Bacteroidetes* in hydrocarbon degradation in various environments especially the Gulf of Mexico has been reported.

The Deepwater Horizon (DWH) oil spill that occurred in the Gulf of Mexico in April 2010 was a major anthropogenic disaster, which caused significant impacts to marine ecosystems and the economies that depend on these. Many investigations followed to try and understand the contribution that microbes played to the oil biodegradation process during this spill. For example, Liu and Liu (2013) showed that clones of *Flavobacteria* were detected in DWH oil spill-derived coastal oil mousse but not in ambient seawater. Redmond and Valentine (2012) compared the changes in the microbial community structure and abundance in the deepwater oil plume to that in surrounding waters. As expected, the major responders to oil intrusion in marine waters and during the early phase of biodegradation were members of the *Gammaproteobacteria* and *Alphaproteobacteria*. However, over time, members of *Bacteroidetes* (genera *Polaribacter* and *Owenweeksia*) became major taxa phyla in the oil-containing plume water by September 2010; the proportion of *Bacteroidetes* in plume water was 10–35%, and it was much higher than that (1–15%) in surrounding seawater (Redmond and Valentine 2012).

In this same study, the authors also used stable-isotope probing (SIP) to investigate the consumption of methane and other low-molecular-weight volatile hydrocarbons (ethane, propane, benzene) using ^{13}C-labeled counterparts of these substrates and showed that some members of the *Bacteroidetes* incorporated the label, hence confirming the involvement of these organisms in the biodegradation of hydrocarbons during the spill (Redmond and Valentine 2012, Fig. 2).

Three months after capping the DWH wellhead, metagenomic analysis was conducted for the top 1 cm of deep-sea sediments in the surrounding area of the wellhead and the transect to approximately 60 km from the wellhead (Handley et al. 2017). The results were consistent with those reported by Redmond and Valentine (2012) in that the majority of genes involved in hydrocarbon degradation originated from *Gammaproteobacteria*. However, 3% of hydrocarbon-degrading genes in the sediment samples originated from *Bacteroidetes* (Table 1). Furthermore, the presence of an alkane hydroxylase gene (*alkB*) was confirmed for *Dyadobacter* (Koo 2013) isolated from the DWH-contaminated area.

In summary, considering a range of evidence, *Bacteroidetes* were found to have contributed to hydrocarbon degradation, estimated at approximately 3% of total hydrocarbon-degradation activity in the area of DWH oil spill.

In soil biopiles, the proportion of *Bacteroidetes* was also reported to increase in the presence of a high level of hydrocarbon contamination or a high concentration of the heavy fraction ($>C_{28}$–C_{40}, Ramadass et al. 2015; Fig. 3).

Other studies provide evidence of the potential role of *Bacteroidetes* in the degradation of hydrocarbons. For example, Wang et al. (2014) reported the diversity

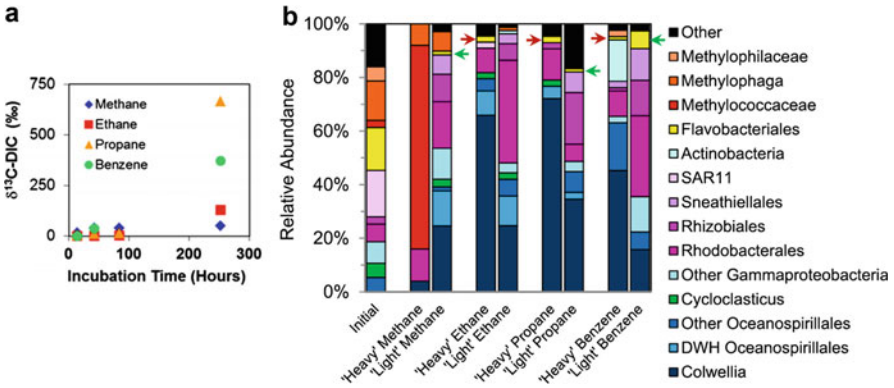

Fig. 2 (**a**) Conversion of ^{13}C ethane, propane, and benzene to ^{13}C-DIC (dissolved inorganic carbon) during DNA-SIP incubations of seawater at 6 °C. (**b**) Relative abundances in 16S rRNA gene clone libraries from heavy (i.e., ^{13}C-labeled) and light (i.e., unlabeled) DNA from SIP incubations with ^{13}C methane, ethane, propane, and benzene and the initial seawater (sample P222) demonstrated that a minor proportion of clones affiliated with *Bacteroidetes* were labeled when incubated with ^{13}C-labeled ethane, ^{13}C-propane, and ^{13}C-benzene. The red and green arrows indicate *Bacteroidetes* in heavy and light DNA, respectively. (Source: Redmond and Valentine (2012). Reprinted with kind permission from Proc Natl Acad Sci)

Table 1 Relative hydrocarbon-degradation (HCD) gene distributions in response to the Deepwater Horizon oil spill. Genome bin and gene identities are given for each row. (Source: Handley et al. (2017). Reprinted with kind permission from Nature Publishing Group)

Group	HCD gene count	Relative abundance (%)	Normalized to CDS (%)[a]
Gammaproteobacteria	180	66	55
Unbinned	67	25	22
Deltaproteobacteria	13	5	11
Bacteroidetes	9	3	7
Alphaproteobacteria	2	1	2
Phage associated	2	1	3
Total	273	100	100

[a]Normalized to total coding DNA sequence (CDS) per group

and relative abundance of oil-degrading bacteria from water samples of the oil spill that occurred in the Dalian coast, China, in July 2010. They isolated 71 strains and revealed that strains affiliated with genera *Algoriphagus*, *Fabibacter*, *Tenacibaculum*, *Roseivirga*, *Winogradskyella*, and *Polaribacter* grew with a mixture of diesel and crude oil. Additional quantitative analysis with *Fabibacter* sp. DLFJ8-5, *Tenacibaculum jejuense* DL3-8, *Winogradskyella* sp. DL5-3, and *Polaribacter dokdonensis* strains DL6-1 and DL8-7 showed high degradation rates in the range of 51–73% after 10 days in the mineral salt medium supplemented with crude oil (Wang et al. 2014).

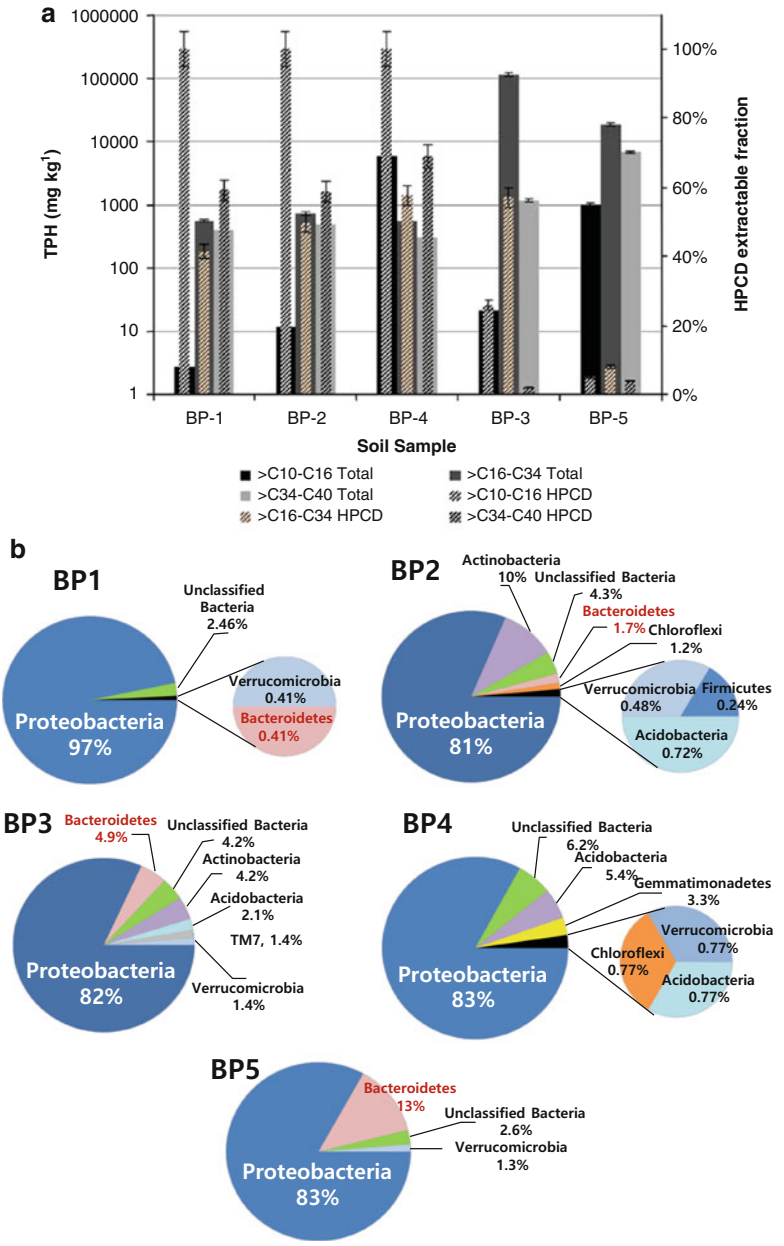

Fig. 3 Comparison of oil contamination characteristics (**a**) and microbial community structure (**b**) in the five biopile method applied soils contaminated with hydrocarbons in the Pilbara region of Western Australia. The relative abundance of *Bacteroidetes* was higher in BP3 (one of the sites with > 100,000 ppm of hydrocarbons) and BP5 (one of the sites with a high proportion of heavy hydrocarbons). (Source: Ramadass et al. (2015). Reprinted with kind permission from Springer)

Several genera of *Bacteroidetes* were identified as dominant taxa by DGGE analysis in lab-based hydrocarbon-enriched experiments, mesocosm studies, and in samples collected from oil-contaminated environments. The identified genera were *Flavobacterium*, *Gaetbulibacter*, *Marivirga*, *Ohtaekwangia*, *Owenweeksia*, *Parasegetibacter*, *Percisivirga*, *Sediminicola*, and *Zunongwangia* (Al-Mailem et al. 2015; Hou et al. 2015; Kappell et al. 2014; Li et al. 2012; Lladó et al. 2012; Sauret et al. 2015; Sherr et al. 2012). *Cloacibacterium* increased in relative abundance in a mesocosm contaminated with naphthalene (Jurelevicius et al. 2013). *Haliscomenobacter* was detected among the bacterial community associated with polyethylene- or polypropylene-plastic debris (Zettler et al. 2013). It should be noted that the identification of these taxa by DGGE does not implicate them in having a direct role in degrading hydrocarbons, but it suggests they may play a role in this respect.

3 Characteristics of Hydrocarbon-Degrading *Bacteroidetes*

Aerobic hydrocarbon-degrading *Bacteroidetes* have been isolated from terrestrial and marine environments contaminated with hydrocarbons. In a previous study, Yetti et al. (2016) showed that the genus *Muricauda* is capable of degrading polycyclic aromatic hydrocarbons (PAH), such as naphthalene, dibenzothiophene, and fluorene. Guo et al. (2008) described that *Chryseobacterium* sp. NCY does not possess a full set of the genes responsible for the process of hydrocarbon biodegradation; however, it degrades carbazole via cooperation with other bacteria. Both culture-dependent and culture-independent methods have revealed the involvement of *Salegentibacter* in phenanthrene degradation in deep-sea environments (Yuan et al. 2015). Thus far, *Bacteroidetes* isolates that are able to utilize hydrocarbon compounds for growth are as follows: *Algoriphagus* sp., *Arenibacter algicola*, *Bergeyella* sp. (formerly identified as *Weeksella* sp.), *Carboxylicivirga flava*, *Chryseobacterium hungaricum*, *Cloacibacterium* sp., *Echinicola* sp., *Fabibacter* sp., *Flavobacterium* sp., *Marivirga* sp., *Mesoflavibacter zeaxanthinifaciens*, *Muricauda* sp., *Myroides pelagius*, *Olleya* sp., *Olivibacter oleidegradans*, *Parapedobacter pyrenivorans*, *Pedobacter cryoconitis*, *Polaribacter* sp., *Roseivirga* sp., *Salegentibacter* sp., *Tenacibaculum* sp., *Winogradskyella* sp., *Yeosuana aromativorans*, and *Zobellia* sp. (Prince et al. 2018 and references in this chapter). Among them, 13 relatively well-characterized isolates in Fig. 4 will be described.

3.1 *Yeosuana aromativorans*

Y. aromativorans GW1–1^T (=KCCM 42019^T = JCM 12862^T) in the family *Flavobacteriaceae* is a Gram-negative, strictly aerobic, non-motile, and rod-shaped bacterium (Kwon et al. 2006). Yellowish-brown colonies are formed on marine agar 2216 (MA) that produce non-diffusible carotenoid pigments; flexirubin-type pigments are absent. The cells, which are 0.7–2.0 μm in length and 0.2–0.3 μm wide,

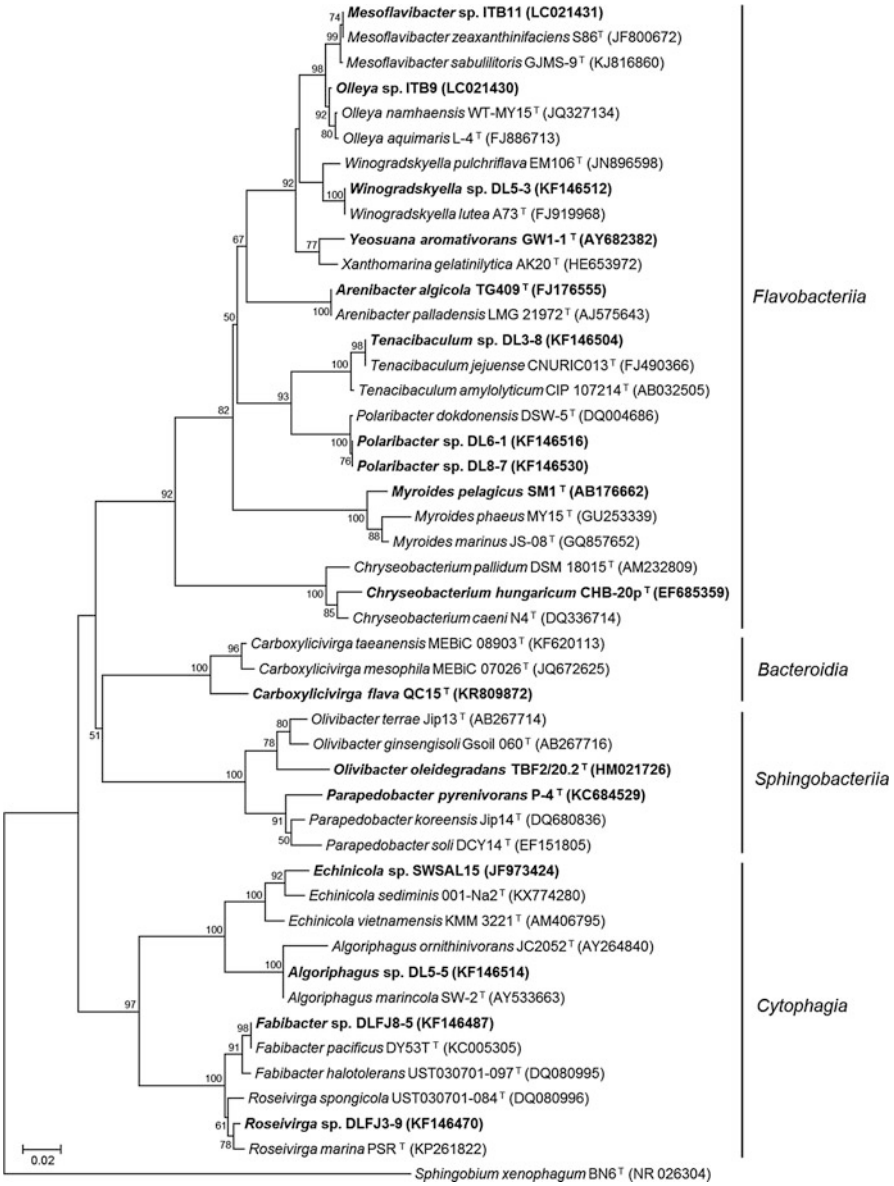

Fig. 4 Phylogeny of hydrocarbonoclastic strains (*bold*) of the phylum *Bacteroidetes* based on 16S rRNA sequences alongside most closely related type strains (*non-bold*). These isolates had been isolated from hydrocarbon-contaminated sites. *Sphingobium xenophagum* BN6^T (NR 026304) was used as the out-group. The numbers at the nodes are bootstrap percentages (only values greater than 50% are shown) based on 1000 replicates. The bar represents 0.02 nucleotide substitutions per site

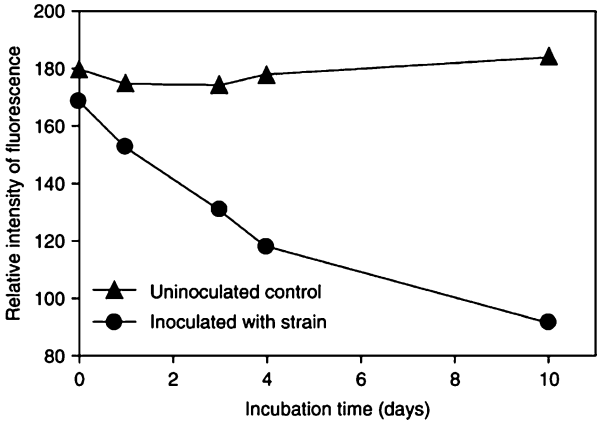

Fig. 5 Degradation rate of benzo[*a*]pyrene B[a]P by *Y. aromativorans* GW1–1^T. The degradation rate was determined by fluorometry (excitation at 293 nm, emission at 406 nm). The relative intensity of fluorescence was measured based on the concentration of remaining B[a]P (0–180 unit = 0–40 ppb B[a]P). *Triangle*, uninoculated control; *circle*, inoculated with strain GW1–1^T. (Source: Kwon et al. (2006). Reprinted with kind permission by the Microbiology Society)

show no gliding motility. Growth occurs at 20–39 °C (optimum temperature 33–36 °C), at pH 5–8 (optimum pH 7), and with 0.5–3.0% (w/v) NaCl (optimum concentration 1%). In addition to NaCl, the cells require either 0.18% (w/v) $CaCl_2$ or 0.59% (w/v) $MgCl_2$ for growth. Oxidase activity is absent, and catalase activity is weak. The GC content of DNA is 51.4 mol%. The major respiratory quinones are MK-5 and MK-6, and the major cellular fatty acids are composed of unsaturated straight and branched chains. The bacterium is capable of degrading PAHs, including pyrene and benzo[*a*]pyrene (B[a]P) (Fig. 5). The strain was isolated from marine sediments collected at Gwangyang Bay of the South Sea, Republic of Korea. Approximately 1 g of the sediment was enriched with 100 ppm each of pyrene and B[a]P in MM2 liquid medium (1 L of aged seawater supplemented with 18 mM $(NH_4)_2SO_4$, 1 μM $FeSO_4 \cdot 7H_2O$, and 100 μL of 1 M KH_2PO_4–Na_2HPO_4) for 2 years at 10–30 °C. Among the various colonies that grew on MA after a 2-year enrichment culture in minimal MM2 liquid medium supplemented with a mixture of B[a]P and pyrene, a tiny, yellowish-brown colony was isolated and named *Y. aromativorans* GW1–1^T.

3.2 *Olivibacter oleidegradans*

O. oleidegradans TBF2/20.2^T (=NCAIM B 02393^T = CCM 7765^T) in the family *Sphingobacteriaceae* is a Gram-negative, obligately aerobic, non-motile, non-sporulating, and rod-shaped bacterium (Szabó et al. 2011). Cells are approximately 0.6 μm in diameter and 1.8–2.0 μm in length (Fig. 6). Growth occurs at

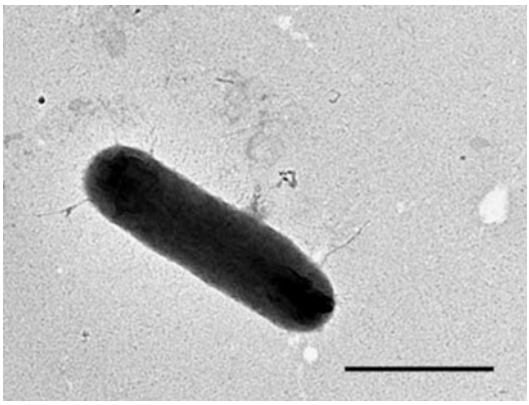

Fig. 6 Transmission electron micrograph of *O. oleidegradans* TBF2/20.2^T cells. The unflagellated rod-shaped cells are about 1.8 μm long and 0.6 μm in diameter. Bar, 1 μm. (Source: Szabó et al. (2011). Reprinted with kind permission by the Microbiology Society)

15–45 °C (optimum temperature 30–37 °C), at pH 6–9 (optimum pH 6.5–7.0), and with >4% (w/v) NaCl. It is oxidase- and catalase-positive. The major fatty acids are iso-$C_{15:0}$ and summed feature 3 (iso-$C_{15:0}$ 2-OH and/or $C_{16:1}\omega7c$, iso-$C_{17:0}$ 3-OH, and $C_{16:0}$). The major respiratory quinone is MK-7, and the polar lipids are phosphatidylethanolamine, two aminophospholipids, three phospholipids, four lipids, and an atypical glycolipid. The GC content of DNA is 41.2 mol%. The strain was isolated from a biofilter cleanup facility set up on a hydrocarbon-contaminated site in Hungary. The degradation experiment was conducted at 25 °C with rotation (120 rpm) for 5 days using pre-diluted and sterilized diesel oil solution inoculated with cells grown in TGY5 broth. More than 70% of the diesel oil was degraded during incubation compared with the control (Szabó et al. 2011).

3.3 *Parapedobacter pyrenivorans*

P. pyrenivorans P-4^T (=NBRC 109113^T = CGMCC 1.12195^T) in the family *Sphingobacteriaceae* is a Gram-negative, non-motile, aerobic, non-sporulating, and rod-shaped bacterium (0.3–0.4 μm in diameter and 0.6–1.3 μm in length) (Zhao et al. 2013). Growth occurs at 15–45 °C (optimum temperature 37 °C), at pH 6–10 (optimum pH 8.5), and with <4% (w/v) NaCl (optimum 0%). It is positive for oxidase, catalase, and flexirubin-type pigment production. The major fatty acids are iso-$C_{15:0}$, iso-$C_{17:0}$ 3-OH, and summed feature 3 ($C_{16:1}\omega7c$ and/or $C_{16:1}\omega6c$). The major respiratory quinone is MK-7, and the major polyamine is homospermidine. The major polar lipids are phosphatidylethanolamine, a sphingolipid, and four unknown glycolipids. The GC content of DNA is 45.4 mol%. The strain was isolated from a PAH-degrading enrichment of polluted soils from a coking chemical plant near Beijing, China. The strain can utilize pyrene, fluoranthene, phenanthrene, and naphthalene as a sole source of carbon and energy for growth (Fig. 7).

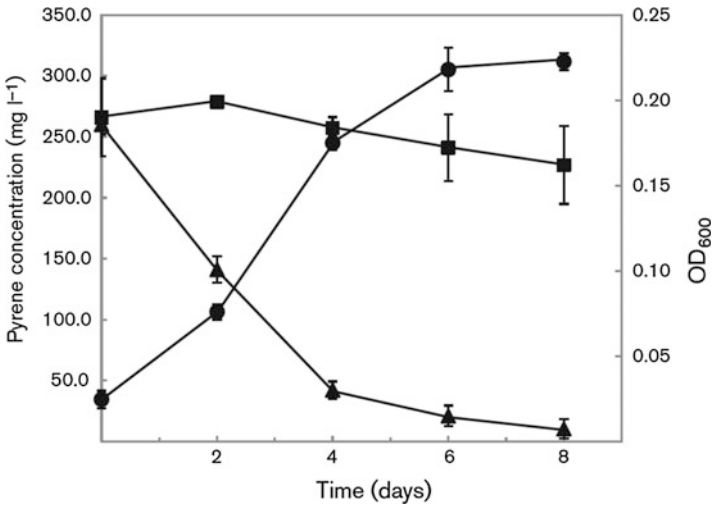

Fig. 7 Growth of *P. pyrenivorans* P-4^T and degradation of pyrene. ■, pyrene concentration in the absence of cells (abiotic control); ▲, pyrene concentration in the presence of cells; ●, OD_{600}. Experiments were conducted using MSM with 200 mg pyrene l^{-1} as sole carbon and energy source. Data shown are the mean of three parallel experiments. Error bars indicate standard deviations. (Source: Zhao et al. (2013). Reprinted with kind permission by the Microbiology Society)

3.4 *Arenibacter algicola*

A. algicola TG409^T (=ACTC BAA-2265^T = DSM 24761^T) in the family *Flavobacteriaceae* is a Gram-negative, aerobic, non-motile but with gliding motility, non-sporulating, and rod-shaped bacterium (Gutierrez et al. 2014). Cells are approximately 0.5 μm in diameter and 2.5–3.0 μm in length, single or in pairs (Fig. 8). Growth occurs at 10–30 °C (optimum temperature 30 °C), at pH 6.5–8.5 (optimum pH 8), and with 0–6% (w/v) NaCl (optimum 0%). It is oxidase-negative and catalase-positive. The major respiratory quinone is MK-6, and the polar lipids are phosphatidylethanolamine, phosphatidic acid, phosphatidylglycerol, and an unidentified phospholipid. The major fatty acids are iso-$C_{15:0}$, iso-$C_{17:0}$ 3OH, iso-$C_{17:1}$, and iso-$C_{15:1}$. The GC content of DNA is 41.9 mol%. The strain was isolated from the marine diatom *Skeletonema costatum* CCAP1077/1C. The cells are capable of mineralizing phenanthrene and naphthalene but not decane, *n*-hexadecane, and anthracene. Furthermore, all six type strains in this genus are capable of degrading phenanthrene and naphthalene, except for the *A. latericius* strain, which is unable to degrade naphthalene (Table 2).

3.5 *Carboxylicivirga flava*

C. flava Q15^T (=CICC 23923^T = KCTC 42707^T) in the family *Marinilabiliaceae* is a Gram-negative, strictly aerobic, rod-shaped bacterium (Wang et al. 2016).

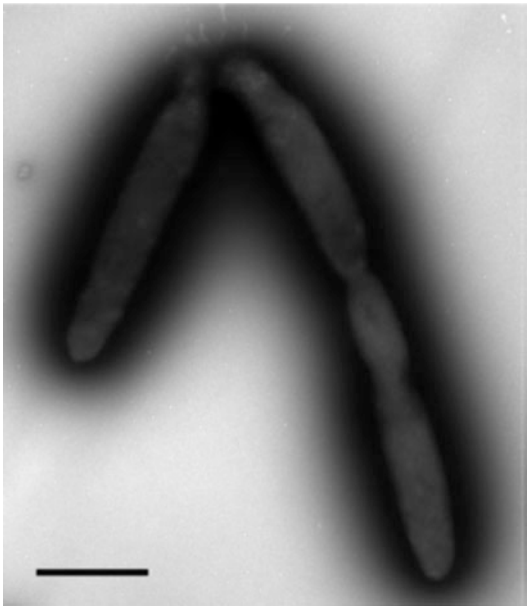

Fig. 8 Transmission electron micrograph of *A. algicola* TG409^T cells. The unflagellated rod-shaped cells are about 2.5 μm long and 0.5 μm in diameter. Bar, 1 μm. (Source: Gutierrez et al. (2014). Reprinted with kind permission by the American Society for Microbiology)

Table 2 Degradation of phenanthrene and naphthalene by strain *A. algicola* TG409^T and closely related type strains when grown in ONR7a medium. All type strains are capable of degrading each compound (except naphthalene by *A. latericius* strain KCTC 12957^T) after 2 weeks. (Source: Gutierrez et al. (2014). Reprinted with kind permission by the American Society for Microbiology)

	Avg % (± SD) of degraded[a]	
Organism	Phenanthrene	Naphthalene
Arenibacter algicola strain TG409^T	3 ± 0.4	10.8 ± 2.9
Arenibacter palladensis strain LMG 21972^T	12.8 ± 0.1	40.1 ± 6.2
Arenibacter echinorum strain KCTC 22013^T	4.2 ± 0.2	19.8 ± 7.3
Arenibacter troitsensis strain KCTC 12362^T	16.1 ± 0.5	17.1 ± 2.9
Arenibacter nanhaiticus strain NH36A^T	10.9 ± 0.6	18.1 ± 5.8
Arenibacter certesii strain KMM 3941^T	9.1 ± 0.1	48.9 ± 9.2
Arenibacter latericius strain KCTC 12957^T	6.1 ± 0.5	0.0 ± 0.0

[a]Values are from triplicate HPLC measurements and expressed as a percentage of compound degraded after 2 weeks relative to uninoculated controls

Colonies grown on MA for 72 h are circular, flat, and orange with a size of 0.5–1.0 mm. Cells are approximately 0.6 μm in diameter and 7.8 μm in length (Fig. 9). Growth occurs at 20–37 °C (optimum temperature 30 °C), at pH 5–10 (optimum pH 7), and with 2–6% (w/v) NaCl (optimum concentration 2%). The major fatty acids are iso-$C_{15:0}$, anteiso-$C_{15:0}$, and iso-$C_{17:0}$ 3-OH, and the major respiratory quinone is MK-7. The polar lipids are phosphatidylethanolamine, an aminolipid, an unidentified phospholipid, and two unknown lipids. The GC content of DNA is 44.7 mol%. The strain was isolated from sediments of the Bohai Sea, north of China.

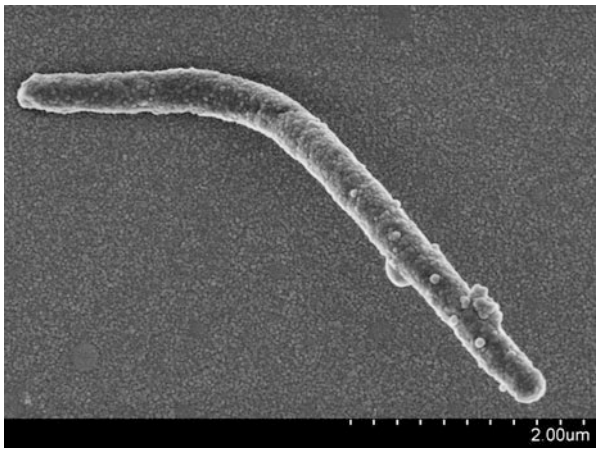

Fig. 9 Scanning electron micrograph of *C. flava* Q15^T cells. The unflagellated rod-shaped cells are about 7.8 μm long and 0.6 μm in diameter. Scale, 2 μm. (Source: Wang et al. (2016). Reprinted with kind permission by the Microbiology Society)

Strain Q15^T was also grown at 30 °C with shaking (180 rpm) using a minimal medium (4 mM $MgSO_4$, 0.5 mM $CaCl_2$, and 85 mM NaCl) supplemented with 400 mg/L (initial concentration) of different hydrocarbons and found to be highly efficient in degrading tetradecane, hexacosane, pristane, and phenanthrene, with a degradation efficiency of 37–58%; however, limited degradation was observed with pyrene with degradation of 16% (Wang et al. 2016, unpublished data).

3.6 *Myroides pelagicus*

M. pelagicus SM1^T (=IAM 15337^T = KCTC 12661^T) in the family *Flavobacteriaceae* is a Gram-negative, aerobic, non-motile, non-sporulating, and short rod-shaped bacterium (Yoon et al. 2006). Colonies grown on LB agar are circular, convex, and yellow-to-orange pigmented with a cell size of 0.2–0.3 μm in diameter and 0.5–1 μm in length. Growth occurs at 10–37 °C, at pH 5–9, and with 0–9% (w/v) NaCl. The major fatty acids are iso-$C_{15:0}$, iso-$C_{17:1}$ $\omega 9c$, and iso-$C_{17:0}$ 3-OH, and the major respiratory quinone is MK-6. The GC content of DNA is 33.6 mol%. The strain was obtained during the isolation of biosurfactant-producing bacteria from seawater, off the coast of Thailand. In a previous study, the strain was shown to produce the crude oil-emulsifying compounds identified as an ornithine lipid in culture supernatant (Maneerat et al. 2006).

3.7 *Pedobacter cryoconitis*

P. cryoconitis A37^T (=DSM 14825^T = LMG 21415^T) in the family *Sphingobacteriaceae* is a Gram-negative, aerobic, non-spore-forming, non-flagellated motile, and rod-shaped bacterium (0.7–0.9 μm in diameter and 0.5–3.0 μm in

length) (Margesin et al. 2003). Growth occurs at 1–25 °C (optimum temperature 20 °C) and at pH 5–8 (optimum pH 7). It is oxidase- and catalase-positive. The major fatty acids are iso-$C_{15:0}$ 2-OH and $C_{16:1}\omega7c$, and the GC content of DNA is 43.4 mol %. The strain was isolated from alpine glacier cryoconite on the Stubaier Glacier in the Tyrolean Alps, Austria. The degradation experiment was conducted to determine the effect of temperature (1–25 °C) and concentration of diesel oil (1–20 g/L) in R2A medium. The degradation extent in medium containing 2 g/L diesel oil was observed to be 53% at 15 °C, 38–40% at 5–10 °C, and 26% at 1 °C after 4 days, and degradation was negligible at both 20 and 25 °C over 8 days (Margesin et al. 2003).

3.8 *Chryseobacterium hungaricum*

C. hungaricum CHB-20p^T (=NCAIM B2269^T = DSM 19684^T) in the family *Flavobacteriaceae* is a Gram-negative, aerobic, non-motile, non-spore-forming, non-flagellated, and rod-shaped bacterium (Szoboszlay et al. 2008). Cells are approximately 0.5 μm in diameter and 0.9 μm in length (Fig. 10). Growth occurs at 5–37 °C (optimum temperature 28–30 °C), at pH 6–10 (optimum pH 6.5–7.5), and with <2% (w/v) NaCl (optimum <1%). It is positive for oxidase, catalase, and flexirubin-type pigment production. The major fatty acids are iso-$C_{15:0}$, iso-$C_{17:0}$ 3-OH, and summed feature 3 ($C_{16:1}\omega7c$ and/or iso-$C_{15:0}$ 2-OH). The major respiratory quinone is MK-6, and the major polar lipids are phosphatidylethanolamine, phosphatidylglycerol, diphosphatidylglycerol, and phosphatidylserine. The GC content of DNA is 37.5 mol%. The strain was isolated from kerosene-contaminated soil in Hungary. The strain can utilize diesel oil as a sole carbon source both in the

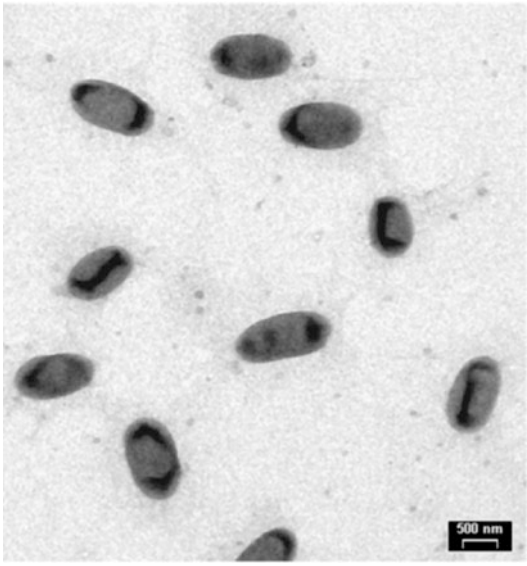

Fig. 10 Transmission electron micrograph of cells. Bar, 0.5 μm. (Source: Szoboszlay et al. (2008). Reprinted with kind permission by the Microbiology Society)

pure compost and the perlite-containing compost media. In addition, the carbon dioxide production by the strain showed a high level of respiration (9.2–11.9 μg CO_2/g/h) in compost or compost-perlite mixture media supplemented with diesel oil (Sebők et al. 2014).

3.9 *Mesoflavibacter* sp. ITB11

Mesoflavibacter sp., a Gram-negative bacterium, was isolated from mud around the Port of Chiba in Tokyo Bay of Japan using ZoBell 2216e medium, and the 16S rRNA sequence was 100% identical to that of *Mesoflavibacter zeaxanthinifaciens* in the phylum *Bacteroidetes* (Okai et al. 2015). Growth experiments revealed that *Mesoflavibacter* sp. ITB11 can utilize B[a]P as the sole carbon and energy source, and most of B[a]P (approximately 86%) was degraded after 42 days. Salicylate was observed to act only as an inducer of B[a]P degradation. Approximately 25% of B[a]P was degraded without salicylate, and the degradation rate was around 40% in the presence of both B[a]P and salicylate (Fig. 11).

3.10 *Olleya* sp. ITB9

Olleya sp. ITB9, a Gram-negative bacterium, was isolated from surface water near a waste-treatment plant in the Tokyo Bay area using R2A + saline medium (Okai et al. 2015). The 16S rRNA gene sequence of the strain was 99% identical to that of *Olleya* sp. in the phylum *Bacteroidetes*. It was found to utilize B[a]P as the sole carbon and energy source, and approximately 79% of B[a]P was degraded after 42 days.

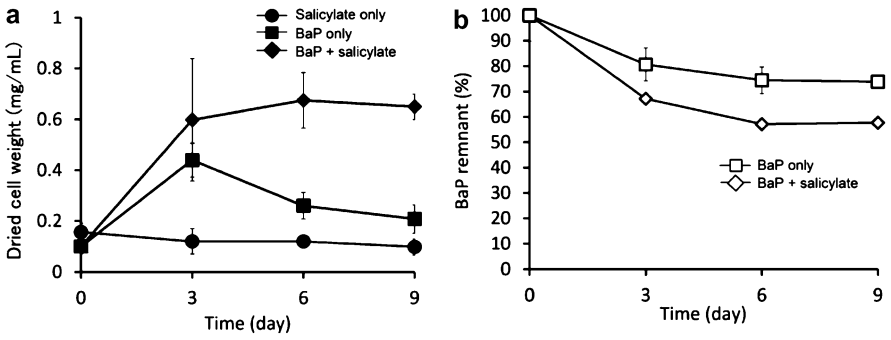

Fig. 11 Growth and degradation of B[a]P and salicylate by *Mesoflavibacter* sp. ITB11. (**a**) Cell dry weights and (**b**) residual concentration of B[a]P. Experiments were conducted using L9 medium supplemented with B[a]P, salicylate, or B[a]P + salicylate as the sole carbon and energy source(s). Error bars indicate standard deviations. (Source: Okai et al. (2015). Reprinted with kind permission by Oxford University Press)

3.11 *Echinicola* sp. SWSAL15

Echinicola sp. SWSAL15, a Gram-negative bacterium, was isolated from hydrocarbon-contaminated coastal water in Salmiya, Kuwait. Based on 16S rRNA gene sequence analysis, strain SWSAL15 was 97.8% identical to that of *Echinicola sedimis* 001-Na2^T in the class *Cytophagia*. Growth experiments revealed that *Echinicola* sp. SWSAL15 can utilize crude oil, C_{18}, and phenanthrene as the sole carbon and energy source. Approximately 23% of crude oil, 18% of C_{18}, and 11% of phenanthrene were degraded after 2 weeks (Al-Awadhi et al. 2012).

3.12 *Bergeyella* sp. RR7

Bergeyella sp. RR7, a Gram-negative and irregular rod-shaped bacterium, was isolated from oil-contaminated soil with enrichments of aromatic and aliphatic hydrocarbons. The 16S rRNA gene sequence of the strain was 95% identical to that of *Bergeyella zoohelcum* (formerly known as *Weeksella zoohelcum*) in the class *Flavobacteriia*. Growth experiments were conducted at 30 °C for 15 days using minimal salts medium supplemented with oil residues such as PAHs, saturated hydrocarbons, and monoaromatic and diaromatic hydrocarbons as the sole carbon and energy source. The strain could degrade long-chain-length alkanes efficiently, but not PAHs (Yuste et al. 2000).

3.13 *Flavobacterium* sp. strain 43

Flavobacterium sp. strain 43 is a Gram-negative, facultative aerobic, multitrichous flagellated, oxidase- and catalase-positive, and rod-shaped bacterium (approximately 0.7 μm in diameter and 1.9 μm in length) (Stucki and Alexander 1987). The strain was isolated from sewage sample of the Ithaca, N.Y., and Marathon, N.Y., sewage treatment plants. The strain can utilize biphenyl and phenanthrene as the sole carbon and energy source for growth. The degradation extent in 162 μM biphenyl- and 84 μM phenanthrene-supplemented medium were 37 and 40%, respectively (Stucki and Alexander 1987).

4 Research Needs

Owing to extensive studies, such as diversity analysis or the identification of novel isolates in the last decade, *Bacteroidetes* have emerged as members of hydrocarbon degrader in natural environments. However, it is still unclear how they respond to hydrocarbons or how they are involved in the process of hydrocarbon degradation. Therefore, it is important to understand the metabolic cooperation, not only among *Bacteroidetes* members but also between different phyla, as demonstrated in the co-metabolism of carbazole by *Chryseobacterium* sp. NCY and *Achromobacter* sp.

NCW. Genomic analysis of isolates and/or metagenomic studies of contaminated environments with isotope probing methods would give us a better understanding of hydrocarbon degradation. Future work could explore combining genomic and physiological approaches to help provide a better understanding on the role of *Bacteroidetes* in degrading hydrocarbons. Direct evidence on tentative hydrocarbon degraders such as *Gaetbulibacter marinus*, *Haliscomenobacter* sp., *Ohtaekwangia* sp., *Owenweeksia hongkongensis*, *Parasegetibacter* sp., *Percisivirga* sp., *Prolixibacter bellariivorans*, *Sediminicola* sp., and *Zunongwangia* sp. also should be obtained.

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Aerobic Hydrocarbon-Degrading *Alphaproteobacteria*: *Rhodobacteraceae* (*Roseobacter*)

5

Alison Buchan, José M. González, and Michelle J. Chua

Contents

Abstract

Members of the *Roseobacter* lineage of bacteria are prevalent in diverse marine environments where they carry out critical biogeochemical processes. Recent reports, based primarily on culture-independent studies and reviewed here, provide compelling evidence that members of this abundant lineage are involved in hydrocarbon degradation in natural systems. Five distinct pathways for the aerobic degradation of aromatic compounds are commonly identified in *Roseobacter* genomes, as are genes encoding alkane hydroxylases and uncharacterized ring-cleaving and ring-hydroxylating dioxygenases. Taken together, these findings suggest roseobacters, a group historically over-

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looked with regard to this physiology, may play important roles in the degradation of hydrocarbons at both naturally occurring and elevated levels in marine environments.

1 Introduction

The *Roseobacter* lineage represents a major marine lineage of biogeochemical importance (reviewed in Buchan et al. 2005; Christie-Oleza and Armengaud 2015; Tang et al. 2016; and Wagner-Döbler and Biebl 2006). This large and phenotypically heterogeneous group of heterotrophic bacteria falls within the family *Rhodobacteraceae* in the order *Rhodobacterales*. With very few exceptions, members of this lineage are exclusively found in marine or hypersaline environments. The *Roseobacter* lineage is well represented across diverse marine habitats with highest abundances typically found in coastal environments or in association with phytoplankton. Yet, members have also been identified as prevalent in marine systems as disparate as hydrothermal vents and polar sea ice. Molecular-based approaches targeting 16S rRNA genes reveal that the *Roseobacter* lineage comprises upward of 20% of coastal and 15% of mixed layer ocean bacterioplankton communities (Buchan et al. 2005). Furthermore, representatives of this group stand out as one of the most readily cultivated of the major marine lineages, making them ideal model organisms for studies aimed at understanding marine bacterial ecology and physiology. Work in this area is facilitated by recent efforts to uncover the genetic repertoire of environmentally relevant marine microbes that have resulted in the production of over 300 complete or draft *Roseobacter* genome sequences (Brinkhoff et al. 2008; Luo and Moran 2014).

2 Evidence for Hydrocarbon Degradation by Roseobacters in Natural Environments

Within the last decade, reports providing evidence for a role of roseobacters in hydrocarbon degradation in marine systems have surfaced. While still relatively small, this body of literature describes compelling data that group members may play pivotal roles in the metabolism of these compounds in diverse natural environments (Table 1 and references within). Most of these studies are rooted in molecular-based analyses of 16S rRNA gene sequences and do not rely on traditional culturing approaches, which may explain, in part, why this group has been overlooked with respect to this physiology until recently. Culture-based approaches report mixed success with pure-culture biodegradation studies. For example, Brito et al. isolated a collection of eight *Roseobacter* strains from mangrove sediments in Brazil by demanding growth on pyrene, naphthalene, or fluoranthrene (Brito et al. 2006). All strains were able to transform these polycyclic aromatic hydrocarbons with varying levels of ability (>10–100%) in pure culture. Since 2010, there have been

Table 1 Representation of roseobacters in bacterial communities exposed to hydrocarbons

Sample source	Treatment	Approach[a]	Roseobacter representation	Reference
North Sea, surface seawaters	Enrichment with crude oil	Clone library	>85%	Brakstad and Lødeng 2005
Gulf of Alaska, crude oil-contaminated surface sediments	Enrichment with crude oil	Clone library	20%	Chang et al. 2000
Thames Estuary, UK, surface seawater	Enrichment with crude oil	Clone library	15%	Coulon et al. 2007
Heita Bay, Japan, surface seawater	Enrichment with hexadecane	DGGE	n.d.[b]	Hara et al. 2003
Etang de Berre, France, crude oil-contaminated microbial mats	None	Clone library	15%	Hernandez-Raquet et al. 2006
Etang de Berre, France, crude oil-contaminated microbial mats	Enrichment with benzothiopene	DGGE	n.d.[b]	Hernandez-Raquet et al. 2006
Thames Estuary, UK, surface seawater	Enrichment with decane	Clone library	15%	McKew et al. 2007
Thames Estuary, UK, surface seawater	Enrichment with hexadecane	Clone library	21%	McKew et al. 2007
Thames Estuary, UK, surface seawater	Enrichment with alkane mix	Clone library	23%	McKew et al. 2007
Thames Estuary, UK, surface seawater	Enrichment with crude oil	Clone library	3%	McKew et al. 2007
Sub-Antarctic, surface seawaters	Enrichment with crude oil	Clone library	45%	Prabagaran et al. 2007
Guanabara Bay, Brazil, surface sediments	Selection on pyrene, naphthalene, and fluoranthrene	Isolation	13%	Brito et al. 2006
Hiroshima Bay, Japan fuel oil-contaminated tidal flat sediments	None	Isolation	50%	Katayama et al. 2003
South China Sea, oil field sediments	None	Isolation	n.d.	Ying et al. 2007

n.d. not determined

[a]Clone library sequence analysis of PCR-amplified 16S rRNA gene sequences from total bacterial community, *DGGE* denaturing gradient gene electrophoresis analysis of PCR-amplified 16S rRNA gene sequences from total bacterial community, isolation cultivation of representative bacteria

[b]Relative representation in 16S rRNA gene amplicon pool not determined but listed as a "major" band apparent in the DGGE profile that was identified by sequence analysis

numerous studies in which roseobacters have been found to be highly represented in hydrocarbon-contaminated environments (Cappello et al. 2012; Chronopoulou et al. 2015; Jimenez et al. 2011; Kimes et al. 2013; Lamendella et al. 2014; Mahjoubi et al. 2013; Mishamandani et al. 2016; Sanni et al. 2015; Todorova et al. 2014;

Viggor et al. 2013; Wang et al. 2016; Yang et al. 2016; Zhou et al. 2009). In addition, isolates have been cultivated from hydrocarbon-contaminated sites. For example, *Tritonibacter horizontis* was isolated from oil-polluted Deepwater Horizon seawater and found to degrade pure hydroxylated aromatic compounds (Giebel 2018; Giebel et al. 2016). Conversely, five *Roseobacter* strains isolated from fuel oil-contaminated tidal bay sediments were unable to grow on pure alkanes (heptane, octane, decane, undecane, dodecane, or hexadecane) or toluene under the conditions tested (Katayama et al. 2003). Caveats attributed to culture-based approaches are particularly relevant to these types of studies, as the appropriate culture conditions for biodegradation in the lab may not adequately reflect favorable conditions found in situ (e.g., concentrations of compounds, whether compounds are provided singly or in mixtures, whether organisms act alone or in concert) (Van Hamme et al. 2003).

The vast majority of studies referenced in Table 1 demonstrate that *Roseobacter* populations respond favorably to hydrocarbon amendment, though general trends concerning preferred substrates or environmental conditions are not yet evident. Brakstad et al. found the relative contribution of *Roseobacter* sequences in bacterial 16S rRNA gene amplicon pools increased from 21% to 89% following exposure to crude oil for 21 days in a near-shore water sample and from <1% to 86% in an offshore water sample collected adjacent to an oil platform (Brakstad and Lødeng 2005). Conversely, decreases in relative abundance of *Roseobacter* have also been reported. For example, a study of the natural bacterial assemblages in sub-Antarctic waters off the coast of Argentina demonstrated that roseobacters comprised nearly 60% of a 16S rRNA gene clone library prior to exposure, yet after 5 days of enrichment of this community with crude oil, their numbers fell to 45% (Prabagaran et al. 2007). Additional studies suggest some *Roseobacter* populations may be stimulated by alkanes but inhibited by other components of crude oil such as polycyclic aromatic hydrocarbons (e.g., Hara et al. 2003; McKew et al. 2007). However, in the absence of quantitative estimates, the precise nature and extent of the *Roseobacter* response remain open. Furthermore, environmental parameters, including nutrient availability, reduction potential, and temperature, are likely to be important drivers in community development overall (e.g., Grayston et al. 2001; Norris et al. 2002). As more comprehensive studies of the total bacterial community, as well as *Roseobacter*-specific, responses to defined substrates are carried out, a clearer picture should emerge.

3 General Features of *Roseobacter* Genomes

Over 300 *Roseobacter* genomes have been sequenced providing a wealth of sequence data to evaluate. Current data indicates that *Roseobacter* genomes range in size from ~2.5 to 5.0 Mbp (Luo et al. 2014, Luo and Moran 2014). The numbers of predicted genes from approximately 20 currently closed genomes range from 3,012 (*Ketogulonicigenium vulgare* SPU B805) to 5,149 (*Octadecabacter arcticus* 238, DSM 13978) (Moran et al. 2007; Swingley et al. 2007; Vollmers et al. 2013). Plasmids appear prevalent in roseobacters, several of which possess genetic

signatures that suggest they may be conjugatively transferred (e.g., Swingley et al. 2007). Strains often possess multiple plasmids of various sizes (<5 to >800 kb) that may be of different conformations (circular or linear) (reviewed in Petersen et al. 2018). Plasmids can house up to 20% of the genome content of individual strains (Brinkhoff et al. 2008; Pradella et al. 2004; Tang et al. 2016). Furthermore, a little understood genetic element, referred to as the gene transfer agent (GTA), has been found in all but one of the sequenced genomes to date and may be another avenue for genetic exchange (Biers et al. 2008; Luo and Moran 2014). While an analysis of the collective genetic complement of roseobacters reveals numerous pathways for biogeochemically relevant metabolisms, including carbon monoxide oxidation, sulfur oxidation, dimethylsulfoniopropionate demethylation, aromatic compound degradation, denitrification, and phosphonate utilization, only a subset of these pathways is present in any single genome. In fact, there is little evidence for a set of unique or predictable genes that would define this group of organisms. This may reflect an adaptation of individuals to the diverse environmental niches roseobacters are known to inhabit (Moran et al. 2007).

4 Prevalence of Pathways Associated with Hydrocarbon Degradation in Sequenced Representatives

As mentioned above, there is a paucity of information in the literature on the ability of *Roseobacter* isolates to utilize hydrocarbons in the laboratory. However, insight into the potential of group members to metabolize specific classes of compounds may be gleaned from genome sequences. To that end, evidence for pathways that may facilitate hydrocarbon degradation in 24 *Roseobacter* strains were identified by homology searches and are presented in Table 2. More updated gene inventories have been presented elsewhere (e.g., Newton et al. 2010; Alejandro-Marin et al. 2014; Nie et al. 2014). These strains represent a wide phylogenetic and phenotypic diversity and were isolated from diverse environments (Brinkhoff et al. 2008). The most prevalent pathways and/or genes identified in these genomes are reviewed below. DMS/DMSP metabolisms are discussed in a separate section (González et al. 2010).

5 Ring-Modifying Pathways

Aerobic bacterial degradation of aromatic hydrocarbons typically proceeds through a limited number of pathways that involve incorporation of molecular oxygen via ring-hydroxylating dioxygenases and/or ring-cleaving dioxygenases (Pérez-Pantoja et al. 2016). Until recently, the paradigm for aerobic aromatic compound degradation involved conversion of a wide array of chemical structures to one of a limited number of di- or trihydroxylated intermediates (e.g., protocatechuate, catechol, gentisate, homoprotocatechuate, homogentisate, hydroxyhydroquinone), followed by oxygen-dependent intra- or extradiol cleavage (Harwood and Parales 1996).

Table 2 Prevalence of catabolic pathways for degradation of hydrocarbon and related compounds in 24 representative *Roseobacter* genome sequences

	Pathways identified[a, e]						
Strain[c]	*pca*	*gdo*	*box*	*hgd*	*paa*	p*AH1*	RHD, misc.
Dinoroseobacter shibae DFL12^T*	+				+[d]	+	+
Jannaschia sp. CCS1*	+	+	+	+	+	+(2)	+
Loktanella vestfoldensis SKA53	+					+	
Maritimibacter alkaliphilus HTCC2654^T	+[b]	+	+	+	+	+(2)	+
Oceanicola batsensis HTCC2597	+					+(2)	+(4)
Oceanicola granulosus HTCC2516^T						+(2)	+(2)
Pelagibaca bermudensis HTCC2601^T	+					+(2)	+(2)
Phaeobacter gallaeciensis BS107^T	+			+	+	+	
Phaeobacter gallaeciensis 2.10	+			+	+		+
Rhodobacterales bacterium HTCC2150	+			+	+	+(2)	+(2)
Rhodobacterales bacterium HTCC2255							
*Roseobacter denitrificans** OCh 114^T	+						
Roseobacter sp. Azwk-3b						+	
Roseobacter sp. CCS2	+					+(2)	+
Roseobacter sp. MED193	+			+	+	+	+(2)
Roseobacter sp. SK209-2-6	+			+	+	+(3)	+(3)
Roseovarius nubinhibens ISM^T	+					+(2)	+
Roseovarius sp. 217	+					+(2)	+(3)
Roseovarius sp. HTCC2601	+					+(2)	+(2)
Roseovarius sp. TM1035						+(2)	+(3)
Ruegeria sp. TM1040*	+			+[d]	+	+	+[d]
Sagittula stellata E-37^T	+	+	+	+	+	+(3)	+(7)
Ruegeria pomeroyi DSS-3^T	+[d]	+(2)[d]	+	+	+	+(2)	+(4)[d]
Sulfitobacter sp. EE-36	+				+	+(2)	
Sulfitobacter sp. NAS-14.1	+				+	+(2)	
Frequency	0.79	0.17	0.17	0.42	0.54	0.88	0.75

[a]Pathway/gene abbreviations are as follows: *pca ortho* cleavage of protocatechuate (exception, [b]*meta*-cleavage of protocatechuate), *gdo ortho*-cleavage of gentisate, *box* benzoyl-CoA pathway, *hgd meta*-cleavage of homoprotocatechuate, *paa* phenylacetyl-CoA pathway, p*AH1* alkane mono-oxygenase, *RHD* ring-hydroxylating dioxygenases, misc. uncharacterized ring-cleaving dioxygenases. Positive signs indicate the presence of ORFs with significant similarity (tBLASTx scores > 30) to complete sets of characterized genes for pathways (*pca*, *gdo*, *box*, *hgd*, *paa*) or individual genes (p*AH1*, RHD, misc.) per references provided in the text. Prevalence of multiple pathways/genes is provided in parenthesis; ORFs putatively encoding subunits of a single RHD are only counted once. Empty cells indicate lack of genomic evidence for a pathway

[c]Refer to Brinkhoff et al. 2008 for information on strain origin. Completed genomes are indicated with an *

[d]Denotes plasmid-encoded genes. Two of the four reports for *R. pomeroyi* DSS3^T in the RHD, misc. category are plasmid-borne

[e]Prevalence of ring-cleaving pathways for *Jannaschia* sp. CCS1, *R. pomeroyi* DSS3^T, and *Ruegeria* sp. TM1040 were previously reported in Moran et al. 2007

[T]Type strain for a genus

However, more recent reports have revealed that CoA activation of the benzene ring followed by non-oxygenolytic ring fission may also be common among aerobic bacteria, including roseobacters (Moran et al. 2007; Newton et al. 2010; Zaar et al. 2004). Regardless of the mechanism of ring cleavage, intermediates from these pathways ultimately feed into the TCA cycle; thus, these compounds typically serve as primary growth substrates for the organisms that utilize them.

Oxygenolytic Ring-Cleaving Pathways: Protocatechuate, Gentisate, and Homoprotocatechuate. The protocatechuate branch of the β-ketoadipate pathway, one of the most broadly distributed pathways for the degradation of aromatic compounds in soil microbes (Harwood and Parales 1996), is prevalent in *Roseobacter* genomes; 80% of genomes analyzed possess this pathway (Table 2). With the exception of genes that may coevolve because they encode subunits of a single enzyme (e.g., *pcaHG* and *pcaIJ*) and in strains demonstrating species-level identity (i.e., >99% 16S rRNA gene similarity), there is little gene synteny among the genomes, and functionally related genes are distributed across multiple loci (Alejandro-Marin et al. 2014; Buchan et al. 2004). A distinguishing feature of this pathway in roseobacters is the presence of a highly conserved open reading frame (ORF; belonging to the PF05853 superfamily found immediately adjacent to the *pcaHG* genes that encode for the *ortho*-cleaving protocatechuate dioxygenase (Alejandro-Marin et al. 2014). Reverse transcription-PCR analysis of RNA from one isolate, *Ruegeria pomeroyi* DSS-3, provides evidence that this ORF is co-expressed with upstream *pca* genes (Buchan et al. 2004). The absence of this ORF in similar bacterial *pca* gene clusters from diverse microbes suggests a niche-specific role for its protein product in *Roseobacter* group members. This enzyme family was recently designated as β-keto acid cleavage enzymes (Bastard et al. 2014), though the specific role in protocatechuate degradation in roseobacters remains unknown.

Three additional oxygenolytic ring-cleaving pathways are found in *Roseobacter* genomes. Genes encoding proteins of the *ortho*-cleavage pathway for gentisate (Adams et al. 2006) are found in four (17%) of the genomes. Interestingly, *Ruegeria pomeroyi* DSS3^T appears to possess two complete copies of this pathway: one plasmid encoded and the other located on the chromosome (Moran et al. 2004). Characterization of the plasmid-encoded gentisate 1,2-dioxygenase from *R. pomeroyi* DSS3^T revealed the active protein exists in an unusual homotrimeric conformation (Liu et al. 2007). The *meta*-cleavage pathway of homoprotocatechuate (Roper et al. 1993) is found in ten (42%) of the genomes analyzed. One single genome sequence, from strain HTCC2654, revealed the presence of genes for the *meta*-cleavage pathway of protocatechuate (Noda et al. 1990) (Table 2). Genes encoding enzymes for either the *ortho*- or *meta*-cleavage of the widespread intermediate catechol are absent from the *Roseobacter* genomes analyzed.

Non-oxygenolytic Ring Fission Pathways: Benzoate and Phenylacetate. Two ring-cleaving pathways that involve CoA thioesterification of the aromatic ring are found in *Roseobacter* genomes. The recently elucidated benzoyl-CoA pathway (Zaar et al. 2004) is found in four (17%) of the genomes, while the

phenylacetic acid pathway (Ferrández et al. 1998) is found in just over half (54%) of the genomes studied (Table 2).

Ring-Hydroxylating and Miscellaneous Ring-Cleaving Dioxygenases. Ring-hydroxylating dioxygenases are responsible for preparing a variety of structurally diverse aromatic compounds for ring cleavage (Butler and Mason 1997). While none of the well-characterized ring-hydroxylating dioxygenases (RHD) (e.g., those involved in the degradation of naphthalene and phenanthrene) were identified in the 24 *Roseobacter* genomes, several uncharacterized genes with signature sequences characteristic of RHD were (e.g., PF00848, PF00866). Seventeen (71%) of the *Roseobacter* genomes possess putative ring-hydroxylating dioxygenases. Furthermore, additional putative ring-cleaving dioxygenases (e.g., PF02900, PF04444, PF00903) were also present in the genomes and may indicate the presence of novel or poorly studied pathways for aromatic compound degradation. For example, PAH-degrading *Celeribacter indicus* P73 possesses genes that encode for an aromatic-ring-hydroxylating dioxygenase, the initial enzyme involved in PAH degradation (Cao et al. 2015).

6 Alkane Hydroxylases

Putative medium-chain-length integral membrane alkane hydroxylase genes (similar to *pAH1*; van Beilen and Funhoff 2005) are found in many *Roseobacter* genomes (Table 2). With the exception of *Sagittula stellata* E-37, *Loktanella vestfoldensis* SKA53, *Roseobacter* sp. MED193, *Pelagibaca bermudensis* HTCC2601, and *Roseovarius* sp. TM1035, these 21 genomes appear to lack genes for either rubredoxin or rubredoxin reductase, both of which are required electron transfer proteins in characterized alkane hydroxlyase systems from *Gammmaproteobacteria* and Gram-positive bacteria (reviewed in van Beilen and Funhoff 2007). Presumably, alternative electron transfer partners would need to be identified in roseobacters. In addition, putative cytochrome P450 CY153 family members have been identified in marine metagenomes and appear to map with *Rhodobacteraceae* homologs in phylogenetic trees (Nie et al. 2014).

Roseobacters harbor a wealth of catabolic pathways for the aerobic degradation of phenolic compounds, a feature that is more reminiscent of soil-borne (e.g., *Pseudomonas* spp., *Rhodococcus* spp.) rather than marine microbes. The diversity and prevalence of ring-cleaving pathways are impressive in the group as a whole and in several strains, in particular. *Sagittula stellata* E-37, *Jannaschia* sp. CCS1, *Ruegeria pomeroyi* DSS-3, and *Maritimibacter alkaliphilus* HTCC2654 possess pathways for the degradation of protocatechuate, gentisate, homoprotocatechuate, benzoate, and phenylacetate. Furthermore, genes showing homology to alkane hydroxylases and ring-hydroxylating and/or additional ring-cleaving dioxygenases are present in these four strains isolated from geographically and physiochemically diverse environments (Brinkhoff et al. 2008), suggesting these may be exceptionally appropriate for future study.

7 Summary and Research Needs

Several lines of compelling evidence indicate a role for roseobacters in the aerobic degradation of hydrocarbons. Culture-independent studies suggest roseobacters in diverse marine systems can be stimulated by enrichment with a range of hydrocarbon-based compounds. The extent of the response and the specific group members or subgroups within the lineage that respond likely depend upon many factors, including composition of the original community, the type and concentration of compound(s) added, and physiochemical nature of the system. Pathways for the oxygen-dependent degradation of aromatic compounds and genes encoding for enzymes involved in alkane degradation are prevalent in *Roseobacter* genomes, though there is significant diversity in the set of pathways present in a given strain, even those within the same species (e.g., *P. gallaeciensis*). Given our current limited understanding of the abilities of specific group members to degrade these structurally diverse compounds, generalizations regarding the catabolic capabilities of cultured representatives are not yet feasible. However, the lack of extradiol cleavage pathways for the degradation of hydrocarbons and related compounds may suggest these organisms employ non traditional approaches for the utilization of these substrates. Research directives aimed at uncovering the genetics and biochemistry of hydrocarbon and related compound catabolism in roseobacters, particularly those for which genome sequences are available, are needed, as are field studies that incorporate quantitative measurements of specific bacterial groups and employ labeled substrates (tracers). Efforts on both complementary fronts will undoubtedly provide a more conclusive understanding of the contribution of this naturally abundant and physiologically versatile group of microbes to hydrocarbon degradation in marine systems.

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Aerobic Hydrocarbon-Degrading *Alphaproteobacteria*: *Sphingomonadales*

6

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Contents

Abstract

The bacterial order *Sphingomonadales* includes many *Sphingomonadaceae* and *Erythrobacteraceae* isolates that have the ability to degrade a wide range of hydrocarbons. Hydrocarbon-degrading members of the *Sphingomonadaceae* (mainly belonging to the genera *Sphingomonas*, *Sphingobium*, *Novosphingobium*, and *Sphingopyxis*) are common Gram-negative aerobic organisms that have been isolated from a wide variety of environments, including temperate and polar soils,

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marine sediments, and plant surfaces and tissues. They degrade a broad range of mono- and polycyclic aromatic compounds, and the genetics and enzymology of these processes have been elucidated in some detail. Recently, the genome sequences of several hydrocarbon-degrading strains have been determined. In sphingomonads, the biodegradation gene loci are often widely distributed in the genome and are not colocalized as in other hydrocarbon-degrading genera, which has hindered genetic manipulation. The relevant degradative genes are very often located on specific large plasmids ("megaplasmids"). Sphingomonad strains are easy to cultivate and are strong candidates for bioremediation applications, but the recent results suggest that their role in the degradation of recalcitrant aromatics in contaminated soils in situ may be less than previously thought.

1 Introduction

Members of the *Sphingomonadaceae* ("sphingomonads") have been known for many years as degraders of aromatic hydrocarbons, and particularly of polycyclic aromatic hydrocarbons in contaminated soil environments. Because these soil sphingomonads are easy to cultivate in vitro, enrichment cultures using two- or three-ring polycyclic and heterocyclic aromatics as carbon source will often yield sphingomonad isolates, and hydrocarbon degradation by several of these strains has been characterized in detail. However, members of the *Sphingomonadaceae* are found in a much wider variety of environments than just soil – large populations of oligotrophic sphingomonads are found in the ocean, and representatives of the family are also present in the deep subsurface, and on plants, both on leaf and root surfaces and as endophytes. There are also a few hydrocarbon-degrading isolates known from the family *Erythrobacteraceae*, which forms the second large family within the *Sphingomonadales*. Here, we present a brief overview of the key hydrocarbon-degrading strains that have been studied to date, and a summary of the ecology and genetic data obtained from this research.

2 Taxonomic Framework for the *Sphingomonadaceae*

Like many other hydrocarbon-degrading families, the sphingomonads have been subject to a number of nomenclature changes over the past few decades. Several well-characterized hydrocarbon-degrading *Sphingomonas* strains were initially classified as pseudomonads or as flavobacteria, mainly because many of them were motile or formed colonies that were yellow in color. The genus *Sphingomonas* was proposed in 1990 (Yabuuchi et al. 1990) – members of the genus are defined as Gram-negative, nonsporulating, rod-shaped organisms, most of which produce deep-yellow-colored colonies. They are obligate aerobes, may be motile or nonmotile, and most characteristically, their outer membranes contain glycosphingolipids instead of lipopolysaccharides, which are otherwise only found in

eukaryotic cells (Gutman et al. 2014). Many, though not all sphingomonads also secrete sphingan exopolysaccharides, a group of polymers including gellan, wellan, and rhamsan, which are important in industrial and food applications (Fialho et al. 2008) and have been used in enhanced oil recovery (Shah and Ashtaputre 1999). Most of the strains included in the original definition were clinical or hospital isolates, but several hydrocarbon-degrading sphingomonads were also known at the time under different names, including *Sphingomonas paucimobilis* (biphenyl or lindane degradation – at that stage called *Pseudomonas paucimobilis*), *Sphingobium yanoikuyae* B1 (PAH degradation – *Beijerinckia* sp.), *Sphingobium chlorophenolicum* (pentachlorophenol degradation – *Flavobacterium* sp.), and *Sphingobium xenophagum* BN6 (arylsulfonate degradation – *Pseudomonas* sp.).

The *Sphingomonadaceae* currently encompass the genera *Sphingomonas*, *Sandaracinobacter*, *Blastomonas*, *Novosphingobium*, *Sphingobium*, *Sphingopyxis*, *Sandarakinorhabdus*, *Sphingosinicella*, *Stakelama*, *Sphingomicrobium*, *Sphingorhabdus,* *Parasphingopyxis*, and *Zymomomonas* (Kosako et al. 2000; Gläser and Kämpfer 2014). These include the genera *Sphingomonas* (*sensu-strictu*), *Novosphingobium,* *Sphingobium*, and *Sphingopyxis*, which are commonly referred to as sphingomonads. These genera were created in 2001 from the originally described genus *Sphingomonas* (*sensu-latu*) by Takeuchi et al. (2001). There was some initial opposition to this subdivision of the genus *Sphingomonas* (*sensu-latu*), arguing that the revised molecular taxonomy was not sufficiently supported by physiological and especially cellular lipid data (Yabuuchi et al. 2002), but the genera *Sphingomonas*, *Sphingobium*, *Novosphingobium*, and *Sphingopyxis* have now achieved general acceptance (Fig. 1) (Aylward et al. 2013; Gan et al. 2015). These genera are distinguished largely on the basis of 16S rRNA phylogeny, though there are also some phenotypic differences between the genera, in particular some variation in 2-hydroxy fatty acid profiles and polyamine patterns, and the inability of *Sphingobium* and *Sphingopyxis* to reduce nitrate. More recently, the genera *Sphingosinicella*, *Sphingomicrobium*, *Sphingorhabdus*, and *Parasphingopyxis* have been described which might also be included in the sphingomonads (Uchida et al. 2012; Jogler et al. 2013).

Many of the *Sphingomonadaceae* show resistance to streptomycin, and this has been used in the past as a preliminary screen in isolation studies to yield mainly *Sphingobium* and *Sphingopyxis* strains (Vanbroekhoven et al. 2004).

3 Taxonomic Framework for the *Erythrobacteraceae*

In many respects, the members of the *Erythrobacteraceae* resemble those belonging to the *Sphingomonadaceae*, as they are also Gram-negative, aerobic, rod-shaped, or pleomorphic coccoid chemo-organotrophic bacteria, which often produce pigments. The separation of the families *Erythrobacteraceae* and *Sphingomonadaceae* was

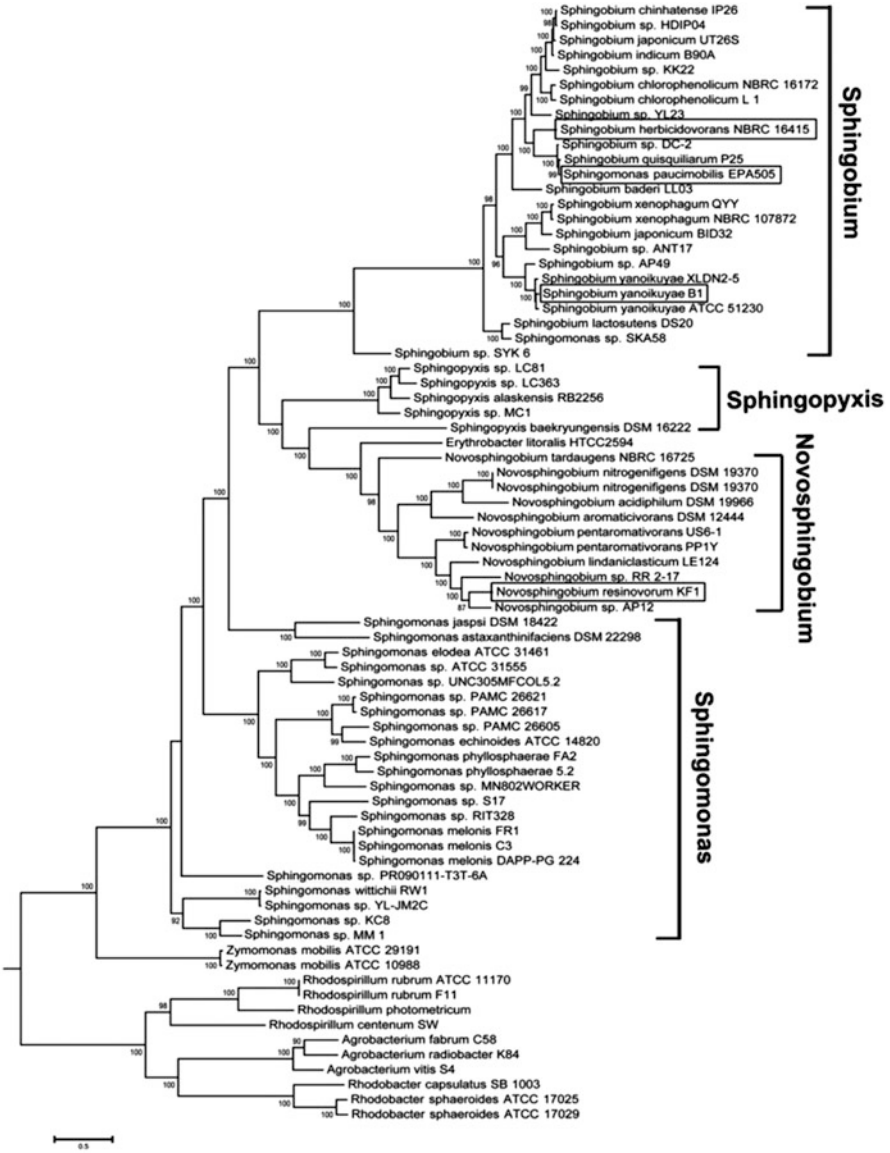

Fig. 1 Phylogenetic tree depicting the evolutionary relationship of currently sequenced sphingomonads based on approximately 400 conserved single-copy genes (Gan et al. 2015, with permission of the author)

mainly based on the formation of two distinct separate clusters observed in 16S rRNA–based phylogenetic trees. Currently, the family *Erythrobacteraceae* encompasses the genera *Erythrobacter*, *Alterythrobacter*, *Erythromicrobium*, *Croceicoccus*, and *Porphyrobacter* (Lee et al. 2005; Tonon et al. 2014).

4 Degradation of Aromatic Hydrocarbons by Key *Sphingomonadaceae* Strains

Members of the *Sphingomonadaceae* have often been isolated from contaminated soils for their ability to use aromatic hydrocarbons as a carbon source, but are less common when selection is for alkane degradation. They are particularly well known for their ability to degrade a broad variety of polycyclic aromatic hydrocarbons (PAHs) (Leys et al. 2004; Waigi et al. 2015) and substituted PAHs (Dimitriou-Christidis et al. 2007; Lamberts et al. 2008) though other studies have concentrated on breakdown of dioxins, pentachlorophenol, carbazole, and lindane (γ-hexachlorocyclohexane). In recent years, there has been an increasing interest in sphingomonads that degrade various pesticides and (putative) endocrine disruptors, such as 2-methyl-4-chlorophenoxyacetic acid (MCPA), estradiol, bisphenol A, and nonylphenol (Roh and Chu 2010; Kolvenbach and Corvini 2012; Nielsen et al. 2013). In addition, increasing numbers of *Sphingomonas* strains have been identified in noncontaminated soil and aquatic environments, and the hydrocarbon-degrading abilities of most of these have not yet been investigated in detail. Nonetheless, most of the research into hydrocarbon degradation by sphingomonads has been done with a few key strains (Table 1). This is reflected by substantially more publications using these strains than for other sphingomonads, and they are described in more detail below.

Sphingobium sp. (*Sphingomonas paucimobilis*) EPA505. Formerly known as *Pseudomonas paucimobilis* EPA505, but 16S rRNA alignment shows it to belong to the *Sphingobium* cluster (Fig. 1). Strain EPA505 was purified from a seven-member bacterial consortium isolated from a creosote waste site, by enrichment with fluoranthene as a carbon source (Mueller et al. 1990). As well as fluoranthene, strain EPA505 degrades many monoaromatic compounds, and a range of four- and five-ring PAHs such as pyrene, benz[*a*]anthracene, chrysene, benzo[*a*]pyrene, benzo [*b*]fluoranthene, and dibenz[*a,h*]anthracene (Ye et al. 1996), though it transforms the higher molecular weight aromatic compounds only cometabolically, and cannot use them as a carbon and energy source for growth (Story et al. 2004). The degradation of PAHs by strain EPA505 has been intensively studied and a specific 16S rRNA primer set for this strain has been designed in order to facilitate studies of its presence and survival (Leys et al. 2005; Adam et al. 2014). The catabolic pathways that catalyze PAH-degradation proceed via initial ring oxygenation, and the genes corresponding to the TOL pathway have been identified and the genome sequence obtained (Pinyakong et al. 2003; Story et al. 2000; Gan et al. 2015).

Sphingomonas wittichii RW1. *Sphingomonas wittichii* RW1 was isolated from dibenzo-*p*-dioxin enrichment cultures inoculated with water samples from the river Elbe (Wittich et al. 1992). The strain is able to utilize dibenzo-*p*-dioxins (DD) and dibenzofurans (DF) as a sole carbon and energy source, and is also able to cometabolize mono-, dichloro-, and also some higher chlorinated derivatives of DD and DF. There is the potential to use strain RW1 in bioaugmentation, as the strain survived after inoculation into contaminated soil, with degradation of diaryl ether compounds (dibenzo-*p*-dioxin, dibenzofuran, and 2-chlorodibenzo-*p*-dioxin).

Table 1 Main sphingomonad strains used in studies of hydrocarbon degradation

Species	Strain designations	Environmental source	Enrichment/isolation substrates	References
Sphingomonas wittichii	RW1 (DSM 6014)	Elbe river	Dibenzo-*p*-dioxin	Wittich et al. (1992)
Sphingomonas sp. (*paucimobilis*)	SYK-6	Kraft pulp effluent	Lignin, vanillate, protocatechuate	Katayama et al. (1988)
Sphingomonas sp.	TTNP3	Activated sludge	Nonylphenol	Tanghe et al. (1999)
Sphingobium sp. (*Sphingomonas paucimobilis*)	EPA505 (DSM 7526)	Creosote waste	Fluoranthene	Mueller et al. (1990)
Sphingobium xenophagum (*Sphingomonas xenophaga*)	BN6 (DSM 6383)	Elbe river	Naphthalenesulfonate	Nörtemann et al. (1986)
Sphingobium sp. (*Sphingomonas* sp.)	UG30	PCP-contaminated industrial site (Ontario, Canada)	Pentachlorophenol	Leung et al. (1997)
Sphingobium japonicum	UT26 (CCM 7287, DSM 9951)	Upland experimental field	Lindane	Imai et al. (1991)
Sphingobium yanoikuyae	B1 (DSM 6900)	Polluted stream	Biphenyl, PAHs	Gibson (1999), Gibson et al. (1973)
Sphingobium chlorophenolicum	L-1 (ATCC 39723, ATCC 53874)	PCP-contaminated freshwater sediment	Pentachlorophenol	Saber and Crawford (1985)
Sphingobium sp. *(chlorophenolicum)*	RA2 (DSM 8671)	Broderick EPA superfund site	Pentachlorophenol	Radehaus and Schmidt (1992)
Sphingobium herbicidovorans	MH (DSM 11019, ATCC 700291)	2,4-DP pretreated soil	Mecoprop	Horvath et al. (1990)
Novosphingobium sp.	KA1	Lagoon sludge	Carbazole	Habe et al. (2002)
Novosphingobium aromaticivorans	F199 (DSM 12444, ATCC 700278)	Underground sediment	Aromatic compounds	Romine et al. (1999)
Novosphingobium pentaromaticivorans	US6-1	Estuarine sediment (South Korea)	Benzo[a]pyrene, pyrene, phenanthrene	Sohn et al. (2004)
Sphingopyxis chilensis	S37 (DSM 14889)	River sediment	Chlorophenol	Godoy et al. (2003)

However, in the presence of higher levels of organic carbon, the degradation rates of the diaryl ether compounds were relatively low (Halden et al. 1999; Nam et al. 2006).

The degradation pathways and the catabolic enzymes for dibenzo-*p*-dioxin and dibenzofuran degradation have been well studied (Armengaud et al. 1998; Bünz and Cook 1993; Happe et al. 1993; Wittich et al. 1992). The genes encoding the dioxin dioxygenase subunits are widely distributed in the genome, and not co-localized (Armengaud et al. 1998). The initial degradation pathways for DD and DF were characterized biochemically (Bünz and Cook 1993), and 2,2′,3-trihydroxybiphenyl dioxygenase, the key enzyme which is responsible for initial *meta*-cleavage of the aromatic ring system in DD and DF, has been genetically and biochemically analyzed (Happe et al. 1993).

The genome sequence of *S. wittichii* RW1 demonstrated the presence of two large plasmids ("megaplasmids") about 220 kbp and 310 kbp in size. It was found that the smaller of these (pSWIT02) contained the genes involved in the initial part of the dioxin degradative pathway, in three separate loci (Miller et al. 2010). Subsequent proteomic and transcriptomic studies demonstrated that during growth with DD and DF, several stress response genes were also upregulated in addition to the relevant degradation genes (Coronado et al. 2012; Hartmann and Armengaud 2014; Chai et al. 2016).

Sphingobium japonicum UT26. Formerly known as *Pseudomonas paucimobilis* UT26. This strain utilizes the pesticide γ-hexachlorocyclohexane (lindane) as a sole carbon source and was isolated from an upland experimental field to which lindane had been applied annually for 12 years (Imai et al. 1991). The lindane degradation pathway proceeds via successive dechlorination steps followed by aromatization – several compounds (e.g., 1,2,4-trichlorobenzene) have been identified as dead-end intermediates branching off this pathway. Degradation of the chlorinated aromatic ring then proceeds by dioxygenation. The enzymes of the pathway and the corresponding *lin* genes have been reviewed (Nagata et al. 1999, 2007). Sequencing of the genome of the organism demonstrated the presence of two circular chromosomes [with sizes of 3.5 Mbp (Chr1) and 682 kbp (Chr2)], a large (mega-)plasmid (191 kbp) and two small plasmids (32 kbp and 5 kbp). The genes involved in the degradation of lindane are dispersed on Chr1, Chr2, and the large plasmid (Nagata et al. 2011; Tabata et al. 2016).

Sphingobium chlorophenolicum. Several pentachlorophenol (PCP) degrading sphingomonads have been independently isolated and initially described as, e.g., *Pseudomonas* sp. or *Flavobacterium* sp. (Ederer et al. 1997). One of these strains, *Sphingobium chlorophenolicum* RA2, was isolated from a PCP-contaminated EPA Superfund site and shown to degrade PCP at concentrations of up to 300 μg/ml in liquid culture (Colores et al. 1995; Radehaus and Schmidt 1992). Bioaugmentation of PCP-contaminated soil with strain RA2 showed it to be an effective colonizer of PCP-contaminated soil, but it did not persist in the absence of PCP (Colores and Schmidt 1999).

The biochemistry and genetics of the degradative pathway for PCP have been studied most intensively with *Sphingobium chlorophenolicum* L-1, and it was found

that PCP is degraded via 2,6-dichlorohydroquinone to maleylacetate. The whole genome sequence of this organism revealed that the genome consists of two chromosomes and a small plasmid. The genes responsible for the degradation of PCP were found on chromosome 2, and evidence was presented that the first three enzymes in the PCP degradative pathway were acquired via two horizontal gene transfer events (Copley et al. 2012).

Sphingobium yanoikuyae B1. Formerly known as *Beijerinckia* sp. B1, this strain was isolated from a polluted stream. Strain B1 is able to utilize polycyclic aromatic compounds such as biphenyl, naphthalene, anthracene, and phenanthrene as the sole carbon source (Gibson et al. 1973). The strain was reclassified as *Sphingobium yanoikuyae* B1 in 1996 (Khan et al. 1996). Many of the genes involved in the metabolism of aromatic compounds have been studied and most of them are grouped together on a 39 kbp fragment of the chromosome (Chadhain et al. 2007). Polycyclic aromatic compounds are degraded by a broad-substrate range initial dioxygenase, leading to dihydroxylated intermediates which are subject to ring cleavage; monoaromatic substrates are degraded via a TOL-like mechanism [reviewed by (Zylstra and Kim 1997)]. Once the degradation pathways are induced, the strain is able to degrade a wide range of aromatic compounds, including dibenzofuran, dibenzothiophene, acenaphthene, acenaphthylene, carbazole, dibenzo-*p*-dioxin, benz[*a*]anthracene, benzo[*a*]pyrene, 3-methylcholanthrene, and 1,2-dihydronaphthalene (Kim and Zylstra 1999). Bioaugmentation studies on PAH-contaminated soil using strain B1 have shown that it was unable to colonize uncontaminated soil, but could survive in PAH-contaminated soils (Cunliffe et al. 2006; Cunliffe and Kertesz 2006).

The genome of this strain harbors 71 transposases encoding genes and the genes for 35 different (putative) dioxygenases (Zhao et al. 2015).

Novosphingobium aromaticivorans F199. Formerly known as *Sphingomonas aromaticivorans*, this strain was isolated from deep sediments (410 m below ground level) and degrades various aromatic compounds, including toluene, naphthalene, dibenzothiophene, salicylate, *p*-cresol, all isomers of xylene, biphenyl, and fluorene (Fredrickson et al. 1991; Romine et al. 1999a, b). The strain harbors two plasmids, pNL1 (~180 kbp) and pNL2 (~480 kbp). The aromatic catabolic genes are associated with the smaller plasmid pNL1 – the sequence of this plasmid has been determined, and of a total of 186 predicted ORFs identified, 79 of which are thought to be associated with the catabolism or transport of aromatic compounds (Romine et al. 1999b). Regions encoding aromatic catabolic genes on pNL1 show a similarity with those of other aromatic degrading sphingomonads, e.g., pSKY4 plasmid in *Sphingomonas* sp. HV3 or the genes involved in the degradation of naphthalenesulfonates by *Sphingobium xenophagum* BN6 (Romine et al. 1999b; Yrjala et al. 1997; Keck et al. 2006).

Other hydrocarbon-degrading sphingomonad strains. The strains described above and in Table 1 have been intensively investigated in order to analyze the degradation of hydrocarbons by sphingomonads. In addition, there are several more hydrocarbon degrading strains among the more than 200 validly described sphingomonads currently known. These include *Novosphingobium naphthalenivorans* (growth with naphthalene (Suzuki and Hiraishi 2007)), *Novosphingobium*

pentaromaticivorans US6-1 (degradation of pyrene, benz[*a*]anthracene, chrysene, benzo[*b*]fluoranthene, and benzo[*a*]pyrene (Sohn et al. 2004; Lyu et al. 2014); *Sphingobium aromaticiconvertens* (growth with monochlorinated dibenzofurans) (Wittich et al. 2007b); *Sphingobium fuliginis* (growth with phenanthrene) (Prakash and Lal 2006); *Sphingomonas* sp. CHY-1 (growth with chrysene) (Willison 2004); and *Sphingomonas haloaromaticamans* (growth with variously substituted polychlorinated phenols) (Wittich et al. 2007a). A comprehensive list of phenanthrene degrading sphingomonads has recently been compiled by Waigi et al. (2015). There is also significant interest in *Sphingobium* sp. SYK-6, which is able to convert various lignin-derived mono- and bicyclic aromatic compounds and thus potentially can be used for commercial exploitation of lignin (Masai et al. 2007; Varman et al. 2016).

5 Ecology of Hydrocarbon-Degrading *Sphingomonadaceae*

The members of the *Sphingomonadaceae* are obligate aerobes and are found in a wide variety of (often oligotrophic) environments, such as different soils, sediments, and pelagic freshwater and marine aquatic environments (Jogler et al. 2011). There is increasing evidence that they play important roles in the (mycor)rhizosphere of plants and they even have been found as dominant members of the rhizosphere of the world's highest growing plants at more than 6000 m above sea level (Boersma et al. 2009; Angel et al. 2016). They have also been reported as common plant endophytes, colonizing several different species, but notably rice and poplar. In rice paddies, they are dominant rhizosphere species in highly acid soils (pH 2.1–3.7), but the hydrocarbon-degrading properties of these strains are not known (Hashidoko et al. 2007). *Sphingomonas* strains isolated from poplar trees growing on hydrocarbon-contaminated soil made up 9% of the endophytic community, and several of them showed the ability to degrade BTEX or other hydrocarbons (Moore et al. 2006). 4-*tert*-Butylphenol-utilizing strains of *Sphingobium fuliginis* have been isolated from the rhizosphere of *Phragmites australis* (Toyama et al. 2010). Furthermore, sphingomonads have been found as the major bacterial group associated with arbuscular mycorrhizal fungi (*Glomeromycota*) within the roots of *Solidago rugosa* plants growing in extremely hydrocarbon-polluted conditions (Iffis et al. 2014). This suggests that these strains have the potential to be useful in phytoremediation applications.

Sphingomonads also have been recovered from the deep subsurface, where they appear to be specialized in the utilization of lignite (Fredrickson et al. 1999), and they are among the most common PAH-degrading organisms obtained from a variety of soil environments (Leys et al. 2004). Hydrocarbon-degrading sphingomonads have not been explicitly described as thermotolerant, and even organisms isolated from the deep subsurface have not been characterized in this way (Fredrickson et al. 1995). Psychrotolerant and psychrophilic sphingomonads, by contrast, have been isolated from several oil-contaminated Antarctic soils (Aislabie et al. 2006).

Taxonomically, they are very similar to mesophilic sphingomonads isolated from elsewhere and do not represent a separate clade.

Sphingomonads are also common marine organisms, and the oligotrophic ultramicrobacterium *Sphingopyxis alaskensis* RB2256 has been studied in detail (Cavicchioli et al. 1999; Williams et al. 2009). Other marine sphingomonads have been isolated from polar to temperate waters, including strains that act as coral pathogens (Cavicchioli et al. 1999, 2003). A recent study of sphingomonad diversity on a range of environmental niches reported that members of the genus *Sphingomicrobium* are particularly strongly associated with marine habitats (Huang et al. 2017). Whereas there is no evidence for hydrocarbon utilization by most of the seawater-derived strains, marine sediments have yielded isolates that degrade PAHs (Gilewicz et al. 1997; Johnson and Hill 2003) or PCBs (Begonja Kolar et al. 2007).

Notably, hydrocarbon contamination has repeatedly been shown to enrich for sphingomonads in soils (Aislabie et al. 2004; Johnsen et al. 2002; Saul et al. 2005; Vinas et al. 2005), and it has been demonstrated that the treatment of PAH-contaminated soil with surfactants results in an increased number of sphingomonads (Wang et al. 2016). While many of these studies have concentrated on polar soils, sphingomonad enrichment is also evident in hydrocarbon-contaminated temperate soils, providing the source of most of the hydrocarbon-degrading sphingomonads reported to date. These organisms can detect PAHs with high sensitivity, and *Sphingobium* (*Sphingomonas*) sp. LB126 has been engineered with the *luxAB* genes to act as a biosensor for a range of PAHs (Bastiaens et al. 2001). Recently, the regulatory system of *Novosphingobium* sp. HR1a has also been used for the construction of biosensors, with the ability to detect PAHs in the μg/L range (Segura et al. 2017).

6 Genetics and Genomics of *Sphingomonadaceae*

The NCBI genome assembly database holds more than 200 entries for sphingomonads. Several hydrocarbon degraders were analyzed in the course of these genome sequencing efforts, including *Novosphingobium aromaticivorans* DSM 12444, *Sphingobium japonicum* UT26, *Novosphingobium pentaromaticivorans* US6-1, *Sphingobium yanoikuyae* B1, *Sphingomonas wittichii* RW1, and *Novosphingobium* sp. PP1Y (D'Argenio et al. 2014; Nagata et al. 2011, Luo et al. 2012; Gan et al. 2013; Miller et al. 2010; Zhao et al. 2015; Choi et al. 2015). There are also several publications which compare the genomes of different sphingomonads in order to identify characteristic features (Aylward et al. 2013; Gan et al. 2013; Verma et al. 2014; Tabata et al. 2016; Kumar et al. 2017). These studies demonstrated typical genome sizes for sphingomonads of 3.4–6.3 Mbp, made up of 1–2 circular chromosomes with 1–3 16S rDNA operons. In addition, in most of the investigated strains, 1–6 plasmids were detected (see below). The genome sequences gave ample evidence for multiple genomic rearrangements, with only a very low degree of a shared gene order was observed among the strains compared. Many of

the genomes also contain a large number of transposons (Aylward et al. 2013; Kumar et al. 2017). The insertion element IS6100 seems to be especially important for the evolution and distribution of the genes responsible for the degradation of the isomeric hexachlorocyclohexanes in sphingomonads (Verma et al. 2014; Tabata et al. 2016). In addition, in many cases, large numbers of genes were found that coded for mono- and dioxygenases involved in the degradation of aromatic compounds.

These sequencing projects, together with earlier characterization of aromatic catabolic genes and gene clusters, revealed that the genes for aromatic degradation are often arranged in a complex manner in sphingomonad genomes. Genes for PAH-degradation pathways, for instance, are not colocalized but are scattered through several clusters (Pinyakong et al. 2003), and a similar phenomenon was found for dioxin degradation genes (Armengaud et al. 1998). Nonetheless, PAH-degradation genes are quite strongly conserved between different sphingomonads (Pinyakong et al. 2003; Basta et al. 2005; Stolz 2009).

Although the hydrocarbon catabolic genes of the terrestrial surface strains *Sphingobium yanoikuyae* B1 and Q1 are chromosomal (Kim et al. 1996), many sphingomonad species carry their hydrocarbon catabolic genes on large plasmids of >100 kbp. A survey of catabolic plasmids in 17 different hydrocarbon-degrading sphingomonads showed that almost all analyzed strains contain two to five circular plasmids in sizes ranging from 50 kbp to 500 kbp (Basta et al. 2004). The near-ubiquitous presence of large plasmids in this group of bacteria was also confirmed in the course of the genome sequencing projects (Aylward et al. 2013). Several of these catabolic plasmids have been sequenced, including the aromatic catabolic plasmid pNL1 from *Novosphingobium aromaticivorans* F199, the carbazole-degradation plasmid pCAR3 from *Novosphingobium* (*Sphingomonas)* sp. KA1, plasmid pCHQ1 from the lindane degrader *Sphingobium japonicum* UT26, plasmid pLA1 from the benzo[*a*]pyrene degrader *Novosphingobium pentaromaticivorans* US6-1, plasmid Mpl from *Novosphingobium* sp. strain PP1Y, and PSWIT02 from the DD-degrader *Sphingomonas wittichii* RW1. These and other plasmids from sphingomonads have been partially classified into different incompatibility groups according to their replication initiation proteins (RepA) and plasmid partitioning proteins (ParAB) (Basta et al. 2004; Stolz 2014).

Most genetic transformations of sphingomonads have been carried out using conjugation methodologies, reflecting the natural abilities of the strains. Thus, plasmid pNL1 from *Novosphingobium aromaticivorans* F199 could be conjugatively transferred to strains from other *Sphingomonadaceae* genera, including *Sphingobium yanoikuyae* B1, *Sphingobium* (*Sphingomonas*) sp. SS3, and *Sphingobium herbicidovorans*, and many mutants have been generated using plasmid-borne transposons, particularly mini-*Tn*5. In *Sphingomonas wittichii* RW1 and other sphingomonads, these mutations appear to be stable (Bünz et al. 1999; Basta et al. 2004). More recently, the construction of a markerless gene deletion system for sphingomonads based on streptomycin-sensitive variants has been described (Kaczmarczyk et al. 2012). Mutant complementation has been reported in *Sphingobium japonicum* using RK2-based plasmids introduced by electroporation (Endo et al. 2007), and using broad host range plasmids of the pBBR1MCS family

in *Sphingobium yanoikuyae* B1 (Cho et al. 2005) and *Sphingobium* (*Sphingomonas*) sp. LB126 (Wattiau et al. 2001) by conjugational transfer. Although intrafamily conjugation is used routinely in laboratory work, it should be noted that sphingomonad plasmids are presumably rarely transferred to other *Proteobacteria*, since high levels of gene flow to other bacteria are not observed (Basta et al. 2004; Stolz 2009).

7 Degradation of Alkanes and Aromatic Hydrocarbons by Members of the *Erythrobacteraceae*

In contrast to the many publications concerned with the degradation of hydrocarbons by sphingomonads, there are only some preliminary reports about *Erythrobacteraceae* isolates with degradative capabilities. Some *Erythrobacter* strains were isolated from weathered crude oil and evidence was obtained that these isolates degraded long-chain alkanes, and also naphthalene, phenanthrene, pristane, and phytane (Harwati et al. 2007). The type-species of *Erythrobacter atlanticus* was obtained from deep-sea sediment after enrichment with a mixture of PAHs and was able to degrade pyrene, phenanthrene, and naphthalene (Zhuang et al. 2015). Furthermore, *Croceicoccus naphthovorans* has been enriched from a marine environment, using phenanthrene as sole source of carbon and energy (Huang et al. 2015), and an isolate belonging to the genus *Porphyrobacter* has been obtained after long-term enrichment with a mixture of high molecular weight PAHs in a two-liquid-phase culture system (Gauthier et al. 2003).

Previously, it was shown that among several tested (nonsphingomonad) *Proteobacteria*, only *Porphyrobacter sanguineus* was able to take up the megaplasmid pNL1 (from *Novosphingobium aromaticivorans*) labeled with a kanamycin resistance cassette (Basta et al. 2004). This might indicate that the ability of some members of the *Erythrobacteraceae* to degrade PAHs might depend on the ability of these organisms to propagate the megaplasmids from the closely related *Sphingomonadaceae*.

8 Research Needs

For many years, sphingomonad species have been regarded as some of the dominant degraders of aromatic hydrocarbons in a variety of terrestrial and sediment environments. This view was based on the ready isolation of members of the *Sphingomonadaceae* from enrichment cultures utilizing a variety of aromatic hydrocarbons as substrate and has led to the characterization of many of the biochemical pathways and genetic components that are involved in hydrocarbon degradation by this family. Experiments using DNA-based stable isotope probing have provided additional evidence that sphingomonads belong to the indigenous microorganisms which participate in situ in the biodegradation of phenanthrene (Li et al. 2017). These organisms have the potential for application as inocula in bioaugmentation applications, and there has been considerable work looking at the survival of sphingomonad

isolates in soil conditions (Cunliffe et al. 2006; Cunliffe and Kertesz 2006; Kastner et al. 1998; Megharaj et al. 1997; van Herwijnen et al. 2006). Despite this work, the challenge for bioremediation still remains the choice of strain or consortium, and optimizing this for survival and effectiveness (Thompson et al. 2005; Horemans et al. 2016). Recently, it has been reported that sphingomonads might enter a (metabolically active) viable but nonculturable-like state directly after the transfer to soil and that it might take several days for them to recover to a culturable state (Fida et al. 2017). More research is required to optimize the survival of introduced sphingomonads under soil conditions, and to improve the rates of hydrocarbon degradation achieved, possibly by utilization of stable mixed consortia rather than single strains, by appropriate pre-treatment of inocula, or by use of surfactants or biosurfactant-producing strains (Wang et al. 2016).

Sphingomonads have an extraordinary ability to degrade a broad variety of man-made organic compounds, most of which have been present in the environment for less than 100 years. This suggests that they can evolve the necessary catabolic pathways more rapidly than most other bacteria, but which molecular characteristics are responsible for this versatility remains an open question. Several explanations have been proposed:

- The presence of a large and diverse set of genes coding for (ring-hydroxylating) (di)-oxygenases.
- The presence of multiple insertion elements and transposons, together with the organization of the degradative genes in several scattered clusters, might allow more efficient combinations of existing genes into novel degradative pathways.
- The existence of a group of large plasmids, which seem to be almost completely restricted to sphingomonads and do not conjugatively transfer to other groups of bacteria might allow a form of "specialized evolution" within this group.
- The presence of glycosphingolipids in the outer membranes of sphingomonads might restrict the conjugative transfer of the megaplasmids to other groups of bacteria, though the mechanism for this remains to be elucidated. It might also result in specific surface structures that allow more efficient degradation of hydrophobic substrates.

The recent rapid progress in genome sequencing was originally expected to provide rapid answers to these questions, but convincing evidence to explain the metabolic versatility of this fascinating group of organisms remains elusive. Although genome sequencing projects have repeatedly demonstrated the presence of various oxygenase genes and insertion elements in the genomes of many sphingomonads, this characteristic is not exclusive to sphingomonads. Therefore, it might be speculated that the metabolic versatility of the sphingomonads is related to the combination of megaplasmids with the almost unique presence of glycosphingolipids in the outer membranes, providing a genetic system which largely restricts the exchange of the degradative capabilities to the sphingomonads. Based on the accumulating genomic data, it should be possible to tackle this problem in the near future and thus to solve this long-standing riddle.

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Hydrocarbon Degradation by *Betaproteobacteria*

7

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Contents

Abstract

Members of the *Betaproteobacteria* are widespread in soil environments. Many isolates are capable of aerobic degradation of aromatic hydrocarbons, as well as chloroaromatic, nitroaromatic, and aminoaromatic compounds. Anaerobic aromatic hydrocarbon degradation under nitrate-reducing or (per)-chlorate-reducing conditions appears to be a process that is carried out mainly by *Betaproteobacteria*. Less is known about the distribution of *Betaproteobacteria* that are capable of utilizing alkanes as carbon and energy sources, but both aerobic and anaerobic alkane-degrading *Betaproteobacteria* have been isolated.

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1 Introduction

The class *Betaproteobacteria* is based on phylogenetic analysis of 16S rRNA sequences. The members are phenotypically, metabolically, and ecologically quite diverse. However, many members of the *Betaproteobacteria* were originally classified as members of the genus *Pseudomonas* based on their morphology and physiology (motile, metabolically versatile respiratory rods (Stanier et al. 1966)). In the 1970s and 1980s, the genus *Pseudomonas* was subdivided into five distinct rRNA homology groups based on rRNA-DNA hybridizations (Palleroni 1984; Palleroni et al. 1973), and subsequent 16S rRNA sequence analysis has placed the majority of members of rRNA groups II and III in the *Betaproteobacteria* (reviewed in Anzai et al. 2000; Kerstens et al. 1996; Palleroni 2003). New genera have been proposed and the nomenclature is still evolving (Vandamme and Coenye 2004); however, every effort has been made to use the most up-to-date strain names. The most common genera in the *Betaproteobacteria* that are known to degrade hydrocarbons and related substituted molecules under oxic conditions include *Acidovorax*, *Burkholderia*, *Comamonas*, *Delftia*, *Polaromonas*, and *Ralstonia* (Tables 1, 2, and 3). Anaerobic degradation of hydrocarbon is primarily carried out by *Aromatoleum, Azoarcus,* and *Thauera* species (Table 1). Most of these genera represent common soil bacteria, but hydrocarbon-degrading *Betaproteobacteria* have also been identified in polluted aquifers (Alfreider and Vogt 2007; Fahy et al. 2006, 2008). In general, only selected strains of each genus are capable of growing with specific subsets of hydrocarbons; i.e., the ability to degrade these compounds is not a general characteristic of these genera or species. In some cases, pathways for the degradation of hydrocarbons are known to be plasmid-encoded in certain *Betaproteobacteria*. This chapter will focus on the ability of *Betaproteobacteria* to grow on and completely mineralize hydrocarbons and related substituted compounds containing chloro-, nitro-, and amino groups.

2 Aerobic Aromatic Hydrocarbon Degradation by *Betaproteobacteria*

For many years, the vast majority of isolates capable of growth on aromatic hydrocarbons featuring one to three aromatic rings (e.g., benzene, toluene, ethylbenzene, biphenyl, naphthalene, phenanthrene, anthracene, etc.) were found to be legitimate members of the genus *Pseudomonas*. These observations were likely due to the common enrichment strategy used: sequential transfers through a minimal medium containing the hydrocarbon as sole carbon source with no added growth factors. Many bacteria, including many *Betaproteobacteria*, grow more slowly than *Pseudomonas* strains and may need one or more growth factors to achieve optimal growth rates. As a result, the commonly used enrichment and isolation strategies routinely detected fast-growing *Pseudomonas* strains. Once direct plating methods and other strategies were developed to identify the range of diverse bacteria capable of aromatic hydrocarbon degradation, it became clear that *Betaproteobacteria* are

Table 1 Representative betaproteobacterial strains capable of growth on single ring substituted and unsubstituted aromatic hydrocarbons

Bacterial strain	Hydrocarbon growth substrate(s)[a]	References
Achromobacter xylosoxidans T7	*o*-, *m*-, *p*-toluidine, aniline	(Hinteregger and Streichsbier 2001)
Acidovorax sp. JS42	2-Nitrotoluene, nitrobenzene	(Haigler et al. 1994; Lessner et al. 2002)
Acidovorax sp. MAK5	Benzene	(Posman et al. 2017)
Acidovorax (formerly *Pseudomonas*) sp. P51	Dichlorobenzenes, 2,4,5-trichlorobenzene	(Fahy et al. 2006; van der Meer et al. 1987)
Acidovorax avenae CBA1	Dichlorobenzenes, 1,2,4-trichlorobenzene	(Monferrán et al. 2005)
Alcaligenes sp. JS871	2,4-Dinitrotoluene	(Nishino et al. 2000)
Alicycliphilus denitrificans BC	Benzene, toluene (+/−O_2/ClO_3^-)	(Oosterkamp et al. 2013; Weelink et al. 2008)
Aromatoleum sp. ToN1	Toluene (−O_2/NO_3^-)	(Rabus and Widdel 1995)
"Aromatoleum aromaticum" EbN1	Toluene, ethylbenzene (−O_2/NO_3^-)	(Rabus et al. 2005; Rabus and Widdel 1995)
"Aromatoleum aromaticum" pCyN1	Toluene, *p*-cymene (−O_2/NO_3^-)	(Harms et al. 1999; Strijkstra et al. 2014)
Azoarcus sp. CIB	Toluene, *m*-xylene (+/−O_2/NO_3^-)	(Juárez et al. 2013; López Barragán et al. 2004)
Azoarcus sp. EB-1	Ethylbenzene (−O_2/NO_3^-)	(Johnson and Spormann 1999)
Azoarcus sp. PbN1	Ethylbenzene, propylbenzene (−O_2/NO_3^-)	(Rabus and Widdel 1995)
Azoarcus sp. T	Toluene, *m*-xylene (−O_2/NO_3^-)	(Krieger et al. 1999)
Azoarcus tolulyticus Tol-4	Toluene (−O_2/ NO_3^-)	(Zhou et al. 1995)
Burkholderia sp. DNT	2,4-Dinitrotoluene	(Spanggord et al. 1991; Suen et al. 1996)
Burkholderia (formerly *Pseudomonas*) sp. JS150	Benzene, toluene, ethylbenzene, naphthalene, chlorobenzene, 1,4-dichlorobenzene	(Haigler et al. 1992)
Burkholderia sp. PS14	Chlorobenzene, dichlorobenzenes, 1,2,4-trichlorobenzene, 1,2,4,5-tetrachlorobenzene	(Rapp and Timmis 1999; Sander et al. 1991)
Burkholderia cepacia JS850	2,6-Dinitrotoluene	(Nishino et al. 2000)
Burkholderia cepacia R34	2,4-Dinitrotoluene	(Nishino et al. 2000)
Burkholderia vietnamiensis G4	Toluene	(Nelson et al. 1987)
Burkholderia xenovorans LB400	Biphenyl, *p*-cymene	(Bopp 1986; Chain et al. 2006)
Comamonas sp. CBN-1	Nitrobenzene, 4-chloronitrobenzene	(Wu et al. 2006)
Comamonas sp. JB	Benzene, toluene, ethylbenzene, *o*-, *m*-, *p*-xylene	(Jiang et al. 2015)
Comamonas sp. JS765	Nitrobenzene, 3-nitrotoluene	(Lessner et al. 2002; Nishino and Spain 1995)

(*continued*)

Table 1 (continued)

Bacterial strain	Hydrocarbon growth substrate(s)[a]	References
Comamonas testosteroni I2	Aniline, 3-chloroaniline	(Boon et al. 2000)
Dechloromonas aromatica RCB	Benzene, toluene (+/−O_2/NO_3^-/ClO_3^-)	(Coates et al. 2001)
Delftia sp. AN3	Aniline	(Liu et al. 2002)
Delftia acidovorans 7N	Aniline	(Urata et al. 2004)
Delftia acidovorans B8c	Aniline, 3-chloroaniline	(Boon et al. 2001)
Delftia acidovorans BN3.1	Aniline, 3-chloroaniline	(Brunsbach and Reineke 1993)
Delftia acidovorans CA28	Aniline, 3-chloroaniline	(Loidl et al. 1990)
Delftia acidovorans LME1	Aniline, 3-chloroaniline	(Boon et al. 2001)
Delftia tsuruhatensis H1	3-, 4-Chloroaniline, 3,4-dichloroaniline, 4-methylaniline	(Zhang et al. 2010)
Diaphorobacter sp. DS1	2-, 3-, 4-Nitrotoluene	(Singh et al. 2014; Singh and Ramanathan 2013)
Diaphorobacter sp. DS2	Nitrobenzene, 2-, 3-, 4-nitrotoluene	(Singh et al. 2014; Singh and Ramanathan 2013)
Diaphorobacter sp. DS3	2-, 3-, 4-Nitrotoluene	(Singh et al. 2014; Singh and Ramanathan 2013)
Georgfuchsia toluolica	Toluene	(Weelink et al. 2009)
Hydrogenophaga sp. Rs71	Benzene, toluene	(Fahy et al. 2008)
Hydrogenophaga palleronii JS863	2,6-Dinitrotoluene	(Nishino et al. 2000)
Pandoraea sp. MCB032	Chlorobenzene	(Jiang et al. 2009)
Ralstonia sp. JS705	Toluene, chlorobenzene	(van der Meer et al. 1998)
Ralstonia sp. sPHS1	Benzene, toluene, ethylbenzene, *o*-xylene	(Lee and Lee 2001; Wilson et al. 2003)
Ralstonia sp. PS12	Chlorobenzene, dichlorobenzenes, 1,2,4-trichlorobenzene, 1,2,4,5-tetrachlorobenzene, toluene, 4-chlorotoluene, dichlorotoluenes	(Beil et al. 1997; Pollmann et al. 2001; Sander et al. 1991)
Ralstonia pickettii PKO1	Benzene, toluene, ethylbenzene	(Kukor 1990)
Thauera sp. DNT-1	Toluene (+/−O_2/NO_3^-)	(Shinoda et al. 2004)
Thauera sp. mXyN1	Toluene, *m*-xylene (−O_2/NO_3^-)	(Rabus and Widdel 1995)
Thauera sp. pCyN2	*p*-cymene (−O_2/NO_3^-)	(Strijkstra et al. 2014)
Thauera aromatica K172	Toluene (−O_2/NO_3^-)	(Anders et al. 1995)
Thauera aromatica T1	Toluene (−O_2/NO_3^-)	(Evans et al. 1991; Song et al. 1998)
Variovorax sp. MAK3	Benzene	(Posman et al. 2017)

[a]Growth is under oxic conditions unless otherwise indicated. "−O_2" indicates growth is in the absence of oxygen; "+/−O_2" indicates that the organism can grow with the listed substrates both aerobically and in the absence of oxygen. Alternative electron acceptors used under anoxic conditions are also shown

Table 2 Representative betaproteobacterial strains capable of growth on multi-ring aromatic hydrocarbons

Bacterial strain	Hydrocarbon growth substrate(s)[a]	References
Acidovorax sp. KKS102	Biphenyl	(Kimbara et al. 1989; Ohtsubo et al. 2006)
Acidovorax sp. NA3	Phenanthrene	(Singleton et al. 2009)
Acidovorax delafieldii P4–1	Phenanthrene	(Samanta et al. 1999)
Acidovorax delafieldii TNA921	Phenanthrene	(Shuttleworth and Cerniglia 1996)
Alcaligenes sp. D-59	Dimethylnaphthalene	(Miyachi et al. 1993)
Alcaligenes faecalis AFK2	Phenanthrene, anthracene, *n*-dodecane	(Kiyohara et al. 1982)
Burkholderia sp. RP007	Naphthalene, phenanthrene, anthracene	(Laurie and Lloyd-Jones 1999)
Burkholderia sp. TNFYE-5	Phenanthrene	(Kang et al. 2003)
Burkholderia sp. TSN101	Biphenyl	(Mukerjee-Dhar et al. 1998)
Burkholderia xenovorans LB400	Biphenyl, *p*-cymene	(Bopp 1986; Chain et al. 2006)
Comamonas testosteroni GZ39	Naphthalene, phenanthrene	(Goyal and Zylstra 1996; Goyal and Zylstra 1997)
Comamonas testosteroni GZ42	Naphthalene	(Goyal and Zylstra 1997)
Cupriavidus necator H850	Biphenyl	(Bedard et al. 1987; Wittich and Wolff 2007)
Diaphorobacter sp. KOTLB	Pyrene, phenanthrene, fluoranthene	(Klankeo et al. 2009)
Pandoraea pnomenusa B-356	Biphenyl	(Ahmad et al. 1990; Vézina et al. 2008)
Polaromonas naphthalenivorans CJ2	Naphthalene	(Jeon et al. 2004; Jeon et al. 2003)
Ralstonia sp. U2	Naphthalene	(Fuenmayor et al. 1998; Zhou et al. 2001)

[a]Growth is under oxic conditions

common contributors to the turnover of aromatic hydrocarbons under oxic conditions (Goyal and Zylstra 1997).

Several *Burkholderia* and *Ralstonia* isolates are able to degrade single-ring aromatic hydrocarbons (Table 1), and a strain of *Hydrogenophaga* was shown to grow on benzene and toluene and partially degrade *m*- and *p*-xylene (Fahy et al. 2008). More recently, a *Comamonas* isolate was shown to degrade benzene, toluene, ethylbenzene and all three xylene isomers (Jiang et al. 2015), and benzene-degrading *Acidovorax* and *Variovorax* isolates have been reported (Posman et al. 2017).

Several betaproteobacterial isolates that are members of the genera *Acidovorax*, *Burkholderia*, and *Comamonas* are also capable of degrading multi-ring polycyclic

Table 3 Representative betaproteobacterial strains capable of growth on linear, cyclic, and substituted alkanes

Bacterial strain	Hydrocarbon growth substrate(s)[a]	References
Acidovorax sp. CHX100	Cyclohexane	(Salamanca and Engesser 2014)
Aromatoleum sp. HxN1	C_6–C_8 *n*-alkanes ($-O_2/NO_3^-$ or NO_2^-)	(Ehrenreich et al. 2000; Zedelius et al. 2011)
Burkholderia cepacia RR10	C_{12}–C_{34} *n*-alkanes phenylhexadecane, pristane	(Yuste et al. 2000)
Polaromonas vacuolata JS666	*cis*-1,2-Dichloroethene, heptane, octane	(Coleman et al. 2002; Mattes et al. 2008)
Strain OcN1 (unknown genus)	C_8–C_{12} *n*-alkanes (+/$-O_2/NO_3^-$ or NO_2^-)	(Ehrenreich et al. 2000; Zedelius et al. 2011)
Thauera butanovorans (formerly *Pseudomonas butanovora*) Bu-B1211	C_2–C_9 *n*-alkanes, isobutene, isopentane	(Anzai et al. 2000; Cooley et al. 2009; Dubbels et al. 2009; Takahashi et al. 1980)

[a]Growth is under oxic conditions unless otherwise indicated. "$-O_2$" indicates growth is in the absence of oxygen; "+/$-O_2$" indicates that the organism can grow with the listed substrates both aerobically and in the absence of oxygen. Alternative electron acceptors used under anoxic conditions are also shown

aromatic hydrocarbons (Table 2), including phenanthrene degradation by several strains of *Acidovorax* and *Burkholderia*. Well-characterized biphenyl degraders include *Acidovorax* sp. KKS102, *Burkholderia xenovorans* LB400, and *Pandoraea pnomenusa*, all of which were originally classified as *Pseudomonas* strains. In addition, *Diaphorobacter* sp. strain KPBTL was reported to carry a megaplasmid that encodes enzymes necessary for pyrene, phenanthrene, and fluoranthene degradation (Klankeo et al. 2009).

Aerobic pathways for the degradation of aromatic hydrocarbons require oxygen as a co-substrate in the initial mono- or dioxygenase-catalyzed step as well as in the ring-cleavage step. One interesting difference in aerobic aromatic degradation pathways that seems to be characteristic to the *Betaproteobacteria* is in the naphthalene degradation pathway. While *Pseudomonas* strains generally convert the naphthalene-degradation intermediate salicylate to catechol and then utilize a *meta*-cleavage pathway to generate TCA cycle intermediates (*nah* pathway), some *Betaproteobacteria,* including *Ralstonia* and *Polaromonas* strains use a pathway (*nag* pathway) in which salicylate is converted to gentisate, which undergoes ring cleavage (Jeon et al. 2006; Zhou et al. 2001). Although most naphthalene degradation pathways are plasmid-encoded, including those in most *Pseudomonas* strains as well as that in *Ralstonia* sp. strain U2 (Fuenmayor and Rodriguez-Lemoine 1992), the *nag* pathway in *Polaromonas naphthalenivorans* CJ2 is encoded on the chromosome (Jeon et al. 2006).

3 Anaerobic Aromatic Hydrocarbon Degradation by *Betaproteobacteria*

Currently, the ability to degrade aromatic hydrocarbons under nitrate-reducing conditions seems to be restricted mainly to the *Betaproteobacteria* (Rabus et al. 2016). Several betaproteobacterial isolates, primarily *Azoarcus, Thauera,* and *Aromatoleum* species, are capable of anaerobic growth with toluene and *m*-xylene under nitrate-reducing conditions (Table 1). These organisms use a novel pathway in which the aromatic substrate is added to fumarate, forming a benzylsuccinate (or similar) intermediate that is ultimately converted to benzoyl-CoA, which undergoes ring reduction and cleavage (reviewed in (Rabus et al. 2016)). *Thauera* sp. strain DNT-1 is of interest because it has pathways for both aerobic and anaerobic toluene degradation (Shinoda et al. 2004). *Azoarcus* sp. strain CIB also degrades toluene and *m*-xylene under both oxic and anoxic conditions (Juárez et al. 2013; López Barragán et al. 2004). Interestingly, unlike other *Azoarcus* strains that are able to catabolize aromatic compounds under anoxic conditions, strain CIB plays a role in plant-bacteria interactions. *Azoarcus* sp. CIB lives as an endophyte in the roots of rice crops (*Oryzae sativa* L.) and is capable of promoting plant growth (Fernández et al. 2014). The authors suggest that the combination of these traits may have potential for economically viable and sustainable phytoremediation approaches.

In contrast to the initial addition of the activated alkylbenzene substrate to fumarate, growth of betaproteobacterial strains on ethylbenzene and propylbenzene (Table 1) involves an oxygen-independent hydroxylation of the alkyl side-chain followed by a series of reactions to generate the same common intermediate benzoyl-CoA (Johnson and Spormann 1999; Kniemeyer and Heider 2001; Rabus and Widdel 1995). It is interesting to note that two closely related bacteria, *Aromatoleum aromaticum* pCyN1 and *Thauera* sp. pCyN2, employ two different strategies to anaerobically degrade *p*-cymene (4-isopropyltoluene) under nitrate-reducing conditions. Whereas *Thauera* sp. pCyN2 adds *p*-cymene to fumarate to form 4-isopropylbenzyl succinate, proteogenomic and metabolite analyses revealed that *Aromatoleum aromaticum* pCyN1 uses a novel pathway that involves the anaerobic hydroxylation of the benzylic methyl group of *p*-cymene by a putative *p*-cymene dehydrogenase (CmdABC) (Strijkstra et al. 2014).

To our knowledge, *Georgfuchsia toluolica* G5C6 is the only documented member of the *Betaproteobacteria* that uses Fe(III), Mn(IV), as well as nitrate as terminal electron acceptors during anaerobic growth on ethylbenzene and toluene via the benzylsuccinate pathway (Weelink et al. 2009). Although this strain is unable to grow on xylene, a recent study reported the presence of *Georgfuchsia*-dominated enrichments with *p*-xylene (Sperfeld et al. 2018). A putative 4-methylbenzoyl-CoA reductase gene cluster was detected in the enrichment culture, which may play a role in the anaerobic degradation of *p*-xylene (Sperfeld et al. 2018).

Anaerobic benzene degradation has been reported, but the mechanism(s) is still under debate. *Dechloromonas aromatica* RCB was reported to grow with benzene in the absence of oxygen under nitrate- and (per)chlorate-reducing conditions as well

as aerobically (Chakraborty et al. 2005; Coates et al. 2001). Benzoate and phenol were detected as intermediates in anaerobic benzene degradation, suggesting the involvement of hydroxylation and carboxylation reactions; however, the enzymes and mechanisms remain unidentified (Chakraborty and Coates 2005). Strain RCB was also reported to grow on toluene both aerobically and under nitrate- and (per) chlorate-reducing conditions, and oxidize ethylbenzene and xylenes anaerobically in the presence of nitrate (Chakraborty et al. 2005). Surprisingly, no genes for anaerobic aromatic compound degradation could be found in the genome of *D. aromatica* RCB, but genes encoding putative benzene/phenol/ toluene monooxygenases were identified. A mechanism of monooxygenase-mediated anaerobic benzene degradation involving the production of oxygen during nitrate or chlorate respiration was proposed but has not been tested (Salinero et al. 2009). Interestingly, *Alicyclus denitrificans* strain BC, which is also capable of anaerobic respiration with nitrate and chlorate, is capable of growth on benzene and toluene both aerobically and under chlorate-reducing conditions but not under nitrate-reducing conditions (Weelink et al. 2008). Analysis of the genome sequence identified genes encoding a multicomponent benzene/phenol monooxygenase, and both *meta*- and *ortho*-cleavage pathways for conversion of catechol to TCA cycle intermediates (Oosterkamp et al. 2013). Two sequential monooxygenation reactions followed by dioxygenase-mediated ring cleavage, all of which require oxygen as a substrate, have been proposed to occur under anoxic conditions via the production of O_2 as an intermediate during chlorate reduction (Weelink et al. 2008). The absence of growth on benzene under nitrate-reducing conditions argues against a similar mechanism of oxygen production from nitrate.

It is possible that *Betaproteobacteria* play an indirect role in the anaerobic degradation of benzene in mixed communities through a syntrophic mechanism. A time-dependent DNA-SIP analysis showed that *Peptococacceae* and *Betaproteobacteria* were enriched in a $^{13}C_6$-benzene-supplemented chemostat. The authors suggested that *Peptococacceae* degraded benzene into H_2 and acetate, which were further consumed by *Betaproteobacteria* under nitrate-reducing conditions (van der Zaan et al. 2012).

4 Degradation of Substituted Aromatic Hydrocarbons by *Betaproteobacteria*

Several strains identified as *Acidovorax*, *Burkholderia, Pandorea,* and *Ralstonia* that degrade mono-, di-, tri-, and tetrachlorobenzenes have been isolated (Table 1). In addition, one of the most well-characterized di- and trichlorobenzene degrading strains, *Pseudomonas* sp. P51 (van der Meer et al. 1987) has been reclassified as a member of the genus *Acidovorax* (Fahy et al. 2006). The ability to grow on chlorobenzenes is not unique to the *Betaproteobacteria*, as numerous other bacterial isolates also degrade chloroaromatic compounds. Similarly, several strains of *Delftia* that degrade aniline have been isolated (Table 1); aniline is also degraded by other *Proteobacteria*, including *Pseudomonas* and *Acinetobacter* strains (Fujii et al. 1997; Fukumori and Saint 1997). In contrast, *Betaproteobacteria* seem to be the

predominant isolates obtained from selections with chloroanilines (Dejonghe et al. 2002; Loidl et al. 1990).

As with many aromatic compounds, *Delftia* strains utilize a ring-hydroxylating dioxygenase in the initial step of aniline and substituted aniline degradation, followed by a ring-cleavage step through either the *meta*- or *ortho*-pathway. *Delftia* sp. AN3 catabolizes aniline via the *meta*-pathway, and the genes required for complete assimilation of aniline are localized on the chromosome (Liu et al. 2002; Zhang et al. 2008). *Delftia tsuruhatensis* H1, in contrast, is able to grow on all three chloroaniline isomers (2-, 3-, and 4-chloroaniline) via the *ortho*-cleavage pathway, even when they are present as a mixture (Zhang et al. 2010). In addition, strain H1 can degrade substituted aniline compounds such as 2,3-, 2,4-, and 3,4-dichloroaniline as well as 4-methylaniline (Zhang et al. 2010).

Pathways for the degradation of chlorobenzenes and anilines are typically plasmid-encoded in *Betaproteobacteria*. In addition, the genes that encode for two initial enzymes for di- and trichlorobenzene degradation in *Acidovorax* sp. P51 (*tcbAaAbAcAdB*) are located on a transposable element, Tn5280, which contains insertion elements (IS1066 and IS1067) at the end of the transposon (van der Meer et al. 1991). These genes are most closely related to genes encoding toluene dioxygenase in *Pseudomonas putida* F1 (*todC1C2BAD*), and benzene dioxygenases in *P. putida* ML2 (*bedC1C2BA*) and *P. putida* 136-R3 (*bnzABCD*) (Irie et al. 1987; Tan 1993; Werlen et al. 1996; Zylstra and Gibson 1989). In the di- and trichlorobenzene degrading *Acidovorax avenae* CBA1, the alpha subunit of chlorobenzene dioxygenase was more closely related to those of isopropylbenzene dioxygenase from *P. putida* RE204 and biphenyl dioxygenase from *Ralstonia oxalatica* than to that from the chlorobenzene dioxygenase in *Acidovorax* sp. P51 (Monferrán et al. 2005). These findings suggest that horizontal gene transfer may have played a role in the evolution of aromatic compound catabolism in bacteria.

Interestingly, most reported nitrobenzene and nitrotoluene-degrading isolates appear to be members of the *Betaproteobacteria*, and include *Burkholderia*, *Comamonas*, *Acidovorax*, and *Diaphorobacter* strains (Table 1) (Lessner et al. 2003; Nishino et al. 2000; Nishino and Spain 1995; Singh and Ramanathan 2013; Suen et al. 1996). The reason for this is not clear; however, the dioxygenase enzyme that catalyzes the initial step in nitroarene compound degradation pathways is more closely related to naphthalene dioxygenases from *Betaproteobacteria* (the *nag* pathway, described above) than those from the *nah*-like pathways in *Pseudomonas* (Parales 2000). It is possible that the genes encoding the dioxygenase in the *nag* pathway were more amenable to the changes necessary to generate an enzyme able to accept nitroarene substrates. Alternatively (or in addition), it is possible that these genes were more mobile in the environment, moving more frequently between related *Betaproteobacteria* and allowing the appropriate mosaic pathways to be generated. In support of this idea, genes encoding 2,4-dinitrotoluene degradation are known to be plasmid encoded in *Burkholderia* sp. strain DNT and *Burkholderia cepacia* R34 (Johnson et al. 2000; Suen and Spain 1993). Similarly, in *Comamonas* sp. strain CNB-1, which grows on nitrobenzene as well as 4-chloronitrobenzene using a partial reductive pathway, the relevant genes are located on a large catabolic

plasmid (Wu et al. 2006). In contrast, genes for nitrobenzene/nitrotoluene degradation in *Diaphorobacter* sp. strains DS1, DS2, and DS3 are located on the chromosome (Singh et al. 2014).

5 Alkane Degradation by *Betaproteobacteria*

Over the last three decades, a handful of betaproteobacterial strains capable of short- and long-chain alkane degradation have been identified (Table 3). *Burkholderia cepacia* RR10 was isolated following enrichment with aromatic oil residue from a refinery (Yuste et al. 2000). It was found to grow aerobically on long-chain alkanes with 12–34 carbons, phenylhexadecane, and the branched alkane pristane. Of seven isolates analyzed from this study, it was the only member of the *Betaproteobacteria*. At the other end of the spectrum, short-chain *n*-alkanes (C_2–C_9) are growth substrates for "*Pseudomonas butanovora*" (Takahashi et al. 1980), which has since been reclassified as *Thauera butanovorans* Bu-B1211 (Dubbels et al. 2009). The ability of this strain to degrade butane has been studied in detail and involves a soluble multicomponent diiron butane monooxygenase (BMO) that is closely related to soluble methane monooxygenase (Sluis et al. 2002). BMO has broad substrate specificity and single amino acid substitutions resulted in variants capable of oxidizing chlorinated ethenes (Halsey et al. 2007). *Polaromonas vacuolata* JS666, in contrast, grows on the *n*-alkanes heptane and octane (Mattes et al. 2008) and can also use *cis*-1,2-dichloroethene as a carbon and energy source under oxic conditions (Coleman et al. 2002). The complete pathway for *cis*-1,2-dichloroethene degradation by this strain has been elucidated, and oxidation is initiated by a cytochrome P450 enzyme rather than a multicomponent monooxygenase (Nishino et al. 2013). More recently, *Acidovorax* sp. CHX100 was reported to degrade cyclohexane through the activity of a novel cytochrome P450 monooxygenase that converted cyclohexane to cyclohexanol, which is subsequently converted to cyclohexanone and fed into the lactone formation pathway (Salamanca and Engesser 2014; Salamanca et al. 2015). The novel monooxygenase was also capable of converting other C5–C8 cycloalkanes into their cyclic alcohol forms (Salamanca et al. 2015).

Anaerobic degradation of *n*-alkanes (reviewed in Wilkes et al. 2016) has also been reported to occur in betaproteobacterial isolates (Table 3). *Aromatoleum* sp. strain HxN1 was isolated for its ability to grow on *n*-hexane under nitrate reducing conditions (Ehrenreich et al. 2000). This strain is capable of growth on C6–C8 *n*-alkanes both aerobically and under nitrate- reducing conditions (Zedelius et al. 2011). The same research group isolated an anaerobic octane-degrading betaproteobacterial strain (Ehrenreich et al. 2000). This strain (designated OcN1) grew on *n*-alkanes with from 8 to 12 carbons under the same conditions (Ehrenreich et al. 2000; Zedelius et al. 2011). Anaerobic *n*-alkane metabolism in these strains is initiated by a benzylsuccinate synthase-like enzyme that adds the C2 carbon of *n*-alkanes to fumarate to form (1-methylalkyl)succinate (Rabus et al. 2001). Interestingly, although unable to grow on toluene, alkane-grown cultures were able to convert toluene to benzylsuccinate, suggesting that the enzyme has broad

specificity for alkylated aromatic compounds as well as alkanes (Rabus et al. 2011). The denitrifying gammaproteobacterial strain HdN1 anaerobically utilizes a wide range of *n*-alkanes (C_6–C_{30}) and apparently employs a denitrification intermediate in a yet unknown mechanism of alkane activation in the absence of external molecular oxygen (Zedelius et al. 2011). Further metabolism by strain HxN1 has been proposed to occur by β-oxidation following CoA esterification, similar to anaerobic benzoate degradation (Wilkes et al. 2016).

6 Conclusions and Research Needs

Betaproteobacteria are clearly important players in the turnover of hydrocarbons, particularly aromatic hydrocarbons and substituted aromatic compounds, in many environments, but especially in soils and aquifers. Although betaproteobacterial strains do not necessarily grow as fast as some *Pseudomonas* isolates on these compounds, they may be more abundant or more efficient degraders in natural environments; their relative contributions in the environment remain to be determined. Further genome sequencing of additional betaproteobacterial strains will also reveal the presence of genes for hydrocarbon degradation pathways in *Betaproteobacteria* that have not been detected to date.

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Marine, Aerobic Hydrocarbon-Degrading *Gammaproteobacteria*: Overview

8

Tony Gutierrez

Contents

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), all obligate hydrocarbonoclastic bacteria (OHCB), represented by the genera *Alcanivorax*, *Cycloclasticus*, *Neptunomonas*, *Oleibacter*, *Oleiphilus*, *Oleispira*, and *Thalassolituus*, are represented within the *Gammaproteobacteria*. Notably, the OHCB appear to be confined to marine environments where they were initially discovered – alluding to an evolutionary genesis in the ocean – and where they are commonly found to become strongly enriched at oil contaminated sites. Prospecting studies aimed in identifying new taxa of hydrocarbonoclastic bacteria from underexplored biotopes in the ocean have uncovered novel strains of OHCB and generalist hydrocarbon degraders

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within the *Gammaproteobacteria*, which has increased the known diversity of hydrocarbonoclastic bacteria that constitute this physiologically and phylogenetically diverse class.

1 Introduction

The *Gammaproteobacteria* is probably the most physiologically and phylogenetically diverse class of bacteria, consisting of 15 orders: *Acidithiobacillales*, *Aeromonadales*, *Alteromonadales*, *Cardiobacteriales*, *Chromatiales*, "Enterobacteriales," *Legionellales*, *Methylococcales*, *Oceanospirillales*, *Pasteurellales*, *Pseudomonadales*, "Salinisphaerales," *Thiotrichales*, "Vibrionales," and *Xanthomonadales* (Garrity et al. 2007). Whilst the class contains several medically important groups of bacteria, principally members of the *Enterobacteriaceae*, *Vibrionaceae*, and *Pseudomonadaceae*, it is the most relevant in that it contains the most important groups of hydrocarbonoclastic bacteria that contribute to the degradation and ultimate removal of hydrocarbon pollutants from marine environments. These include the obligate hydrocarbonoclastic bacteria (OHCB) that are distinguished by their ability to utilize hydrocarbons almost exclusively as a sole source of carbon and energy. This is exemplified by the inability of these organisms to grow on any of the 95 substrates of the BIOLOG® system, with the exception of Tween 40 and Tween 80 – substrates that contain long-chain alkyl groups – and some substrates of central metabolism, such as acetate, propionate, succinate, and/or some amino acids (Yakimov et al. 2007 and references therein). 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*. Several other OHCB (*Algiphilus aromaticivorans*, *Polycyclovorans algicola*, and *Porticoccus hydrocarbonoclasticus*) were recently discovered from an underexplored biotope in the ocean (i.e., the phycosphere of eukaryotic phytoplankton), thus adding to the diversity of known OHCB. A hydrocarbon-degrading *Methylophaga* was also reported recently (Mishamandani et al. 2014), which is intriguing considering that, hitherto, the known substrate spectrum for the *Methylophaga* group had been confined exclusively to C1 sources (methanol, methylamine, dimethylsulfide) as sole carbon and energy sources, with the exception of some species that are also capable of utilizing fructose (Janvier and Grimont 1995).

The *Gammaproteobacteria* also comprises diverse groups of aerobic "generalist" hydrocarbon degraders that can utilize hydrocarbons and nonhydrocarbon substrates as a source of carbon and energy. In contrast to the OHCB, which tend to be highly specialized for utilizing hydrocarbons as a carbon and energy source, generalist hydrocarbon degraders are more metabolically versatile and capable of utilizing a much wider spectrum of organic substrates. Quite often, generalists are overlooked because evidence to substantiate their involvement in the degradation of hydrocarbons may be lacking. For example, the enrichment of a particular taxonomic group in the presence of hydrocarbons, such as from its detection and dominant

representation in a sequencing survey of oil-contaminated seawater, is certainly indicative but not sufficient evidence to substantiate that it plays a direct (i.e., partake initial steps in the degradation of hydrocarbon compounds) role in degrading oil hydrocarbons in that environment. Nonetheless, generalist *Gammaproteobacteria* can constitute a significant proportion of a hydrocarbon-degrading community in marine environments, and they include, but not limited to, members within the genera *Acinetobacter*, *Colwellia*, *Glaciecola*, *Halomonas*, *Marinobacter*, *Marinomonas*, *Methylomonas*, *Pseudoalteromonas*, *Pseudomonas*, *Rhodanobacter*, *Shewanella*, *Stenotrophomonas*, and *Vibrio*. *Marinobacter* is possibly the most important of the generalists as members of this genus are commonly found enriched in oil-rich seawater and sediment. This genus, however, is classified at one extreme of the utilizable-substrate spectrum for the generalists, since although its members can grow on non-hydrocarbon substrates (e.g., in marine medium 2216 without added hydrocarbons) they exhibit an almost exclusive preference for *n*-alkanes (Duran 2010). Hence, the genus *Marinobacter* could be considered both generalist and OHCB since its members are recognized for thriving on hydrocarbons (like the OHCB) and also being somewhat metabolically versatile (like the generalists).

This chapter provides an overview on hydrocarbon-degrading *Gammaproteobacteria* comprising the OHCB and generalists that are found in marine environments. Whilst many of these taxa will be discussed in more detail in other chapters of this volume, the physiology, ecology and phylogenetic affiliations of recently discovered hydrocarbonoclastic *Gammaproteobacteria* is presented here.

2 Obligate Hydrocarbonoclastic *Gammaproteobacteria*

The OHCB are specialists with respect to their ability to utilize hydrocarbons almost exclusively as a carbon source. These fastidious hydrocarbon "specialists" appear to be solely confined to the marine environment since, to the best of our current knowledge, they are found nowhere else on earth. The importance of the OHCB to help purging the oceans and seas is evidenced in the wealth of reports documenting their enrichment from abundances near undetectable levels (<0.1%) to constituting up to 90% of the total bacterial community at oil-impacted sites (Head et al. 2006; Yakimov et al. 2007, and references therein). Of the currently established genera of OHCB, most comprise members that specialize in the degradation of linear or branched saturated hydrocarbons (*Alcanivorax*, *Oleiphilus*, *Oleispira*, *Thalassolitus*), whereas two genera, *Cycloclasticus* and *Neptunomonas*, comprise species that specialize in the degradation of polycyclic aromatic hydrocarbons (PAHs) – all are members of the *Gammaproteobacteria* and are covered in detail elsewhere in this volume and/or in the first edition of the *Handbook of Hydrocarbon and Lipid Microbiology* (Timmis et al. 2010). The phylogenetic relationship between these organisms is shown in Fig. 1.

It is worthy to note that although *Alcanivorax* isolates degrade alkane hydrocarbons, none have been described to metabolize PAHs (Schneiker et al. 2006; Gutierrez et al. 2013). *Alcanivorax* has previously been detected in bacterial

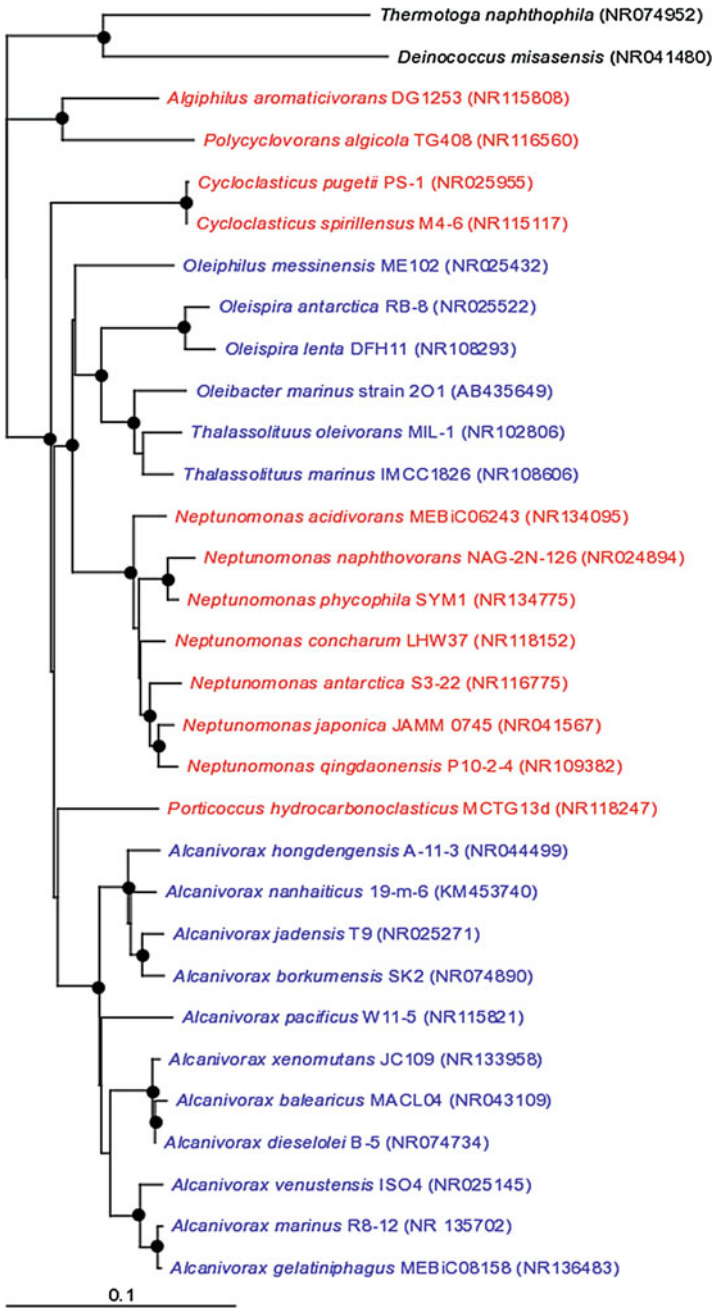

Fig. 1 Phylogenetic tree showing the affiliations of OHCB type strains in the class *Gammaproteobacteria* based on the 16S rRNA gene. Those that specialize in the degradation of saturated hydrocarbons (e.g., *n*-alkanes) are shown in *blue*, whereas those in *red* specialize in the degradation

consortia growing on pyrene as the sole carbon source (Wang et al. 2007; Lai et al. 2011), thus supporting a broader versatility in *Alcanivorax* for degrading not only alkanes but also PAHs, potentially by participating in bacterial consortia that fully metabolize PAHs.

Of importance to also highlight is that the genus *Cycloclasticus* should no longer be considered to have an exclusive appetite for aromatic hydrocarbons since a recent study has revealed that members of this genus, found living in symbiosis with mussels and sponges in deep-sea gas and oil seeps, can utilize short-chain alkanes (Rubin-Blum et al. 2017).

Other OHCB that have recently been discovered (*Algiphilus aromaticivorans*, *Polycyclovorans algicola*, and *Porticoccus hydrocarbonoclasticus*) are less well studied and are presented elsewhere in this volume.

3 Uncultured Putative OHCB

There are likely many more OHCB that remain to be discovered, but either we have not yet searched for them in the right place or they have not yet been coaxed into cultivation in the laboratory so that we may then have the opportunity to directly interrogate them for their hydrocarbon-degrading potential. With the exception of *Planomicrobium alkanoclasticum* of the Firmicutes, all OHCB taxonomic groups that have to-date been characterized constitute genera within the class *Gammaproteobacteria*, principally within the order *Oceanospirillales* (*Alcanivorax*, *Oleiphilus*, *Oleispira*, *Oleibacter Neptunomonas*, *Thalassolituus*) and to a lesser extent of the orders *Xanthomonadales* (*Algiphilus*, *Polycyclovorans*), *Thiotrichales* (*Cycloclasticus*) and *Cellvibrionalles* (*Porticoccus*). The future may still see to the discovery of new OHCB taxa within the *Gammaproteobacteria* as it constitutes one of the most diverse classes of bacteria in terms of physiology and phylogeny. The literature provides a multitude of studies that report on the enrichment, based mainly from microbial community sequencing surveys, of phyla affiliated to the *Oceanospirillales*, *Xanthomonadales*, and *Thiotrichales* at oil-contaminated marine sites. However, this sort of community analysis falls short of providing direct evidence on whether taxa found enriched in such environments can degrade hydrocarbons, and if so whether they are OHCB or generalists.

During the Deepwater Horizon oil spill, the organisms that responded to the massive influx of crude oil into the Gulf of Mexico were principally members of the class *Gammaproteobacteria*. Gene sequencing approaches, such as PCR, clone library construction, and sequencing of 16S rRNA genes (Hazen et al. 2010;

Fig. 1 (continued) of polycyclic aromatic hydrocarbons. The tree was constructed using the neighbor-joining algorithm. *Thermotoga naphthophila* and *Deinococcus misasensis* served as outgroups. Nodes with bootstrap support of at least 80% are marked with a *black circle*. *Bar* represents 0.1 nucleotide substitutions per site

Valentine et al. 2010; Redmond and Valentine 2012), revealed an initial enrichment of a specific cluster within the *Oceanospirillales* (termed DWH *Oceanospirillales*) that was found to dominate an oil plume that formed in the subsurface at 1000–1300 m depth. This cluster constituted up to 90% of bacterial 16S rRNA gene clone libraries (Redmond and Valentine 2012) and pyrosequence libraries (Yang et al. 2016). Other groups of *Gammaproteobacteria* dominated the remaining percentage relative abundance of these oil plume libraries. Genome sequencing of isolated single cells of the DWH *Oceanospirillales* present in oil plume water samples during the DWH spill revealed that they possessed genes encoding *n*-alkane and cycloalkane degradation, including a complete cyclohexane oxidation pathway (Mason et al. 2012). Closest well-characterized relatives (>97% 16S rRNA identity) to this DWH *Oceanospirillales* were *Oleispira antarctica* and *Thalassolituus oleivorans* (Yakimov et al. 2003, 2004), both of which are OHCB with an almost exclusive preference for utilizing aliphatic hydrocarbons as a sole source of carbon and energy. Other noncultivated and less well-characterized members of the *Oceanospirillales* have been detected in high relative abundance at oil-contaminated marine environments (see, for example, Lanfranconi et al. 2010; Coulon et al. 2012) and may represent new taxa of OHCB. However, in the absence of cultivated representatives, substantiating their hydrocarbon-degrading qualities, as in whether they are "true" OHCB, may require exploring genome sequencing approaches.

4 Generalist Hydrocarbonoclastic *Gammaproteobacteria*

A number of gammaproteobacterial generalists, such as *Acinetobacter*, *Marinobacter*, *Pseudomonas* and many others, contribute to hydrocarbon degradation in marine environments. Hydrocarbonoclastic generalists are commonly found in oxygenated environments, such as the sea surface, subsurface, and surficial sediment. Whereas the OHCB are recognized as a key group that responds and becomes strongly enriched following oil contamination in the marine environment, the role that generalists play and contribute to the degradation of oil hydrocarbons is often overlooked. Generalist hydrocarbonoclastic bacteria can in fact constitute a significant proportion of a hydrocarbon-degrading community. To exemplify, generalist oil-degraders became significantly enriched during the DWH oil spill. Almost 2 months after the onset of the spill, the initial dominance of DWH *Oceanospirillales* in the subsurface oil plume was then succeeded by members affiliated to *Cycloclasticus* and *Colwellia* (Valentine et al. 2010; Yang et al. 2016). The genus *Colwellia* had not been recognized for degrading hydrocarbons, so its enrichment in the plume during the active phase of the spill was surrounded by conjecture as to its direct role in degrading the oil. However, a representative strain designated RC25 was isolated from an uncontaminated deep water sample (at plume depth near the DWH site) and confirmed by empirical means to be capable of degrading hydrocarbons (Bælum et al. 2012). In sea surface oil slicks in the Gulf of Mexico during the DWH spill, other hydrocarbonoclastic generalists of the *Gammaproteobacteria* became enriched, in particular *Halomonas*, *Alteromonas*, and *Pseudoalteromonas*

(Hazen et al. 2010; Valentine et al. 2010; Gutierrez et al. 2013), although the OHCB *Cycloclasticus* dominated these contaminated surface waters (Gutierrez et al. 2013; Yang et al. 2016).

Like for the OHCB, many of the generalist oil-degraders also fall into the order *Oceanospirillales*. In San Diego Bay sediments, members of the *Halomonas*, *Marinobacter*, *Marinomonas*, *Pseudoalteromonas*, and *Vibrio* were isolated and found able to grow on phenanthrene or chrysene (Melcher et al. 2002). Possibly the most well-known gammaproteobacterial oil-degrading genus is *Pseudomonas*. During the early stages of the DWH oil spill, members of the *Pseudomonas* became enriched in subsurface waters of the Gulf of Mexico (Dubinsky et al. 2013), and enrichment of Baltic Sea sediment with phenanthrene revealed a large diversity of cultivated and uncultivable putative PAH-degrading *Gammaproteobacteria* that included members of the genera *Pseudomonas*, as well as *Methylomonas* and *Shewanella* (Edlund and Jansson 2008). In a study by Chronopoulou et al. (2015), which aimed to determine the effect of light crude oil on the bacterial communities in the water column of the North Sea, no OHCB were isolated or detected, whereas the marine generalist *Pseudoalteromonas* was identified as the most common hydrocarbon degrader in this environment. Other oil-degrading gammaproteobacterial generalists include members of the *Stenotrophomonas* (Luo et al. 2009) of the order *Xanthomonadales*, *Acinetobacter* (van Beilen and Funhoff 2007; Rojo 2009) of the order *Pseudomonadales*, and *Arhodomonas* (Dalvi et al. 2014) of the order *Chromatiales*. During a survey for flavin-binding monooxygenase genes (*almA*) – involved in *n*-alkane hydrocarbon degradation – in surface seawaters of the Xiamen coastal area and the Atlantic Ocean, several bacterial isolates were identified with this gene, including a *Salinisphaera* sp. (designated strain w510-1) which was capable of degrading diesel oil and paraffin wax (Wang and Shao 2012).

5 Research Needs

The class *Gammaproteobacteria* contain the most important and widely recognized hydrocarbonoclastic bacteria that are commonly found enriched and to contribute importantly to the degradation of petrochemicals in the marine environment. Many of these organisms have been isolated and described in detail. However, quite often the methods used to isolate or detect these organism are biased towards those that, respectively, can be cultivated in the laboratory or that bloom sufficiently to be easily detected, such as by 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. For example, the PAH-degrading specialists *A. aromaticivorans*, *P. algicola*, and *P. hydrocarbonoclasticus* that are found associated with species of eukaryotic phytoplankton are not well represented in the pool of 16S rRNA sequence data that is currently available (i.e., in the GenBank and RDP databases). This may be because their occurrence in the marine environment is possibly confined to a life associated with certain species of phytoplankton from where they were originally isolated, and

their abundance per phytoplankton host cell may be sufficiently low to escape their detection by sequencing approaches (e.g., Sanger sequencing of clone libraries, MiSeq, or pyrosequencing). These peculiar OHCB were identified because of prospecting in the right place (i.e., the bacterial community associated with non-axenic marine phytoplankton) and using targeted enrichment approaches. Many new taxa of hydrocarbonoclastic bacteria are likely to exist in seawater and sediments of the global ocean that await discovery.

A multitude of *Gammaproteobacteria* have been isolated and taxonomically characterized without having been reported to degrade hydrocarbons. This is because in most studies this specific phenotype (i.e., ability to degrade hydrocarbons) was not a test that was included for evaluating the substrate spectrum by the strain under study. One way to expand our knowledge of the known diversity of marine hydrocarbonoclastic bacteria is to encourage strain repositories and research groups that work on marine bacteria to include this test as part of their characterization program. Appreciably, scientists with a vested interest in the field of oil pollution microbiology and the isolation of hydrocarbon-degrading bacteria are likely to take this on board.

In summary, more work is needed to better understand the association of these fastidious and somewhat elusive OHCB with their eukaryotic hosts, as well as their function and ecology in the wider context of oil degradation in the ocean. The demonstration that laboratory cultures of phytoplankton are a source of novel hydrocarbonoclastic bacteria should encourage more detailed study of these organisms and their occurrence with different species of phytoplankton comprising the different lineages. This should also be expanded to investigate the diversity of these organisms when associated with natural populations of phytoplankton in the ocean. Other biotopes in the ocean may be found to harbor novel taxa of OHCB and generalist hydrocarbon-degrading bacteria that await discovery.

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Aerobic Hydrocarbon-Degrading *Gammaproteobacteria*: *Oleiphilaceae* and Relatives

9

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Abstract

Despite the ubiquity of marine hydrocarbon-degrading bacteria from the family *Oleiphilaceae,* until now there is only one strain from this family with a *v*alidly published name and fully assembled genome, *Oleiphilus messinensis* strain ME102 (= DSM 13489). The availability of draft genomes of 27 other isolates gave us the opportunity to get an insight into the genome evolution and speciation patterns within this group. Whole-genome alignments and genome-to-genome distance calculation data demonstrated that *Oleiphilaceae* consists of four distinct genome clusters that correspond to the species level. Furthermore, we suggest that all known *Oleiphilaceae* genomes cluster into two genera, the first one being *Oleiphilus*, which includes *O. messinensis* ME102 and the second represented by bacteria isolated near Hawaii. The *Oleiphilaceae* pangenome of 1796 core gene clusters roughly corresponds to the two-thirds of an *Oleiphilaceae* genome. All high-quality genomes had double copies of *alm*A coding for flavin-binding family monooxygenase linked with degradation of long-chain alkanes. Alkane monooxygenases with pairwise identities between 43% and 86.5% were encoded by four genomes, with two of them having double loci. Cytochromes P450 were present in all genomes and were assigned to two distinct clusters, which, together with the low redundancy of alkane monooxygenases, points at different microorganisms as the sources of acquisition of alkane-monooxygenation enzymes by *Oleiphilaceae*.

1 Introduction

Hydrocarbonoclastic bacteria (HCB) represent wide group of microorganisms involved in degradation of oil and oil derivatives, which are common pollutants in marine environments. HCB are present in *Proteobacteria, Actinobacteria, Firmicutes, FCB (Fibrobacteres, Chlorobi* and *Bacteroidetes)* group bacteria, etc. In turn, known obligate HCB, for which hydrocarbons are sole, or almost sole, source of energy and carbon, are represented only by taxa within *Gammaproteobacteria* with the exception of *Thalassospira (Alphaproteobacteria)* (Berry and Gutierrez 2017). To date, despite the availability of a vast number of metagenome-derived genome sequences through public genome databases, only a small number of studies involved comparative genomics and/or detailed genome-based analysis of phylogenetic diversity within different groups of obligate HCB. One of such examples must be the family *Oleiphilaceae,* which includes *Oleiphilus messinensis* – one of the first discovered obligate alkane degraders, isolated and described in 1998 (Yakimov et al. 1998) and sequenced only recently in 2017 (Toshchakov et al. 2017).

Here we present a brief analysis of publicly available *Oleiphilaceae* genomes, including verification of taxonomic attribution based on 16S rRNA genes, average nucleotide identity, and pangenome analysis.

2 Type Strain, Global Distribution, and Genomic Properties of *Oleiphilaceae*

The type strain, *O. messinensis* strain ME102 (= DSM 13489), was isolated in Messina harbor, Italy (38° 11′ 22" N; 15° 33′ 55″ E) from sediments polluted by hydrocarbons from heavy marine traffic, and formed a new genus, *Oleiphilus*, within the novel family *Oleiphilaceae* in the order *Oceanospirillales* (*Gammaproteobacteria*) (Golyshin et al. 2002). For many years, *O. messinensis ME102* was the only strain in the *Oleiphilaceae* with a validly published name, despite the presence of *Oleiphilus*-related bacteria, e.g., in:

1. Subtidal sediments of the North Atlantic coast of Spain impacted by tanker Prestige oil spill (JQ580103.1, Rodas Beach, 42° 13′ 56″ N; 8° 53′ 50″ W and JQ579692.1, Figueiras Beach, 42° 8′ 8″ N; 8° 32′ 6″ W) (Acosta-González et al. 2013)
2. Chronically contaminated coastal sediments of Etang de Berre lagoon (FM242233.1, France, 43° 28′ 00″ N; 5° 10′ 00″ E) (Yakimov and Golyshin 2014)
3. 100-m-deep water sampled from the Southern Ocean iron fertilization experiment (JX530194.1, Southern Ocean, 47° 30′ 05″ S, 15° 26′ 42″ W) (Singh et al. 2015)
4. Enrichment cultures, derived from samples taken from the bottom of euphotic zone (deep chlorophyll maximum, 95 m depth) and the upper mesopelagic zone (250 m depth) near Hawaii islands (22° 46′ 41.5″ N, 158° 04′ 10.2″ W) (Sosa et al. 2017)

Thus, *Oleiphilaceae* can be found in a wide range of marine environments from tropical to moderate, in both the water column and sediments, showing high potential for adaptation to environmental stimuli in diverse and rapidly changing marine environments (Fig. 1).

The complete genome of *O. messinensis* ME102^T was sequenced and analyzed recently, showing the presence of genes necessary for both short- and long-chain alkane catabolism, as well as coding potential for utilization of other alkane derivatives, e.g., gene for haloalkane dehalogenase (Toshchakov et al. 2017). Notably, strain ME102^T showed an unprecedented level of genome mobility for OHCB, stressing the importance of wider genome analysis of *Oleiphilaceae* representatives to better understand their biology and diversity. In 2017, the family *Oleiphilaceae* gained 27 draft genomes, acquired during a study of bacterial degraders of phosphonates associated with high-molecular-weight dissolved organic matter (HMWDOM) produced by photosynthetic microorganisms of surface waters. Water samples for that study were taken near Hawaii Islands from the bottom of euphotic zone (deep chlorophyll maximum, 95 m depth) and the upper mesopelagic zone (250 m depth). These samples were used for setting up series of enrichments amended with HMWDOM collected by ultrafiltration of seawater collected at 20 m

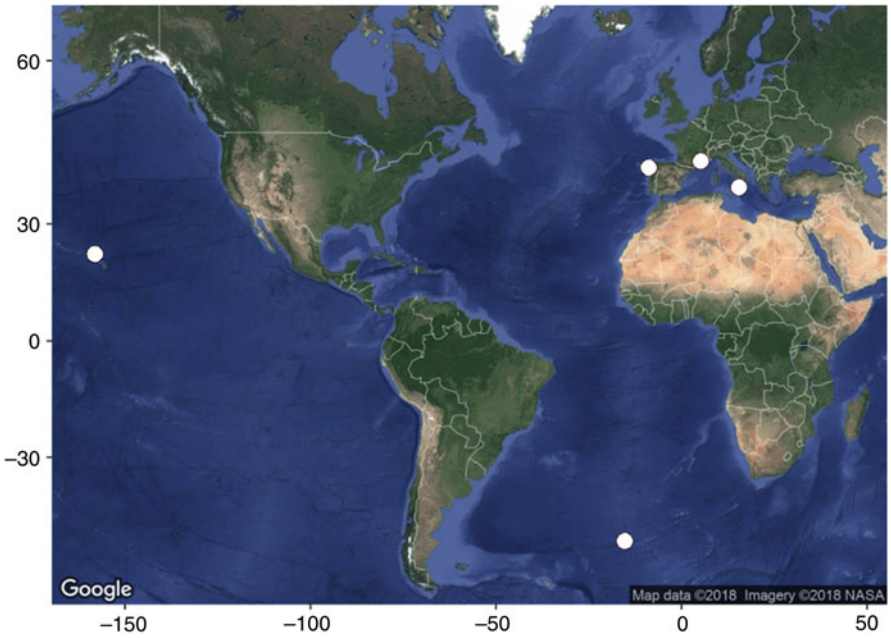

Fig. 1 Sampling sites where *Oleiphilaceae* bacteria were detected. White circles mark sampling sites, where 16S rRNA gene sequences of *Oleiphiliaceae* bacteria were detected, in particular: sediments of Messina harbor, Sicily, site of *O. messinensis* type strain isolation (CP021425); subtidal sediments of the North Atlantic coast of Spain (JQ580103 and JQ579692), chronically contaminated coastal sediments of Etang de Berre lagoon (FM242233), 100 m-deep water sampled from the Southern ocean iron fertilization experiment (JX530194)

depth. High-throughput sequencing of the obtained cultures showed that the most abundant group was *Oleiphilus*-related *Gammaproteobacteria*, which was specifically enriched in mesopelagic samples. Analysis of correlation of abundance of *Oleiphilus*-related OTUs with depth showed that it continuously increased with sampling depth, showing maximum at >200 m depth. The authors also reported that the growth rate of *Oleiphilaceae* isolates had been significantly stimulated by addition of HMWDOM, hydrocarbon compounds, and fatty acids, reflecting a broad substrate specificity of *Oleiphilaceae* representatives (Sosa et al. 2017).

3 Genome-Based Assessment of Taxonomic Relatedness in *Oleiphilaceae*

As of January 2019, 28 genome assemblies attributed to *Oleiphilus* were deposited in NCBI GenBank databases, with only one complete genome belonging to *O. messinensis* ME102^T (Table 1). All available genome assemblies were assessed for completeness and contamination using CheckM (Parks et al. 2014) with *Oceanospirillales* marker set. *O. messinensis* ME102^T showed 99.13% completeness lacking only three marker genes and having five duplicated marker genes, which can be

Table 1 ***Oleiphilaceae*** **genome assemblies** available by January 2019 in NCBI Assembly database

Cluster	GenBank accession	Strain	Contig number	Assembly length	N50	Completeness, %	Contamination, %	Strain heterogeneity, %
4	GCA_002162375.1	*O. messinensis* ME102	1	6379281	6379281	99.13	1.67	0
3	GCA_001635535.1	HI0009	2650	6316839	16347	99.53	89.45	88.08
1	GCA_001634935.1	HI0043	1975	5472755	25101	99.31	49.91	93.57
1	GCA_001635615.1	HI0050	2036	4819895	5000	97.29	38.33	89.22
1	GCA_001635675.1	HI0061	1885	5095272	8507	99.04	40.59	89.59
2	GCA_001635715.1	HI0065	2693	6398614	28355	99.56	93.42	96.27
3	GCA_001635725.1	HI0066	1887	5044867	22586	99.31	56.3	93.89
3	GCA_001635735.1	HI0067	1752	4840075	20427	99.53	49.52	93.1
1	GCA_001635765.1	HI0068	2001	4842151	5172	98.34	29.58	87.58
1	GCA_001635795.1	HI0069	2238	5901752	14127	99.25	60.24	90.16
2	GCA_001635805.1	HI0071	2289	5698317	24676	99.56	70.33	95.28
1	GCA_001634975.1	HI0072	2192	4921543	4687	98.82	36.27	90.05
2	GCA_001634985.1	HI0073	2628	6103244	56040	99.34	90.21	96.07
1	GCA_001635075.1	HI0078	2530	4431832	2905	96.47	20.89	76.11
2	GCA_001635105.1	HI0079	1448	4407017	13007	99.56	33.79	91.33
2	GCA_001635135.1	HI0080	1249	3938001	8128	99.56	22.59	87.96

(*continued*)

Table 1 (continued)

Cluster	GenBank accession	Strain	Contig number	Assembly length	N50	Completeness, %	Contamination, %	Strain heterogeneity, %
1	GCA_001635145.1	HI0081	2463	4402732	3030	98.43	23.99	88.06
1	GCA_001635215.1	HI0085	3230	4482542	2080	97.01	28.47	83.23
1	GCA_001635225.1	HI0086	1534	4753747	10771	99.25	28.81	90.32
1	GCA_001635235.1	HI0117	1521	4620570	7176	98.49	19.8	88.19
2	GCA_001635265.1	HI0118	2826	6560891	15926	99.56	99.67	94.23
2	GCA_001635305.1	HI0122	2490	6039240	37188	99.56	82.6	94.25
1	GCA_001635315.1	HI0123	1909	4468126	5022	98.21	21.72	85.12
3	GCA_001635355.1	HI0125	1531	4553828	19709	99.53	47.16	92.75
1	GCA_001635365.1	HI0128	1636	4885910	11631	98.49	36.07	92.75
2	GCA_001635385.1	HI0130	2064	5194401	10326	99.56	52.88	96.11
1	GCA_001635845.1	HI0132	1901	5170237	9473	98.82	36	92.27
2	GCA_001635835.1	HI0133	2393	5728633	47727	99.56	73.76	94.93

Table 2 Reassembled *Oleiphilaceae* draft genomes, representing distinct clusters, revealed with ANI analysis and clustering

Cluster	Strain	Contig number	Assembly length	N50	Completeness, %	Contamination, %	Strain heterogeneity, %
3	HI0009	235	3593725	41038	99.53	4.27	0
1	HI0043	504	3686850	12403	98.60	2.65	9.09
2	HI0118	98	3382672	119088	99.56	0.96	0

explained by extensive gene transfer and a large number of mobile elements in the genome (Toshchakov et al. 2017). Initial assemblies of the "Hawaiian group" had mean completeness 98.92 ± 0.85% and mean contamination 49.35 ± 24.88% (contamination ranged from 19.80% to 99.67%). Genome contamination of more than 15% usually is considered as "very high" and occurs mostly in single-cell assemblies and metagenome-derived genomes (Parks et al. 2017). Detailed information about all publicly available *Oleiphilaceae* genome assemblies is shown in Table 1.

Because of the poor quality of the "Hawaiian group" genome assemblies, draft genomes were reassembled using raw sequencing reads from the study of Sosa and coauthors, deposited in NCBI Sequence Read Archive (Kodama et al. 2011). Sequencing reads were trimmed by quality and filtered by length using fastq-mcf tool from ea-utils package (Aronesty 2013). Overlapping paired reads were merged using seqprep tool (https://github.com/jstjohn/SeqPrep). De novo genome assembly was performed using SPAdes 3.10 (Bankevich et al. 2012) in "careful" mode with default settings and automatic choice of k-mer length. Reassembled genomes showed much better levels of completeness and contamination, indicating the possibility of minor contamination of dilution-to-extinction enrichment cultures obtained by Sosa et al., which for some reasons significantly compromised assembly quality. Thus, initial assemblies had a mean length of 5.15 Mbp with significant levels of contamination and strain heterogeneity (Table 1), while reassembled genomes had a mean length of 3.39 Mbp, mean completeness of 94.06 ± 7.07%, and mean contamination of 3.20 ± 1.51% (Table 2). To assess correspondence of two assembly pipelines, average nucleotide identities (ANI) between original genome assemblies (Sosa et al. 2017) and respective reassembled genomes were calculated using ani.rb script from the "enveomics" tool collection (https://github.com/lmrodriguezr/enveomics; Rodriguez-R and Konstantinidis 2016). Analysis showed that all ANI values were close to 100%, indicating that biological conclusions made by Sosa and coauthors shouldn't be compromised by the low levels of homogeneity of their assemblies.

4 Four Distinct Genomic Clusters Within *Oleiphilaceae*

Reassembled genomes from 27 "Hawaiian" strains (Sosa et al. 2017) and genome of *O. messinensis* ME102 (Toshchakov et al. 2017) were clustered on the basis of ANI (calculated as described before) in R environment using hierarchical cluster

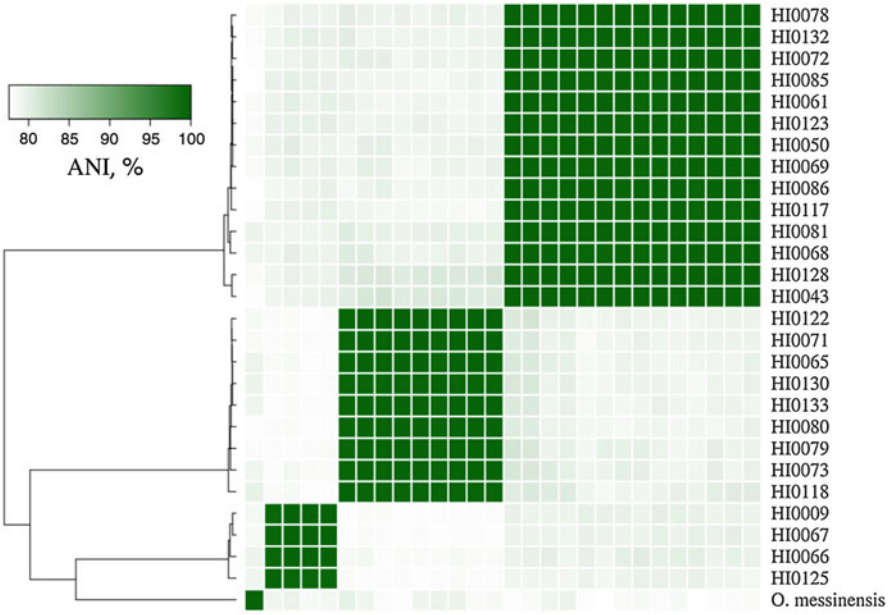

Fig. 2 Average nucleotide identity between *Oleiphilaceae* genomes. Heatmap displays hierarchical clustering of *Oleiphilaceae* genomes based on average nucleotide identities between genome assemblies. Color represents the ANI value (white corresponding to 77.61% ANI and dark green to almost identical genomes with 99.99% ANI)

analysis (Fig. 2). ANI values were in the range from 77.61% to 99.99%. Hierarchical clustering resulted in four distinct genome clusters (Fig. 2). The minimal intra-cluster ANI value was 99.41 ± 0.36%, which leads to the conclusion that each cluster contains genomes of same subspecies. For several intra-cluster pairs, digital DNA–DNA hybridization (dDDH) was performed using genome-to-genome distance calculator (Meier-Kolthoff et al. 2013) resulting in values close to 100% (data not shown). Clustering of reassembled genomes by ANI value showed the same results as clustering of original assemblies (data not shown).

For further analysis, one genomic assembly was selected from each cluster on the basis of high genome completeness, number of contigs, N50 value, and level of contamination: *Oleiphilus* sp. HI0043, *Oleiphilus* sp. HI0118, *Oleiphilus* sp. HI0009, and *O. messinensis* strain ME102^T. 16S rRNA genes were identified using Infernal tool (Nawrocki and Eddy 2013) and rfam database (Nawrocki et al. 2014) and aligned against each other using BLASTn (Altschul et al. 1990). *O. messinensis* ME102^T had the most distant 16S rRNA gene sequence showing just 93.37% average identity to Hawaiian strains (Table 3). Meanwhile, 16S rRNA genes of HI0043, HI0118, HI0009 had an identity of more than 96.5% (Table 3). dDDH were performed for all possible pairs of the four genomes, and showed that no one pair of genomes could be considered as the same species (data not shown).

Table 3 16S rRNA gene identity matrix, values at intersections equals to gene identity in percent

	Oleiphilus sp. HI0043	*Oleiphilus* sp. HI0118	*O. messinensis* ME102^{TT}
Oleiphilus sp. HI0009	96.74	98.17	93.56
Oleiphilus sp. HI0043		96.54	92.91
Oleiphilus sp. HI0118			93.63

5 New Uncultivated Taxa Within *Oleiphilaceae*

According to 95% 16S rRNA gene identity threshold for genus delimitation (Stackebrandt and Goebel 1994), we suppose that *Oleiphilus* sp. HI0043, *Oleiphilus* sp. HI0118, and *Oleiphilus* sp. HI0009 form a new genus of *Oleiphilaceae.* Analysis of ANI values and dDDH estimation suggest that these genomes represent three different species. Thus, while 16S rRNA gene sequences of *Oleiphilus* sp. HI0118 and *Oleiphilus* sp. HI0009 have 98.17% identity, falling close to the 98.65% 16S rRNA identity threshold (Kim et al. 2014), the ANI value between these genomes is significantly less than the 95% threshold for ANI-based species delimitation proposed by Richter and Rossello-Mora (2009).

The 16S rRNA sequences of *O. messinensis*, representatives of the "Hawaiian group" and the closest 16S rRNA gene sequences from uncultivated microorganisms found in NCBI nr nucleotide database, were used for phylogenetic reconstruction of *Oleiphilaceae* (Fig. 3). Multiple sequence alignment was performed using MUSCLE (Edgar 2004) and a phylogenetic tree was inferred using RAxML (Stamatakis 2014) with GTR-Gamma model. The phylogenetic reconstruction supports our suggestions and suggests the possible presence of 3–4 genus-level and 5–6 species-level lineages based on 16S rRNA gene sequences.

6 Pangenome, Hydrocarbon Utilization, and Osmoprotection

Reassembled genomes of strains HI0043, HI0118, and HI0009 and the genome of *O. messinensis* strain ME102 were used for prediction of protein-coding genes and sequential pangenome analysis. Protein-coding sequences were predicted using prodigal in single-genome mode (Hyatt et al. 2010). Pangenome analysis was made using orthoMCL according to instructions provided by software developers (Li et al. 2003). The results of pangenome analysis were visualized using ClusterVenn web server (Wang et al. 2015). *Oleiphilaceae* pangenome consists of 3117 clusters including 11,488 protein-coding genes, 1686 of them were single-copy gene clusters. Furthermore, clusters containing genes from only one genome were

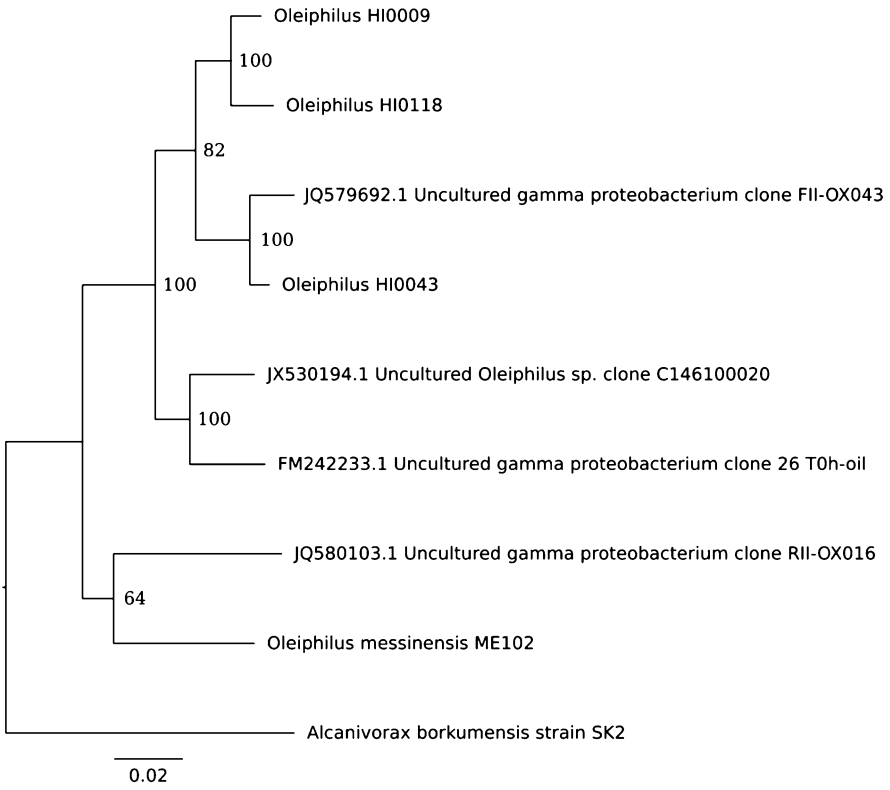

Fig. 3 Phylogenetic reconstruction of *Oleiphilaceae* based on 16S rRNA gene sequence. Acc. No. JQ580103.1 clone RII-OX016 and acc. no. JQ579692.1 clone FII-OX043 from subtidal sediments of North Atlantic coast of Spain impacted by tanker Prestige oil spill, acc.no. FM242233.1 clone 26 T0 h-oil from chronically contaminated coastal sediments of Etang de Berre lagoon, France, acc. no. JX530194.1 clone C146100020 from 100 m depth water samples derived from the Southern Ocean iron fertilization experiment (Singh et al. 2015). Bootstrap support values displayed next to nodes (based on 100 resamplings). 16S rRNA gene sequence of *A. borkumensis* was used as an out-group. The scale bar represents estimated number of nucleotide substitutions per site

found: 198 such clusters for *O. messinensis* strain ME102, 25 for HI0009, 24 for HI0043, 12 for HI0118. These clusters mainly consist of mobile elements or proteins with unknown function, supporting the remote phylogenetic position of *O. messinensis* ME102^T relative to "Hawaii strains." 3997 genes were found to be singletons (825 for HI0043, 840 for HI0009, 338 for HI0118, 1994 for ME102, which is in good agreement with genome size). Strains HI0009 and HI0118 have the closest proteomes, supporting the 16S rRNA-based phylogenetic reconstruction: 2370 shared gene clusters, 228 and 273 unique clusters, respectively (Fig. 4).

We conducted the analysis of gene clusters responsible for hydrocarbon utilization in genomes of *Oleiphilaceae* bacteria. Genes of enzymes involved in hydrocarbon utilization and ectoine biosynthesis were identified either by alignment against

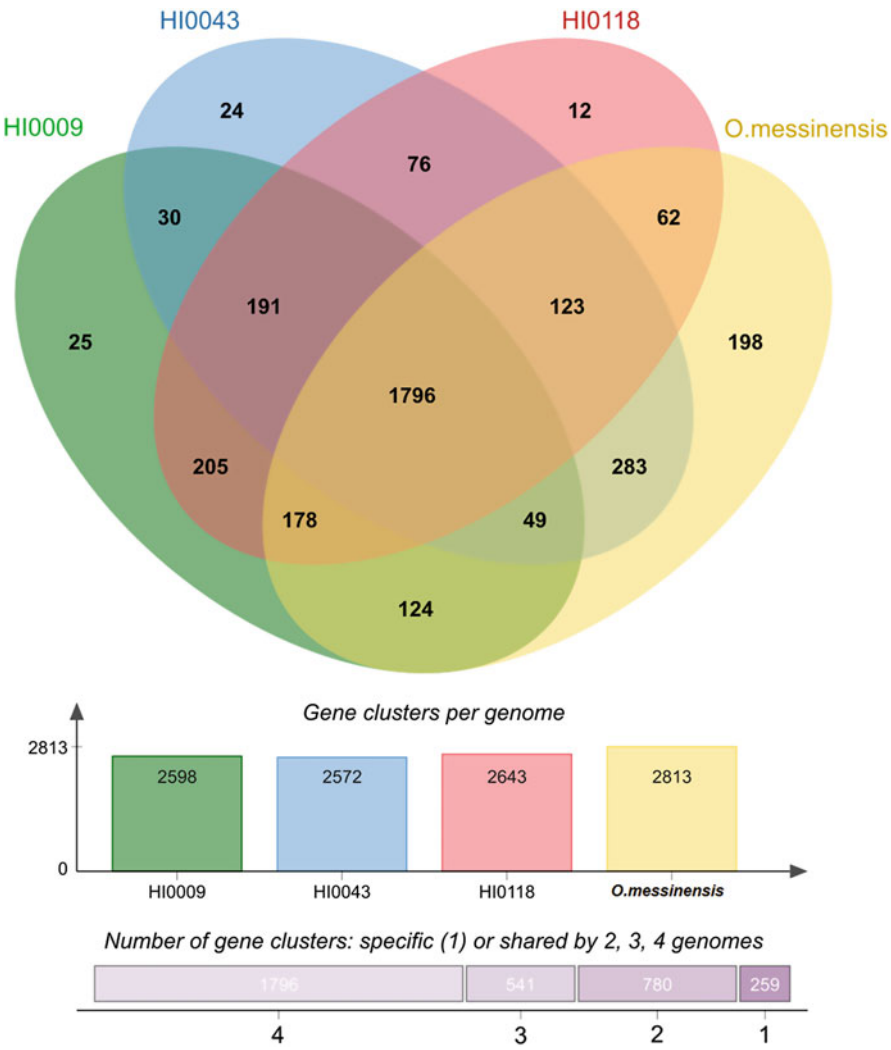

Fig. 4 ***Oleiphilaceae*** **pangenome**. Top chart: shared and unique gene clusters in *Oleiphilaceae* genomes. Middle panel: gene clusters number shown for each genome. Bottom panel: numbers of gene clusters shared by 4, 3, or 2 genomes and specific gene clusters

annotated sequences from NCBI nr protein database using BLASTp algorithm (Altschul et al. 1997) or by alignment against respective pfam hmm profile (Finn et al. 2015) using hmmsearch tool from HMMER package (Eddy 2011). Two clusters of cytochrome P450 monooxygenases were identified: one containing proteins presented in all four genomes analyzed and the second presented in only three genomes (HI0009, HI0118, and *O. messinensis* 102^T). Notably, *O. messinensis* $ME102^T$ genome contains three genes of CYP450, one of which is disrupted by an active IS4 mobile element, therefore accentuating the dynamic nature of

Oleiphilaceae genomes (Toshchakov et al. 2017). The gene *almA* coding for flavin-binding family monooxygenase, linked with long-chain alkanes degradation (Shao and Wang 2013), was found twice in all genomes except that from strain HI0043. All seven sequences formed one orthologous cluster. Alkane monooxygenases were present as single copies in *O. messinensis* strain ME102^T and strain HI0009, and double copies in strains HI0043 and HI0118.

Ectoine is a compatible solute which protects bacteria against osmotic stress (Louis and Galinski 1997). To study possible differences in adaptation to lower temperatures, high osmosis, or elevated hydrostatic pressure, we analyzed genetic loci responsible for ectoine biosynthesis. The ectoine synthesis operon *ectABCR* includes genes coding for: diaminobutyric acid (DABA) aminotransferase (EctB), DABA acetyltransferase (EctA), ectoine synthase (EctC), and MarR-like transcriptional regulatory protein (EctR). This operon was found in all *Oleiphilaceae* genomes except HI0009, which was sampled from a depth of 95 mbsf as opposed to HI0043 and HI0118, sampled from 250 mbsf (Sosa et al. 2017). Increased level of transcription of ectoine biosynthesis operon at elevated hydrostatic pressure was reported for *Alcanivorax borkumensis* (Scoma et al. 2016). Despite the unclear role of ectoine in the response to stress induced by hydrostatic pressure, the lack of an ectoine biosynthesis operon in shallow-water strain HI0009 gives an opportunity to speculate that this solute might be utilized by *Oleiphilaceae* not only as an osmoprotector, but also as a piezoprotector.

7 Conclusion

In this study, we analyzed all publicly available (by the beginning of 2019) *Oleiphilaceae* genomes and closest environmental 16S rRNA gene sequences. Whole-genome alignments and digital DNA–DNA hybridization demonstrated that *Oleiphilaceae* includes four distinct genome clusters that correspond to a species. Additional 16S rRNA gene alignment and phylogenetic reconstruction showed that *Oleiphilaceae* genomes could be divided into two genera: the first includes *O. messinensis* ME102 and the second is represented by bacteria isolated near Hawaii islands. Low quality of assembly and high level of contamination motivated us to reassemble the genomes of the "Hawaiian group" from raw reads available at NCBI SRA. Resulting assemblies had higher completeness, lower contamination, improved N50 metrics, and smaller contig number.

Pangenome analysis of complete and reassembled genomes of *Oleiphilaceae* resulted in 1796 core gene clusters, which roughly correspond to the two-thirds of *Oleiphilaceae* genome. The number of unique genes in the genome is in good agreement with genome size and distance from other genomes. All genomes of *Oleiphilaceae* bacteria have genes responsible for alkane degradation, such as genes coding for alkane monooxygenase, flavin-binding family monooxygenase, and cytochrome P450 monooxygenase.

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Marine, Aerobic Hydrocarbon-Degrading *Gammaproteobacteria*: The Family *Alcanivoracaceae*

10

Michail M. Yakimov, Peter N. Golyshin, Francesca Crisafi, Renata Denaro, and Laura Giuliano

Contents

Abstract

The family *Alcanivoracaceae* contains the group of hydrocarbon-degrading bacteria that, due to their ability to use hydrocarbons as main carbon source, have been described as *obligate hydrocarbonoclastic bacteria (OHCB)*. Currently, the family includes the validly published genera *Alcanivorax* and

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Ketobacter. Most family members are highly specialized in degrading linear and branched alkanes of different origin. They typically dominate marine environments suffering from oil contamination and, through their highly adapted metabolic capabilities, are extremely efficient in the cleanup of marine oil spills. In particular, according to the results of the genome sequence analyses of nine species, they are proficient at scavenging nutrients and microelements, especially iron. They produce biosurfactants and can form biofilms around oil droplets and at the oil–water interface. Recent studies, discussed in this chapter with emphasis on the sequencing surveys, have expanded our knowledge and understanding of the diversity of *Alcanivoracaceae* bacteria, their wide distribution in the natural marine and terrestrial environments (both oil-contaminated and non-contaminated), and their possible association with various marine invertebrates and microalgae.

1 Introduction

Oil pollution, which represents one of the major threats to ocean life, is mainly caused by man's activities at sea, including tanker and pipeline leaks, accidental ocean discharges, and numerous spills of different scales. According to some assessments, oil spills represent around 10% of global marine oil pollution, while most of the oil enters the sea from less obvious pathways, including natural seeps, discharges of tanker ballast water, and off-shore petroleum platform leakages (GESAMP 2007). Oil inputs to marine environments damage resident organisms, due to chemical toxicity and by physical coating and smothering of wildlife. Petroleum hydrocarbons can have short- and long-term effects on the marine food-web.

The ability to degrade hydrocarbons is widespread among the marine prokaryotes. The majority of specialized hydrocarbon-degrading microorganisms belong to *Gammaproteobacteria*, one the most diverse bacterial class both from a physiological and a phylogenetic perspective. It consists of 15 orders, and four of them, namely, *Cellvibrionales*, *Nevskiales*, *Oceanospirillales,* and *Thiotrichales*, comprise important groups of marine crude oil degraders, coined as *obligate hydrocarbonoclastic bacteria* (OHCB). These organisms are distinguished by their ability to utilize almost exclusively hydrocarbons as a sole source of carbon and energy (Head et al. 2006; Yakimov et al. 2007; McGenity et al. 2012; Joye et al. 2016). Another characteristic feature of OHCB is being almost undetectable in pristine marine environments. OHCB typically bloom after an oil spill and can be easily isolated from chronically polluted marine sites. This unusual feature was first recognized after the isolation of strain SK2 from seawater/sediment samples collected near the Isle of Borkum (North Sea), leading to subsequent isolation of many more OHCB. Initial material was enriched on a selective medium with Mihagol-S (a mixture of tetra- and pentadecane) as principal carbon source (Yakimov et al. 1998). According to physiological and phylogenetic analyses, SK2 was described as the novel species *Alcanivorax borkumensis* of the novel genus *Alcanivorax* of the novel family *Alcanivoracaceae*.

This chapter provides an overview on current taxonomy and phylogenetic affiliations of the members of the family *Alcanivoracaceae* along with their physiology, ecology, and biogeography.

2 Descriptive Information and Current Taxonomy

Al.ca.ni.vo.ra'.ca.ce.ae. M.L. masc. n. *Alcanivorax* type and only validly described genus of the family; *-aceae* ending to denote family; M.L. masc. pl. n. *Alcanivoraceae* the *Alcanivorax* family. *Al.ca.ni.vo'.rax.* M.L. masc. n. *alcanum* alkane, aliphatic hydrocarbon; L. adj. *vorax* voracious, gluttonous; M.L. masc. n. *Alcanivorax* alkane-devouring.

Since the first description in Bergey's Manual of Systematics of Archaea and Bacteria (Golyshin et al. 2005), the family *Alcanivoracaceae* did not change its structure and content at the genus level until very recently (Kim et al. 2018). Currently this family contains two genera, *Alcanivorax* and *Ketobacter*. The family name is defined by the type genus. Gram-negative rods are generally 0.6–0.8 μm × 1.6–2.5 μm in size, depending on growth substrate. Some isolates are capable of anaerobic growth, via the first step of denitrification, the reduction of nitrate to nitrite. All members of this family use linear- and branched-chain C_9-C_{30} alkanes and their corresponding alcohols and acids as principal carbon and energy sources. Only a few simple organic compounds are used as an alternative carbon and energy source (formate, acetate, propionate, [methyl]pyruvate, and ketoglutarate), albeit uptake of simple sugars and degradation of gelatine was demonstrated for some species. The optimal NaCl content for growth, typical of moderately halophilic bacteria, is between 30 and 100 g L^{-1}. Strains of all recognized species and recent isolates are mesophilic, with the temperature optima around 25–30 °C. Some isolates produce glucolipid biosurfactants. The main phenotypic characteristics of currently recognized *Alcanivorax* species are listed in Table 1.

The type and validly published genus of the *Alcanivoracaceae* family was first proposed by Yakimov et al. (1998) with *A. borkumensis* as type species. The description of *Alcanivorax* genus was later amended by Fernández-Martínez et al. (2003). Second validly published genus of the *Alcanivoracaceae* family is *Ketobacter* with *K. alkanivorans* as type and only one species (Kim et al. 2018).

The list of prokaryotic names with standing in nomenclature (LPSN) currently keeps four genera in the *Alcanivoracaceae* as follows: *Alcanivorax*, *Fundibacter*, *Kangiella*, and *Pleionea* (www.bacterio.net). Apparently this status needs revision, since there are many discrepancies. Firstly, the single representative species of the genus *Fundibacter* (Bruns and Berthe-Corti 1999) was reclassified later as *Alcanivorax jadensis* (Fernández-Martínez et al. 2003). Secondly, based on molecular analyses of 16S rRNA gene sequences, Silveira and Thompson (2014) originally assigned the genus *Kangiella* to *Alcanivoracaceae*. However, owing to follow-up phylogenetic, chemotaxonomic, and physiological characteristics, this genus, together with the genus *Pleionea,* was integrated into the new

Table 1 Differential phenotypic characteristics among 12 species of the genus *Alcanivorax*

	1.	2.	3.	4.	5.	6.	7.	8.	9.	10.	11.	12.
Flagella	+	–	+	+	–	–	+	–	–	+	+	+
Na^+ requirement	–	+	+	+	+	+	+	+	+	+	+	+
Growth at 170 g L^{-1}	–	–	w	+	–	–	–	–	–	+	+	–
Tween 20 hydrolysis	–	+	+	+	+	+	+	ND	ND	+	+	+
NO_3^- reduction	ND	+	+	–	+	+	–	–	+	–	–	+
Gelatinase	–	–	–	+	+	–	–	–	ND	–	–	–
Growth on[a]:												
Glucose	w	–	–	–	–	–	–	–	+	–	–	–
Arabinose	w	–	–	–	–	–	–	–	+	–	+	–
Acetate	w	+	+	+	+	+	+	+	+	+	+	+
Lactate	–	–	+	+	+	w	–	–	+	w	–	ND
Propionate	w	+	–	+	+	+	+	+	+	+	+	–

Taxa: 1. *A. balearicus* (Rivas et al. 2007); 2. *A. borkumensis* (Yakimov et al. 1998); 3. *A. diselolei* (Liu and Shao 2005); 4. *A. gelatiniphagus* (Kwon et al. 2015); 5. *A. hongdengensis* (Wu et al. 2009); 6. *A. jadensis* (Bruns and Berthe-Corti 1999); 7. *A. marinus* (Lai et al. 2013); 8. *A. nanhaiticus* (Lai et al. 2016); 9. *A. pacificus* (Lai et al. 2011); 10. *A. venustensis* (Fernández-Martínez et al. 2003); 11. *A. xenomutans* (Rahul et al. 2014); 12. *A. mobilis* (Yang et al. 2018)
[a]as sole source of carbon and energy; *ND* not demonstrated; *w* weak growth

family, *Kangiellaceae* fam. nov., in the order *Oceanospirillales* (Wang et al. 2015). In the NCBI Taxonomy Database, status of the family *Alcanivoracaceae* is also somehow vague and along with genus *Alcanivorax* currently includes the validly published genera *Marinicella* and *Ketobacter*. To date, the genus *Marinicella* contains two recognized and one proposed species *M. litoralis*, *M. pacifica,* and *M. marina*, respectively. It should be noted that the genus *Marinicella* originally was considered to be more closely related to the genera *Kangiella* and *Pleionea* and only distantly related to the genus *Alcanivorax* (Romanenko et al. 2010, 2012; Wang et al. 2016). We agree with this assessment, since accordingly to the phenotypic characteristics, all members of the genus *Marinicella* are versatile heterotrophic strict aerobes, capable of growth on marine agar 2216, which strongly distinguishes them from representatives of the *Alcanivoracaceae*. Additionally, we performed a phylogenetic analysis of nearly complete 16S rRNA sequences using various algorithms (Fig. 1) and found that *Marinicella* strains form a distinct monophyletic lineage in a clade consisting of members of the genera *Aliikangiella*, *Kangiella,* and *Pleionea* (*Kangiellaceae* family). We also claim that *Alcanivorax*, the type genus of the family *Alcanivoracaceae*, does not form a monophyletic group with the *Kangiella* group but rather forms a robust clade (98% of 1000 bootstraps) with the *n*-alkane-degrading bacterium, isolated from a surface seawater sample collected from Garorim Bay (West Sea, Republic of Korea) (Fig. 1). On the basis of the results of phenotypic, chemotaxonomic, and phylogenetic studies, this type strain GI5^T represents a novel species of a novel genus of the family *Alcanivoracaceae*, *Ketobacter alkanivorans* (Kim et al. 2018).

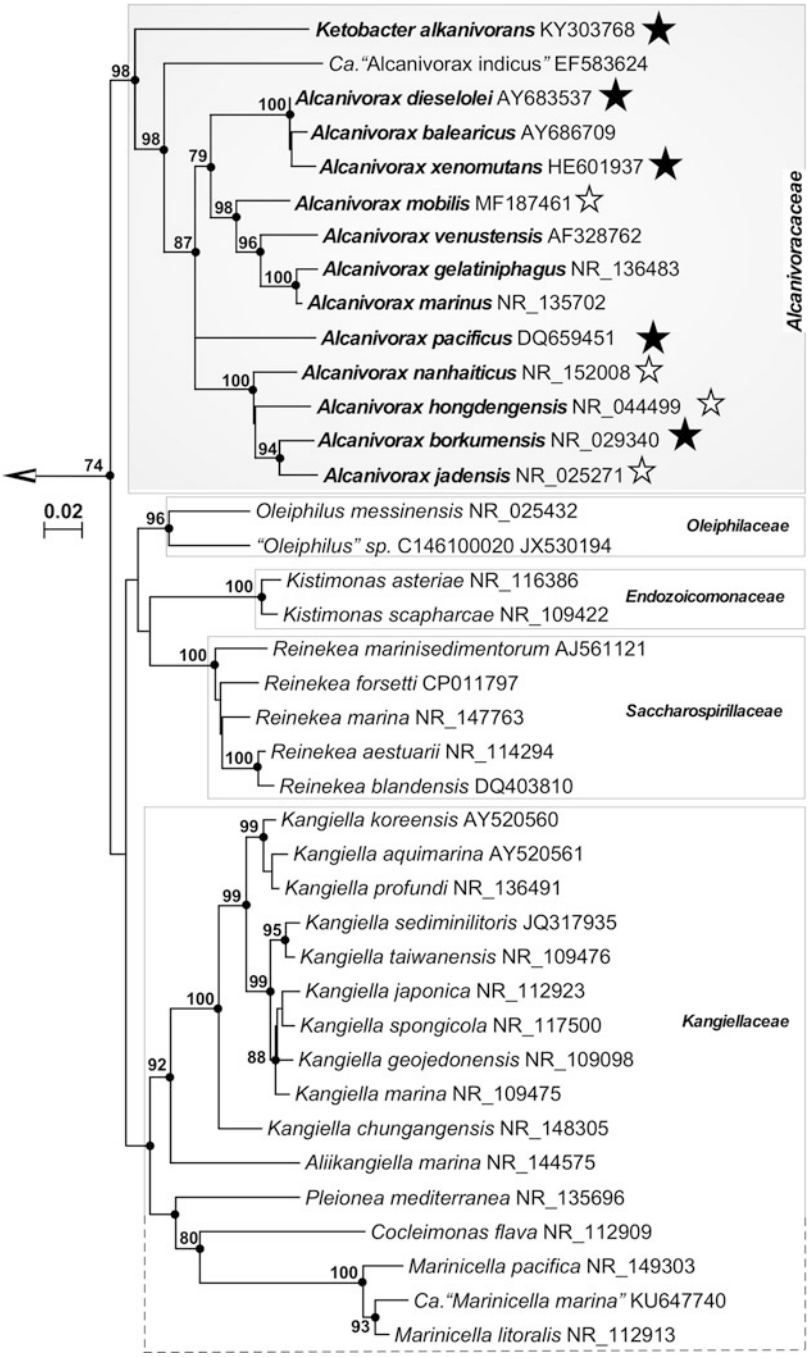

Fig. 1 16S rRNA gene-based Bayesian phylogenetic reconstruction, showing the position of the family *Alcanivoracaceae* and the families *Endozoicomonaceae, Kangiellaceae*, *Oleiphilaceae*, and

3 Biogeography and Ecophysiology

Currently, the genus *Alcanivorax* comprises 12 recognized (Table 1 and Fig. 1) and one proposed species Ca. "A. indicus" (GenBank accession number EF583624). The vast majority of them (*A. borkumensis*, *A. diselolei*, *A. gelatiniphagus, A. hongdengensis*, *A. jadensis*, *A. venustensis*, and Ca."A. indicus") were isolated worldwide from surficial seawater and/or tidal sediments, either accidentally or chronically contaminated with petroleum hydrocarbons. Four species, namely, *A. marinus*, *A. mobilis, A. nanhaiticus*, and *A. pacificus*, were obtained during the screening of oil-degrading bacteria from deep seawater and/or sediment samples, collected in Indian and Pacific Oceans and South China Sea. The two remaining species, *A. balearicus* and *A. xenomutans*, were isolated from nonmarine environments, namely, from a subterranean saline Lake Martel (Balearic Islands, Spain) and from the sediment sample of a shrimp cultivation pond (Ramnad, Tamil Nadu, India). Recently, an additional strain P40 of *A. xenomutans* was isolated from the deep seawater of the Indian Ocean after enrichment with petroleum and diesel as carbon source (Fu et al. 2018). At the time of writing this chapter, the NCBI Taxonomy Data Base additionally contains more than 530 16S rRNA gene sequences belonging to the genus *Alcanivorax*, recovered either directly from various environments (here termed: environmental riboclones) or from enrichments amended with petroleum hydrocarbons (here termed: culture clones), as well as isolates that have not been validly described. The vast majority of these 16S rRNA gene sequences originated from various types of marine environments, including surficial and deep seawater, shallow and deep marine sediments, whale carcasses and ridge flank crustal fluids, mud volcanoes and deep-sea hypersaline lakes, hydrothermal vents, and marine aerosols. A special clade of *Alcanivorax*-related 16S rRNA gene sequences has been retrieved from microbial communities associated with various marine invertebrates, including algae, namely, bryozoans, polychaetes, sponges, dinoflagellates, and cyanobacteria (Table 2). Although the association with diatoms is still to be clarified, there is some evidence indicating *Alcanivorax* as the main component of a diatom-dominated floating biofilm (Coulon et al. 2012). It is noteworthy that the spread of *Alcanivorax* also extends to a few terrestrial environments, which possess features important for its functioning, namely, moderate salinity and sufficient amount hydrocarbons, for example: saline and brackish lakes, saline subsurface water bodies, potash mine wastes, oil-contaminated soils, and hydrocarbon-seeping geothermal areas (Table 2).

Fig. 1 (continued) Saccharospirillaceae. *Alcanivoracaceae* species with sequenced genomes are highlighted by solid stars (completed genomes) and open stars (not assembled). The numbers at the nodes indicate the bootstrap values >70% (1000 bootstrap iterations). Filled circles indicate that the corresponding nodes were also recovered in the tree generated with the maximum likelihood and parsimony algorithms. *Escherichia coli* ATCC 11775^{T} (GenBank accession no. X80725) was used as an out-group. Bar, 0.02 nucleotide substitutions per nucleotide position

Table 2 Sources of 16S rRNA gene sequences of *Alcanivorax*-affiliated bacteria isolated or detected in environmental samples, other than seawater and sediments

Source	Designation	GenBank accession n°.	Reference
Hypersaline microbial mat	L21-PYE-C1	KJ187993	Spring et al. (2015)
Marine bryozoan, *M. membranacea*	BB31	FR693301	Unpublished
Marine cyanobacteria, *Prochlorococcus* NATL2A	EZ46	EU591711	Morris et al. (2008)
Marine sponge, Phylum *Porifera*	D529	AY362015	Sfanos et al. (2005)
Marine polychaete, *Branchiomma luctuosum*	A52	JX298540	Unpublished
Dinoflagellate, *Gymnodinium catenatum*	DG812	AY258104	Green et al. (2004)
Contaminated soil	6–5/B5	AY918109	Kleinsteuber et al. (2006)
Potash mine waste material	BI06	KX344924	Unpublished
Nullarbor caves (Australia)	wb1_C04	AF317760	Holmes et al. (2001)
Saline mud volcano at San Biagio-Belpasso, Mt. Etna (Italy)	STET1	AJ416686	Yakimov et al. (2002)
Lake Martel, Mallorca Island (Spain)	MACL04	AY686709	Rivas et al. (2007)
Lonar Crater Lake (India)	KBDL8	GU392037	Deshmukh et al. (2011)
Selenium-contaminated hypersaline evaporation pond	MPD-12	AF348709	De Souza et al. (2001)

Recent functional genomic, biochemical, and physiological analyses have revealed the underlying basis of the ecological success of *Alcanivorax* (Harayama et al. 2004; Schneiker et al. 2006; Sabirova et al. 2006, 2008; references in Table 3). *Alcanivorax* species possess a multitude of adaptations to access oil at various temperatures and salinities (synthesis of biosurfactants and biofilm formation on oil droplets (Schneiker et al. 2006)) and to survive in open marine environments at the oil–water interface (scavenging nutrients and resistance to ultraviolet light (Schneiker et al. 2006; Sabirova et al. 2008)). More recently, the analysis of the supernatant of *A. borkumensis* SK2 culture has shown the presence of an extracellular type of iron-chelating molecule with structural similarity to pseudomonin (Denaro et al. 2014) This molecule, combined with the membrane-associated amphiphilic tetrapeptidic siderophore amphibactin, provides a highly performing siderophore-based iron-uptake system that provides the necessary iron supply to *A. borkumensis* and other oil-associated microbes, even in iron-depleted marine habitats (Kem et al. 2014; Denaro et al. 2014). Iron is not only a component of various oxygenases that are essential for alkane activation, but also of iron-containing heme, which forms part of many electron transport systems protecting *Alcanivorax* cells against oxidative stress (Sabirova et al. 2011).

Table 3 Main genomic features of *Alcanivoracaceae* species with sequenced genomes

	A. borkumensis^T	*A. diselolei*	*A. jadensis*	*A. hongdengensis*	*A. mobilis*	*A. nanhaiticus*	*A. pacificus*	*A. xenomutans*	*K. alkanivorans*
Size, bp	3,120,143	4,928,223	3,629,371	3,664,876	4,099,910	4,132,804	4,168,427	4,733,951	4,914,503
Accession N°	NC_008260	CP003466	ARXU01000000	AMRJ00000000	NMQZ01000000	ARXV00000000	CP004387	CP012331	CP022684
Genome status	Circular	Circular	Not assembled	Not assembled	Not assembled	Not assembled	Circular	Circular	Circular
Scaffolds	0	0	31	94	93	36	0	0	0
Plasmid	None	None	None	None	None	None	None	None	None
Proteins	2,750	4,362	3,266	3,416	3,657	3,778	3,669	4,275	4,131
GC content %	54.7	61.6	58.4	60.7	63.4	56.4	62.6	61.5	49.7
Isolation source	Seawater North Sea	Seawater Bohai Sea	Sediments North Sea	Seawater Strait of Malaca	Deep-sea sediments Indian Ocean	Deep-sea sediments South China sea	Deep-sea sediments Pacific Ocean	Deep seawater Indian Ocean	Seawater West Sea
Reference	Schneiker et al. (2006)	Lai et al. (2012)	Parks et al. (2017)	Lai and Shao (2012a)	Unpublished	Unpublished	Lai and Shao (2012b)	Fu et al. (2018)	Kim et al. (2018)

Despite lacking catabolic versatility, many members of the genus *Alcanivorax* possess several alternative enzymatic systems for terminal hydroxylation of alkanes, including two nonheme iron-dependent alkane monooxygenases and three cytochrome P450 alkane hydrolases (Schneiker et al. 2006; Liu et al. 2011). It is worthy of mention that, besides aliphatic hydrocarbons, some *Alcanivorax* spp. are capable of degradation of mono-aromatic compounds, such as benzene, toluene, and xylene (Hassan et al. 2012; Rahul et al. 2014). In contrast to well-studied hydrolase-initiated alkane degradation pathways, the early responses of *Alcanivorax* cells to alkane exposure (alkane sensing, signal transduction, and cross-membrane transport) were poorly understood before the study published by Wang and Shao (2014). A signal transduction network was proposed to be associated with regulation of gene expression and alkane degradation in *Alcanivorax diselolei* B5. This network begins with the outer membrane receptor proteins OmpS and OmpT1/2/3 (capable of recognizing and transporting across the outer plasma membrane hydrocarbons of diverse types and lengths), triggering the expression of the alkane-sensing chemotaxis complex proteins CheA/CheW and MCP (methyl-accepting chemotaxis protein). Successively, the CheW protein activates the expression of cytochrome o terminal oxidase (Cyo), which in turn downregulates the expression of AlmR protein, the negative regulator of the initial steps of alkane degradation pathway.

4 Genomic Features of *Alcanivoraceae* Species

Currently (January 2018), the genomes of nine species of the family *Alcanivoraceae* are sequenced. The assembly and annotation of five genomes are completed, whereas four remaining are represented in form of 31 to 94 contigs (Table 3). All genome structures validate the highly specialized eco-physiological traits of *Alcanivorax*. In particular, the analyzed genomes possess multiple alkane mono-oxygenases, cytochrome P450, and *almA* genes. They all harbor many glycosyltransferases, conjugative and Type IV pili, translocons, and Type II secretion system genes, which allow the biofilm formation at the oil–water interface. All genomes are enriched by various permeases, high-affinity ABC-type, and many other transporters, which may be involved in nutrient and trace elements scavenging and heavy-metal resistance. It is noteworthy that all genomes harbor a considerable number of genes related to "stress response" (i.e., *A. xenomutans* has 136 such genes (Fu et al. 2018)). The presence of the genes involved in synthesis and direct uptake of compatible solutes (choline and glycine betaine) mediates an effective strategy to maintain osmotic balance across a membrane at different salinities. There are many genes encoding cold/heat proteins (i.e., 10 and 21 in *A. borkumensis* and *A. xenomutans* genomes, respectively (Schneiker et al. 2006; Fu et al. 2018)), which could protect the cells from extreme temperatures. A number of genes that respond to oxidative stress were also identified in *Alcanivorax* genomes, such as genes coding for catalase and superoxide dismutase. All these genomic adaptations allow *Alcanivorax* to flourish in oil-contaminated marine and other saline environments worldwide.

5 Research Needs

Our current knowledge on the diversity, eco-physiology, and broad adaptive capacities of *Alcanivoraceae* in the ocean has significantly increased in recent years. However, further studies are necessary to develop our understanding of the molecular basis for oligotrophic growth, e.g., by scavenging nutrients, trace elements, especially iron, as well as biosurfactant production, and formation of biofilms. Unraveling the regulatory processes that control multiple alkane degradation systems and global alkane regulatory and sensing network present in *Alcanivoracaceae* should help to shed light on ecological success of these hydrocarbon-degrading bacteria. Many hydrocarbonoclastic bacteria of this family continue to be discovered and isolated from various environments, including deep-sea sediments (Yang et al. 2018), hypersaline and soda lakes (Hassan et al. 2012), whereas many others likely still escape cultivation. New findings will facilitate understanding of how these bacteria, while being highly restricted by the range of accessible substrates, remain extremely competitive and advantaged in natural habitats. Of special interest is the recently evidenced interaction of hydrocarbonoclastic bacteria with invertebrates and all three major phytoplankton lineages (see Thompson et al. 2018 for further references). There are questions still to be resolved – are they transient or permanent residents, and do they play a role in the physiology of the host or are they simply hitch-hiking consumers?

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Aerobic Hydrocarbon-Degrading *Gammaproteobacteria*: *Porticoccus*

11

Tony Gutierrez

Contents

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

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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 $C_{16:0}$, $C_{16:1}\omega7c$, and $C_{18:1}\omega7c$.

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

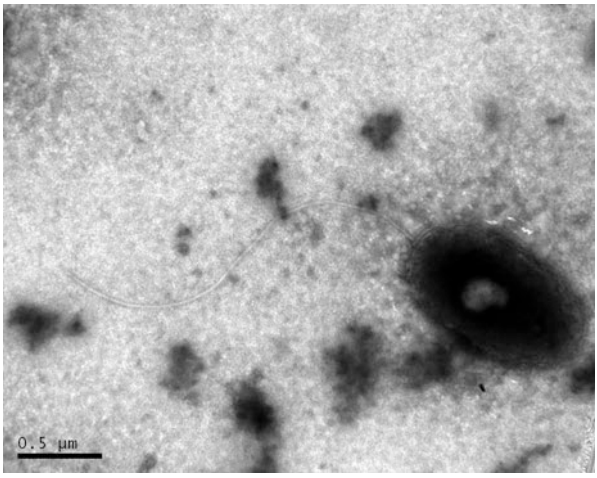

Fig. 1 Transmission electron micrograph of a cell with negative staining of strain *Porticoccus hydrocarbonoclasticus* MCTG13d^T. Bar, 0.5 μm (Source: Gutierrez et al. (2012). Reprinted with the permission from Appl. Environ. Microbiol)

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

From a BLAST analysis in 2016, the highest levels (≥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*, *Halieceae*, *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

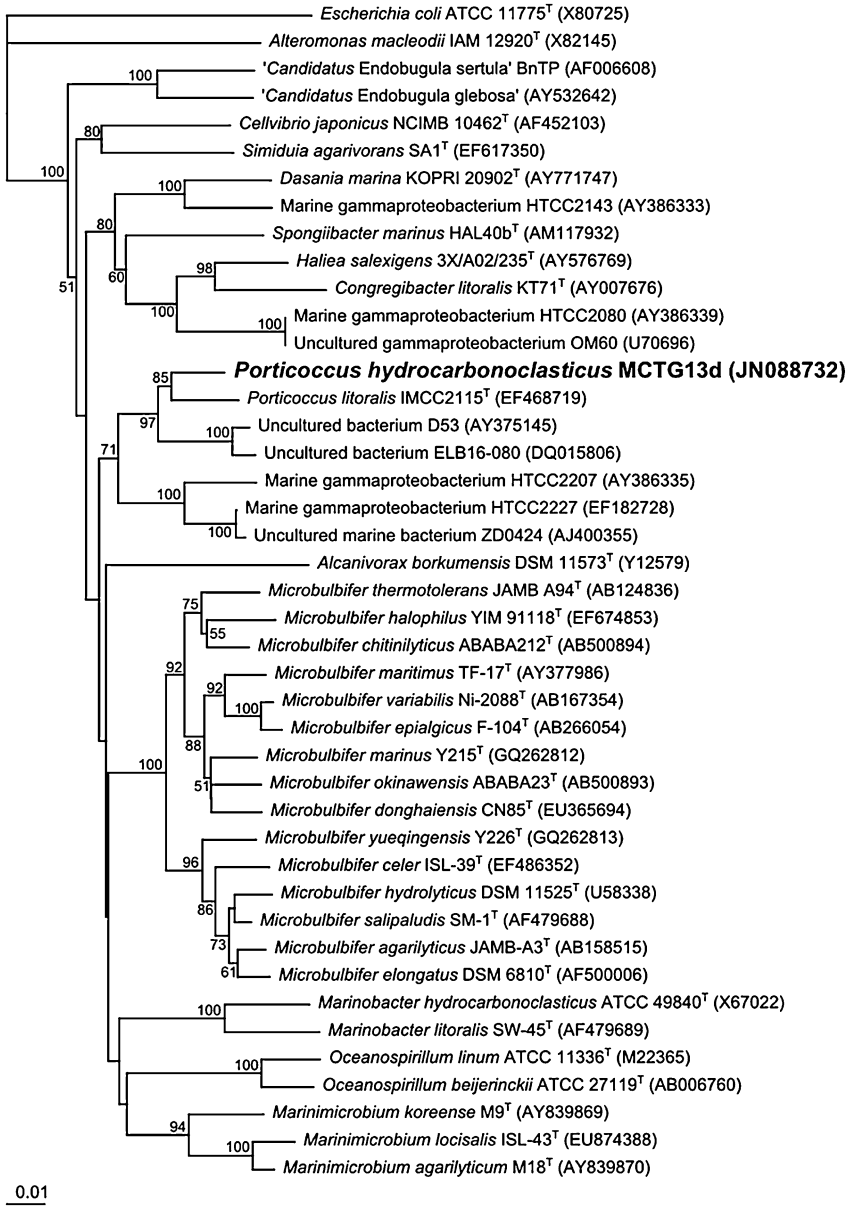

Fig. 2 Neighbour-joining phylogenetic tree, based on 16S rRNA gene sequences (>1200 bp), showing the relationships between *P. hydrocarbonoclasticus* strain MCTG13d^T and representative type strains and environmental clones. *Escherichia coli* ATCC 11775^T and *Alteromonas macleodii* IAM 12920^T were 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

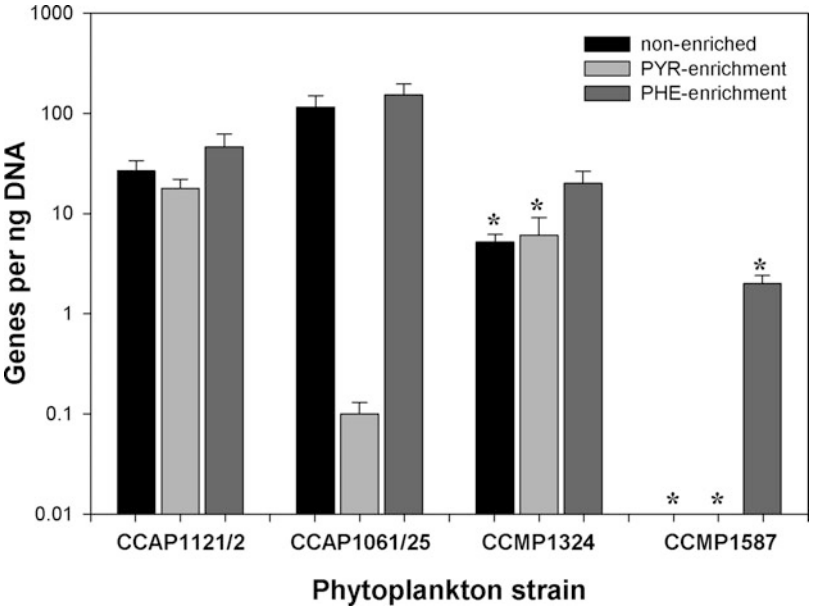

Fig. 3 Abundance of *P. hydrocarbonoclasticus* MCTG13d^T 16S rRNA genes in various marine phytoplankton species using PCR-targeted probes. Phytoplankton cultures were enriched with pyruvate (PYR-enriched), phenanthrene (PHE-enriched), or no added carbon source (unenriched). Bars are the average and standard 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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Aerobic Hydrocarbon-Degrading *Gammaproteobacteria*: *Xanthomonadales*

12

Tony Gutierrez

Contents

Abstract

The *Xanthomonadales* comprises a morphologically and physiologically diverse order of bacteria, though its classification has been contentious pertaining to its division into families. Currently, the order is divided into the two families, *Xanthomonadaceae* and *Rhodanobacteraceae*, that collectively contain members from approximately 29 genera. Hydrocarbon degraders of the family *Xanthomonadaceae* include members of the genera *Arenimonas*, *Luteimonas*, *Pseudoxanthomonas*, *Stenotrophomonas*, *Xanthomonas*, and *Xylella*, whereas those of the family *Rhodanobacteraceae* include *Dokdonella*, *Dyella*, *Frateuria*, *Luteibacter*, *Oleiagrimonas*, *Rhodanobacter*, and *Rudaea*. These organisms are categorized as generalist hydrocarbon-degraders based on their ability to also utilize various other carbon substrates as a sole source of carbon and energy. To-date, of the nine recognized genera of obligate hydrocarbonoclastic bacteria that are able to degrade hydrocarbons almost exclusively as a preferred carbon and energy source, only *Algiphilus* and *Polycyclovorans* are represented within the

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order *Xanthomonadales*, principally within the family *Xanthomonadaceae*. The type species of these two genera are *Algiphilus aromaticivorans* and *Polycyclovorans algicola*, which respectively were originally isolated from the phycosphere of a marine dinoflagellate and diatom. Members of these two genera have also been identified living associated with various other species of marine eukaryotic phytoplankton, and sequencing surveys have identified their presence in a wide variety of environments that include oil-contaminated and non-contaminated marine and terrestrial environments as well as human skin.

1 Introduction

The *Xanthomonadales* are a bacterial order within the *Gammaproteobacteria* whose members are all Gram-negative, catalase positive, and nonspore forming obligate aerobes. Some members are nonmotile, whereas others are motile by means of flagella. The order contains the largest and most important species of phytopathogens, such as *Xanthomonas citri*, *Xanthomonas euvesicatoria*, *Xanthomonas oryzae*, and *Xylella fastidiosa*, that can cause significant agricultural losses worldwide. *Xanthomonas campestris* is another important plant pathogen, but it is better known for its enormous commercial value through its production of the high-molecular-weight polysaccharide xanthan gum. Many species within the order are also human pathogens and include species of *Stenotrophomonas* that have been reported to express multidrug resistance. The order contains 28 validly named genera that are divided into two major families – the *Xanthomonadaceae* comprising 13 genera and the *Rhodanobacteraceae* comprising 14 genera. Additional families (*Algiphilaceae*, *Solimonadaceae*, *Nevskiaceae*, *Sinobacteraceae*) within this order have been proposed through the primary portal for the classification of microorganisms (Int. J. Syst. Evol. Microbiol.). Classification of some families within this order, however, remains contentious and *incertae sedis* (Naushad et al. 2015). A previous chapter in the last edition of this handbook discussed the genera within the family *Xanthomonadaceae* that have been identified to either directly or indirectly be involved in degrading petroleum hydrocarbons (Chang and Zylstra 2010). This chapter focuses on the OHCB within the *Xanthomonadales*, including an overview of various generalist hydrocarbon-degraders comprising this order.

2 Obligate Hydrocarbonoclastic Members of the *Xanthomonadales*

Obligate hydrocarbonoclastic bacteria (OHCB) are specialists with respect to their ability to utilize hydrocarbons almost exclusively as a carbon and energy source. Although some genera of OHCB have been identified in terrestrial environments, their occurrence is largely confined to the marine environment. These organisms are often present in very low cell abundances (<1%) in seawater and sediment but can

become strongly enriched for when presented with crude oil or other form of petrochemical input (Head et al. 2006; Yakimov et al. 2007). With the exception of *Planomicrobium alkanoclasticum* (a Gram-positive of the Firmicutes), most of the recognized obligate hydrocarbonoclastic bacteria (OHCB) are classified within the order *Oceanospirillales* of the class *Gammaproteobacteria* – these are members of the genera *Alcanivorax*, *Cycloclasticus*, *Neptunomonas*, *Oleibacter*, *Oleiphilus*, *Oleispira*, and *Thalassolituus*. The remaining two genera of known OHCB are *Algiphilus* and *Polycyclovorans* which are represented in the family *Xanthomonadaceae* of the order *Xanthomonadales*. The type species of these two genera, *Algiphilus aromaticivorans* and *Polycyclovorans algicola*, were originally isolated from laboratory cultures of marine eukaryotic phytoplankton (micro-algae) but are poorly represented in 16S rRNA gene sequence databases. They are thus less well studied and are presented here together with an overview on generalist hydrocarbon-degraders within the *Xanthomonadales*.

2.1 *Polycyclovorans algicola*

P. algicola TG408^T (=ATCC BAA-2242^T = KCTC 23940^T) is a halotolerant, Gram-stain-negative, rod-shaped bacterium. The strain was isolated from a non-axenic laboratory culture of the marine diatom *Skeletonema costatum* (CCAP 1077/1C), which was originally isolated from the North Sea (Gutierrez et al. 2013).

On ONR7a agar plates amended with pyruvate as the sole carbon and energy source, colonies of *P. algicola* develop within 3 weeks of incubation at 28 °C. They appear flat, smooth, and translucent with irregular edges and diameters of 3–4 mm. Colonies allowed to grow out for longer appeared off-white and slightly umbonate. On ONR7a agar supplemented with phenanthrene that was sprayed as a thin film on the agar surface (Gutierrez 2017), colonies appeared surrounded by clearing zones that was indicative for degradation of polycyclic aromatic hydrocarbons (PAHs). Under the microscope, cells are small, nonspore-forming rods, 1.0–1.2 × 0.5 μm in average size, that occur singly or in pairs when grown on pyruvate (Fig. 1). They are motile by means of a single polar flagellum, and cells can be found showing the presence of blebs at their surface and extracellularly.

P. algicola is an obligate aerobe that produces catalase and oxidase, and nitrate is not reduced to nitrite. The G+C content of the organism's DNA is 64.3 mol%. The predominant isoprenoid quinone is Q-8, and the dominant fatty acids are $C_{16:0}$, $C_{16:1}\omega7c$, and $C_{18:1}\omega7c$.

The bacterium is able to grow at 10–30 °C (optimal, 25–30 °C) but does not grow at temperatures ≥30 °C. The pH range for growth is 6.5–8.5 (optimal, pH 8.3). The organism produces a positive result for lipase (Tween 80) and phosphatase activity, gives a negative result in tests for gelatinase and for fermentation of glucose and various other sugars, and does not accumulate PHB granules. It is able to grow well in a medium containing NaCl at concentrations of 0–9% (optimal, 3%), whereas growth is completely inhibited in the presence of NaCl at concentrations >9%. Hence, *P. algicola* is a slightly halotolerant bacterium.

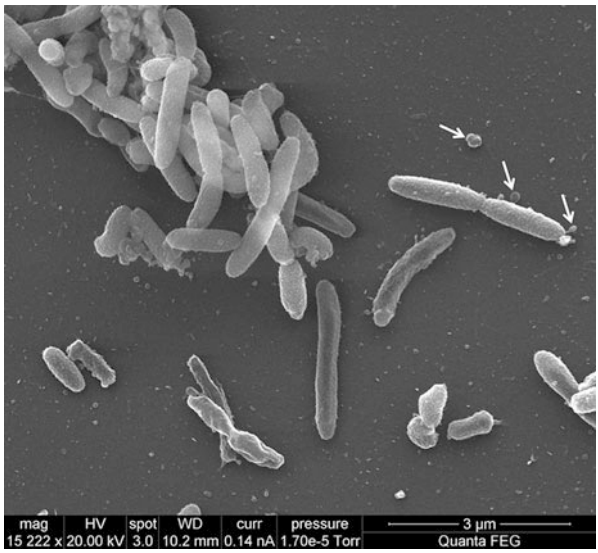

Fig. 1 Scanning electron micrograph of *Polycyclovorans algicola* strain TG408^T. Arrows indicate cell-surface and extracellularly-released blebs. Bar, 3 µm (Source: Gutierrez et al. (2013). Reprinted with the permission from Appl. Environ. Microbiol)

Substituting Na^+ for K^+ in growth medium inhibited growth, and growth was completely inhibited at KCl concentrations ≥3%.

From a BLAST analysis in 2016, the highest levels (≥95%) of sequence similarity for *P. algicola* were to 23 environmental clones (Table 1). The highest levels of sequence similarity to type strains is with *Alkanibacter difficilis* MN154.3^T and *Hydrocarboniphaga daqingensis* B2-9^T (91.7%), *Nevskia soli* GR15-1^T (91.5%), *Hydrocarboniphaga effusa* AP103^T (91.4%), *Sinobacter flavus* CW-KD4^T and *Solimonas variicoloris* MN28^T (91.3%), *Nevskia ramosa* Soe^T (90.8%), and *Solimonas soli* DCY12^T (90.7%). The most closely related clones and type strains originated from contaminated soil, human skin, fresh and marine waters, or polluted environments, and they are represented alongside *P. algicola* in the phylogenetic tree shown in Fig. 2.

Although *P. algicola* clustered most closely with *Solimonas* and *Sinobacter* (bootstrap value of 85%), its highest level of sequence similarity was to *A. difficilis* MN154.3^T. All of the related clones and type strains form a deeply rooted lineage – the so-called *Hydrocarboniphaga-Nevskia-Sinobacter* (HNS) clade – that is separated from the family *Xanthomonadaceae* (Zhou et al. 2008). However, they have been allocated to this family exclusively on the basis of 16S rRNA gene sequence data (Palleroni et al. 2004; Saddler and Bradbury 2005). In the systematic description of *S. flavus* CW-KD4^T, Zhou et al. (2008) proposed a new family – *Sinobacteraceae* – in order to distinguish this strain and members of the HNS clade from the family *Xanthomonadaceae* on the basis of distinct phenotypic characteristics. Analysis by Classifier in RDP-II (Wang et al. 2007) indicated that TG408 is affiliated with the family *Sinobacteraceae*. This was further supported by the strain's DNA G+C content of 64.3 mol%, which is similar to that of most members of the *Sinobacteraceae* family (60–65 mol%).

Table 1 Sources and characteristics of 16S rRNA gene sequences with highest sequence similarity to *Polycyclovorans algicola* strain TG408^T (Updated from Gutierrez et al. 2013)

Source	Designation[a]	GenBank accession no.	% similarity	Reference
Zhongyuan oil field, China	BP76	HQ190544	99%	Unpublished
Zhongyuan oil field, China	BP24	HQ190510	99%	Unpublished
Surface marine sediment	NS096	JX391811	99%	Unpublished
Indian ocean water column	YD1000-110	JX441430	99%	Jiang and Jiao (2016)
Surface marine sediment	NF018	JX391637	99%	Unpublished
Surface marine sediment	NF012	JX391631	99%	Unpublished
Oil contaminated soil	B194	EU328045	99%	Unpublished
ND[b]	DSW25-19	HQ263242	99%	Unpublished
Rancho La Brea Tar Pits, Los Angeles, California	101-95	EF157240	99%	Kim and Crowley (2007)
Contaminated soil	TERI-KL22	JN217160	99%	Unpublished
Terrestrial mud volcano	SYNH02-ew01B-031	JQ245626	99%	Cheng et al. (2012)
Human skin	nbw777e05c1	GQ009517	99%	Grice et al. (2009)
Human skin	nbw779c06c1	GQ009776	99%	Grice et al. (2009)
Human skin	ncd1111g03c1	HM337987	99%	Kong et al. (2012)
Contaminated coastal water	DVASW-J249	KF722502	99%	Patel et al. (2014)
Undefined reservoir	A27	KC442838	99%	Unpublished
Nitrifying bioreactor	NCAAH 15N22	KR537254	98%	Unpublished
Southern Ocean, Antarctica	C114Chl069	JX525449	98%	Singh et al. (2015)
Mountain brackish lake	ch-xj4	JQ327935	98%	Zeng et al. (2014)
Contaminated soil	TERI-KL4	JN217179	97%	Unpublished
Arabian Sea oxygen minimum zone	ASTS-FIM-1000m-402	KJ590004	97%	Unpublished
Borax hot springs of Ladakh, India	TPB-GMAT-SPRING12-40	HF677147	96%	Unpublished
Yellow Sea water	D13W-30	HM057752	95%	Unpublished

[a]Sequence designation from the BLASTN database
[b]ND, no data available

The following hydrocarbons are degraded and serve as sole carbon sources for growth and/or are mineralized by *P. algicola*: decane, pristane, *n*-hexadecane, benzene, toluene, *p*-xylene, biphenyl, naphthalene, fluorene, anthracene, phenanthrene, and dibenzothiophene. No growth is produced on the following hydrocarbons: hexane, pentane, decane, phytane, pyrene, and fluoranthene. The strain is able

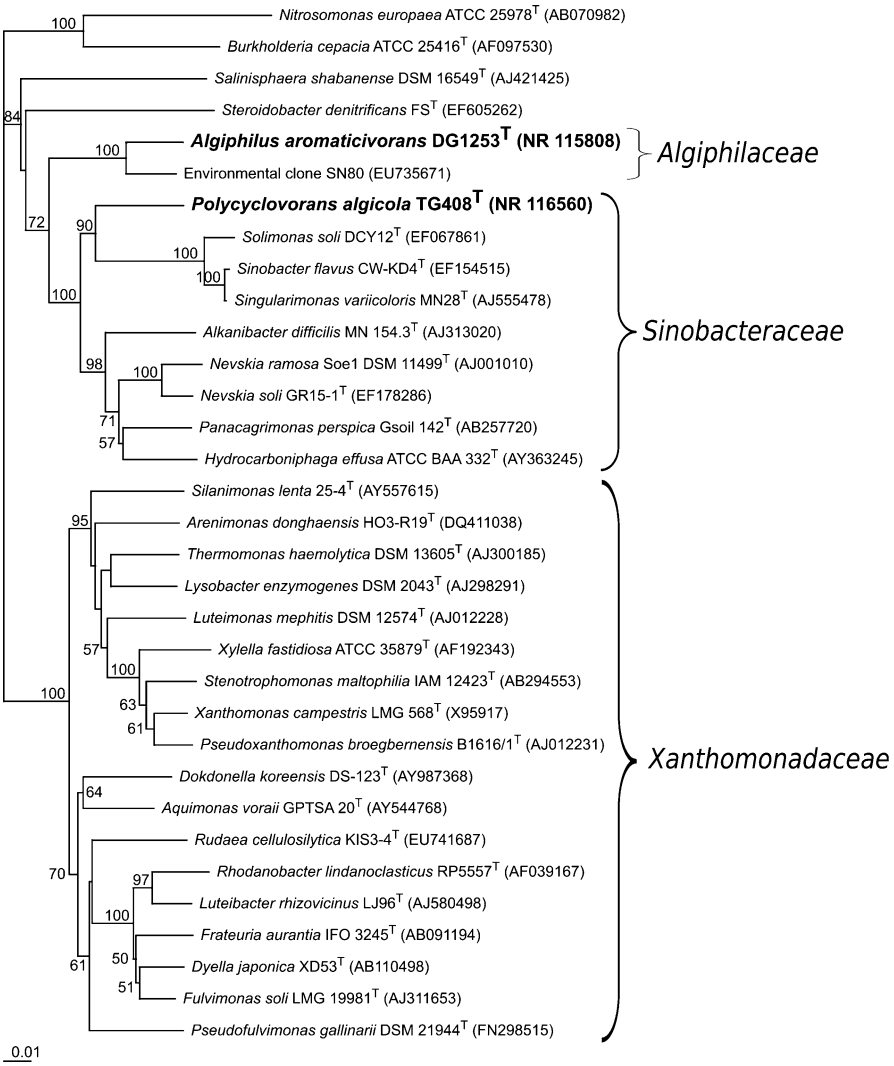

Fig. 2 Neighbour-joining phylogenetic tree, based on 16S rRNA gene sequences (>1200 bp), showing the relationships between *Polycyclovorans algicola* strain TG408^T and *Algiphilus aromaticivorans* strain DG1253^T, and representative type strains within the order *Xanthomonadales*. *Nitrosomonas europaea* ATCC 25978^T and *Burkholderia cepacia* ATCC 25416^T in the class *Betaproteobacteria* served as the outgroup. Bootstrap values (expressed as percentages of 1000 replications) >50% are shown at each node. GenBank accession numbers are shown in parentheses. Bar represents 0.01 substitutions per nucleotide position. Note that the genus *Sinobacter* was reclassified as synonymous with *Solimonas* (Sheu et al. 2011)

to grow on pyruvate, succinate, acetate, and propionate as sole sources of carbon, with weak growth on arabinose. No growth is produced on sugar/sugar alcohols, such as mannitol, fructose, glucose, mannose, sucrose, and xylose. The organism does not grow in rich nutrient marine medium, such as marine 2216 medium, unless the medium is amended with pyruvate or other carbon source within the nutritional spectrum for the strain. *P. algicola* utilizes hydrocarbons as its preferred source of carbon over other naturally occurring organic substrates. On the basis of its preference for hydrocarbon substrates, in particular, aromatic compounds, the organism represents a new genus of OHCB.

It is not presently clear why *P. algicola* has not been identified in previous studies, except that its natural biotope may be confined to living in association with marine phytoplankton, which is still somewhat largely unexplored. The organism has been shown to be associated with a range of other species of diatoms, dinoflagellates (Gutierrez et al. 2013; Fig. 3), and coccolithophores (unpublished data).

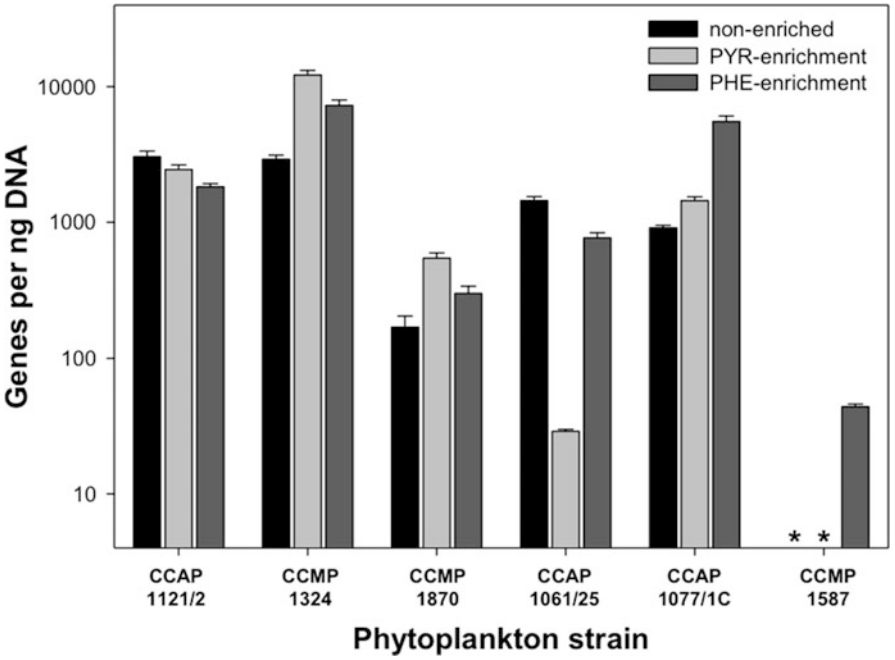

Fig. 3 Abundance of *P. algicola* TG408^T 16S rRNA genes in various marine phytoplankton species using PCR-targeted probes. Phytoplankton cultures were enriched with pyruvate (PYR-enriched), phenanthrene (PHE-enriched), or no added carbon source (unenriched). Bars are the average and standard deviation of duplicate qPCR reactions measuring the abundance of *P. algicola*-specific 16S rRNA genes per ng DNA. Phytoplankton strains used were *Lingulodinium polyedrum* CCAP1121/2, *Isochrysis* sp. CCMP1324, *Heterosigma akashiwo* CCMP1870, *Pseudo-Nitzschia* CCAP1061/25, *Skeletonema costatum* CCAP1077/1C, and *Thalassiosira weissflogii* CCMP1587. Asterisks represent values that were below the quantification limit (<3 gene copies per reaction) of the assay (Source: Gutierrez et al. (2013). Reprinted with the permission from Appl. Environ. Microbiol)

2.2 *Algiphilus aromaticivorans*

A. aromaticivorans DG1253^T (=ATCC BAA-2243^T = DSM 24793^T) is a halotolerant, Gram-stain-negative, rod-shaped bacterium. The strain was isolated from a non-axenic laboratory culture of *Lingulodinium polyedrum* (CCAP 1121/2), which is a marine dinoflagellate originally isolated from Loch Creran in Argyll, Scotland (Gutierrez et al. 2012).

On ONR7a agar plates amended with pyruvate as the sole carbon and energy source, colonies of *A. aromaticivorans* develop within 10 days of incubation at 30 °C. They appear off-white, circular, slightly umbonate, entire, and approximately 0.5–1.0 mm in diameter. Under the microscope, cells are small, nonspore-forming, of size 0.8–1.36 × 0.4–0.8 μm, and motile by means of a single polar flagellum (Fig. 4). Some cells show the presence of surface blebs.

A. aromaticivorans is an obligate aerobe that produces catalase, oxidase, and nitrate is reduced to nitrite. The DNA G+C content is 63.6 mol%. The dominant fatty acid is $C_{18:1}\omega7c$, and the predominant isoprenoid quinone is Q-8.

The bacterium is able to grow at 4–37 °C (optimal, 30 °C), but growth at 4 °C is significantly decreased compared with that at 10 °C. The pH range for growth is 6.5–8.5 (optimal, pH 7.6). The organism produces a positive result for lipase (Tween 80) and phosphatase activity, gives a negative result in tests for gelatinase and for fermentation of glucose and various other sugars, and does not accumulate PHB granules. It exhibits slight halotolerance, due to its ability to grow well in a medium containing NaCl at concentrations of 0–12% (optimal, 3%), whereas growth is completely inhibited in the presence of NaCl at concentrations >15%. Hence,

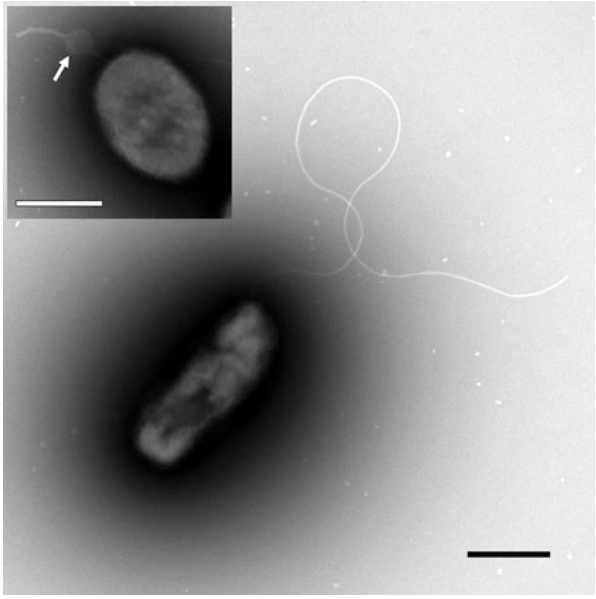

Fig. 4 Transmission electron micrograph of a cell of *A. aromaticivorans* DG1253^T. Inset shows bleb formation at the cell surface (arrow). Bars, 0.5 μm (Source: Gutierrez et al. (2012). Reprinted with the permission from Int. J. Syst. Evol. Microbiol)

A. aromaticivorans is a slightly halotolerant and slightly halophilic bacterium and can be considered a marine strain (Larsen 1986). Substituting Na^+ for K^+ in growth medium inhibited growth, and growth was completely inhibited at KCl concentrations ≥3%.

From a BLAST search in 2016, the highest level (≥95%) of 16S rRNA gene sequence similarity for *A. aromaticivorans* strain DG1253^T was to a single environmental clone, SN80 (EU735671), that originated from a soil sample located 50 m from the Jidong Oilfield near Bohai Bay in China (Liu et al. 2009; Fig. 2). Of unpublished sequences in GenBank with ≥95% identity to DG1253^T, only four exist which are environmental clones originating from petroleum-contaminated saline-alkali soil or saltern on the Black Sea coast. The closest type strains are *Nevskia soli* GR15-1^T and *A. difficilis* MN154.3^T, both of which shared 89.9% 16S rRNA gene sequence similarity with DG1253^T. The genera *Alkanibacter* and *Nevskia*, together with a few other genera, form a deeply rooted lineage – the so-called *Hydrocarboniphaga–Nevskia–Sinobacter* clade – that is defined by the family *Sinobacteraceae* (Zhou et al. 2008) and separated from the family *Xanthomonadaceae* (Fig. 2). Since the phylogenetic position of strain DG1253^T within the family *Sinobacteraceae* is poorly supported (low bootstrap value of <50%) and Classifier analysis in RDP-II (Wang et al. 2007) indicates the strain is only moderately affiliated with this family (confidence threshold of <89%), the phylogenetic position of the strain in the order *Xanthomonadales* is unique, distinct, and represents a novel family, *Algiphilaceae*.

The following hydrocarbons serve as sole carbon sources for growth and/or are mineralized by *A. aromaticivorans*: decane, pristane, *n*-hexadecane, benzene, toluene, *p*-xylene, biphenyl, naphthalene, anthracene, phenanthrene, phenol, phytane, salicylate, benzoate and dibenzothiophene. No growth is produced on the following hydrocarbons: hexane, pentane, fluorene, pyrene, and fluoranthene. The strain is able to grow on pyruvate, acetate and propionate as sole sources of carbon, with weak growth on arabinose. No growth is produced on sugar/sugar alcohols, such as mannitol, fructose, glucose, mannose, sucrose, and xylose. The organism grows poorly in rich nutrient marine medium, such as marine agar 2216 medium, unless the medium is amended with pyruvate or other carbon source within the nutritional spectrum for the strain.

Based on the narrow nutritional spectrum exhibited by *A. aromaticivorans*, wherein it prefers to utilize mono- and polycyclic aromatic hydrocarbons, as well as some *n*-alkanes (e.g., decane, *n*-hexadecane) and small organic acids, this organism can be described as a marine OHCB. Like for *P. algicola*, *A. aromaticivorans* has also been shown to be associated with a range of species of diatoms, dinoflagellates, and coccolithophores using qPCR-specific probes targeting this organism (unpublished data).

3 Generalist Hydrocarbonoclastic *Xanthomonadales*

Isolated or characterized generalist hydrocarbonoclastic bacteria of the *Xanthomonadales*, that are able to degrade substituted/unsubstituted alkane or aromatic hydrocarbons, are listed in Table 2. The list also includes the only two

Table 2 Representative genera of obligate and generalist hydrocarbon-degrading bacteria within the order *Xanthomonadales*

Genus	Environmental source	Hydrocarbons degraded	References
Algiphilus aromaticivorans[a]	Marine dinoflagellate	Aromatics and some alkanes	Gutierrez et al. (2012)
Alkanibacter	Biofilter from an oil mill	Hexane	Friedrich and Lipski (2008)
Dyella	Mangrove sediment; activated sludge; contaminated soil	Phenanthrene; biphenyl	Muangchinda et al. (2013); Li et al. (2009); Uhlik et al. (2012)
Hydrocarboniphaga	Oil-contaminated soil	Alkanes and aromatics	Palleroni et al. (2004)
Luteibacter	Mangrove sediment; contaminated marine sediment, seawater or biofilm	Phenanthrene; crude oil	Muangchinda et al. (2013); Mahjoubi et al. (2013)
Lysobacter [b]	Wheat rhizosphere	Chloroalkane, chloroalkene, ethylbenzene, toluene, bisphenol, PAHs; crude oil	Yi et al. (2015); Susilaningsih et al. (2013)
Nevskia	Compost	Toluene	Juteau et al. (1999)
Polycyclovorans [a]	Marine diatoms	Aromatics and some alkanes	Gutierrez et al. (2013)
Pseudoxanthomonas	Gasoline/petroleum-contaminated soil; hydrocarbon-contaminated sediment	Benzene, toluene, ethylbenzene, *o*-, *m*-, and *p*-xylene; diesel, crude oil, *n*-tetradecane, *n*-hexadecane; phenanthrene	Choi et al. (2013); Nopcharoenkul et al. (2013); Patel et al. (2012); Cébron et al. (2011)
Rhodanobacter	Soil; hydrocarbon-contaminated site	Benzo[*a*]pyrene; biphenyl; naphthalene; lindane	Kanaly et al. (2002)[c]; Nalin et al. (1999); Uhlik et al. (2012)[d]
Rudaea	Contaminated soil	Biphenyl	Uhlik et al. (2012)
Singularimonas	Biofilter from an oil mill	Hexane	Friedrich and Lipski (2008)
Stenotrophomonas	Contaminated marine sediment, seawater or biofilm; e-waste desmantling area; biofilter for the removal of mono-aromatic hydrocarbons; brackish water lagoon; contaminated soil	Pyrene; benzo[a] pyrene; benzene, toluene, ethylbenzene, xylene; phenanthrene and pyrene; trichloroethylene; dibenzothiophen; 2-chlorobiphenyl	Chen et al. (2013, 2014); Lee et al. (2002); Juhasz et al. (2000); Mangwani et al. (2014); Mukherjee and Roy (2013); Papizadeh et al. (2011); Somaraja et al. (2013)

(*continued*)

Table 2 (continued)

Genus	Environmental source	Hydrocarbons degraded	References
Xanthomonas	Contaminated soil	Phenanthrene, anthracene, naphthalene; isoprene; γ-hexachlorocyclo-hexane	Braaz et al. (2005); Hamann et al. (1999); Manickam et al. (2007)
Xylella	Oil-contaminated soil	Alkanes	Yuste et al. (2000)

[a]Species that have been confirmed as obligate hydrocarbonoclastic bacteria (*OHCB*)
[b]The hydrocarbons degraded are based on an analysis of the genome of *Lysobacter capsici* strain X2-3, as reported by Yi et al. (2015)
[c]In the paper by Kanaly et al. (2002), *Rhodanobacter* contributed to the biodegradation of benzo[*a*] pyrene only within a consortium
[d]In the paper by Uhlik et al. (2012), *Rhodanobacter* that degrade biphenyl and naphthalene were identified by DNA-based stable isotope probing contributed using ^{13}C-labelled forms of these substrates

species within this order that have been confirmed as OHCB (i.e., *Algiphilus aromaticivorans* and *Polycyclovorans algicola*).

A number of studies have also reported the enrichment of members of the *Xanthomonadales* to petrochemicals, though without substantiating their hydrocarbon-degrading potential. In a study investigating PAH-degrading microbial communities in tsunami sediments in Miyagi, Japan, following the Great East Japan Earthquake, the dominant members of the microbial community after enrichment with PAHs included *Dokdonella* and *Luteimonas* (Bacosa and Inoue 2015). However, degradation of the PAHs by representative strains of these genera was not confirmed in this study. Other studies have isolated species of *Luteimonas* from oil-contaminated seawater or soil, but the focus of these studies was primarily a polyphasic characterization of the respective strains and was not evaluated for whether they could degrade hydrocarbons (Xin et al. 2014; Zhang et al. 2010). Young et al. (2007) isolated from oil-contaminated soil a member of the genus *Arenimonas* but did not confirm it to degrade hydrocarbons. In a paper by Sivakumar et al. (2012), the authors reported the isolation of a *Frateuria* sp. and show its production of EPS to be enhanced in media amended with xylene, although the ability of the strain to degrade xylene and other hydrocarbon substrates was not evaluated. In a study by Fang et al. (2015), the authors conducted a polyphasic characterization on a strain of *Oleiagrimonas* isolated from oil-contaminated saline soil. Although the authors did not determine whether the strain could degrade hydrocarbons, they suggested it was involved in aromatic hydrocarbon degradation with other members of the halophilic bacterial community. In a study by Luo et al. (2009), enhanced degradation of benzo[*a*]pyrene occurred when strains of *Stenotrophomonas* and *Pseudomonas* were combined than when tested individually.

4 Research Needs

Within the class *Gammaproteobacteria*, the order *Xanthomonadales* contains some important hydrocarbon-degrading bacteria that are commonly found enriched in environments (terrestrial and aquatic) that are contaminated with petrochemicals. Many of these organisms are generalist hydrocarbon degraders. However, the order contains two OHCB – namely, *Algiphilus aromaticivorans* and *Polycyclovorans algicola* – that were isolated from marine eukaryotic phytoplankton and characterized with the rare ability to use hydrocarbons (preferably aromatics) almost exclusively as their sole source of carbon and energy. These organisms were identified because they were targeted for in laboratory cultures of non-axenic marine phytoplankton by enrichment with PAHs. Further work to explore the hydrocarbon-degrading communities living associated with eukaryotic phytoplankton may uncover new taxa of OHCB within the order *Xanthomonadales*, as indeed affiliated to other major groups.

It may be inferred that they have hitherto eluded cultivation because they occupy a specific biotope in the ocean (i.e., the phycosphere of phytoplankton), which has not been sufficiently explored in this respect. The OHCB confer a narrow nutritional spectrum, primarily for hydrocarbons as growth substrates, and by nature of this fastidious quality some have been found difficult to isolate; it would not be unreasonable to assume that many others, including novel taxa, remain recalcitrant to cultivation. Comprehensive research, such as the use of targeted approaches for the cultivation of these types of organisms from the environment, is required to help expand our current knowledge on the diversity and ecological niches of hydrocarbon-degraders of the order *Xanthomonadales*.

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Anaerobic Hydrocarbon-Degrading *Deltaproteobacteria*

13

Irene A. Davidova, Christopher R. Marks, and Joseph M. Suflita

Contents

Abstract

Despite being a remarkably biodiverse taxon, the class *Deltaproteobacteria* includes relatively few model hydrocarbonoclastic isolates. These organisms as well as notable enrichment cultures containing *Deltaproteobacteria* are able to anaerobically metabolize a wide variety of normal, iso-, and cyclic alkanes, as well as mono- and polycyclic aromatic hydrocarbons. The isolates are mostly recovered from marine environments and can readily couple the metabolism of

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their parent substrate with the consumption of sulfate as a terminal electron acceptor. As a consequence, the organisms reduce sulfate (and often other sulfur oxyanions) to sulfide. Fe(III) can also serve as electron acceptor for the biodegradation of aromatic compounds, and alkynes can be fermented by *Pelobacter* strains. In the absence of a respiratory electron acceptor, the hydrocarbonoclastic *Deltaproteobacteria* can participate as members of syntrophic consortia that transfer reducing equivalents ultimately to methanogenic *Archaea*. Under the latter circumstances, the major consequence is the transformation of parent hydrocarbons to the stoichiometrically expected quantity of methane. The mechanism of hydrocarbon activation by *Deltaproteobacteria* is fundamentally different from hydrocarbonoclastic aerobes in that molecular oxygen is not a co-reactant. Rather, there are at least four biochemical strategies governing the primary attack on parent substrates. Identification of some functional genes has allowed for the detection and greater appreciation for the diversity of anaerobes capable of hydrocarbon metabolism. Despite the recognition that such organisms reside in varied environments, most cultivated representatives reflect rather limited environmental tolerance ranges. Given the importance of hydrocarbonoclastic *Deltaproteobacteria* in the biogeochemical cycling of carbon, the biocorrosion of steel, and prospects for methane recovery, studies on the full metabolic diversity inherent in this group of organisms seem particularly warranted.

1 Introduction

The *Deltaproteobacteria* is a physiologically and morphologically diverse class within the *Proteobacteria*. It is comprised of gram-negative, nonspore-forming bacteria that conserve energy by respiring with oxygen or other electron acceptors, such as sulfur oxyanions or ferric iron. Some *Deltaproteobacteria* can also ferment. This chapter focuses on anaerobic members of the *Deltaproteobacteria* that are able to oxidize hydrocarbons with electron acceptors other than oxygen or can degrade hydrocarbons by transferring reducing equivalents (electrons) to other microbes. The latter cooperative interaction between at least two microbial species to biodegrade a single substrate is called syntrophism. Such syntrophic cocultures often have a thermodynamic basis, and hydrogen-utilizing microbes are typically required to maintain low H_2 levels that effectively allow energetically unfavorable reactions to proceed. However, H_2-consuming organisms generally are not involved in the initial or primary catalytic steps.

A small number of *Deltaproteobacteria* genera able to biodegrade hydrocarbons under strictly anaerobic conditions have been isolated and characterized over the past two decades. Most of the described isolates metabolize hydrocarbons using sulfate or other sulfur oxyanions as terminal electron acceptors. A few organisms are known to use ferric iron as an electron acceptor. To date, there are no *Deltaproteobacteria* that couple hydrocarbon metabolism with nitrate respiration. Hydrocarbon-degrading sulfate-reducing bacteria seem to be most versatile in terms of the substrates utilized. They are known to biodegrade alkanes, alkenes,

monoaromatic compounds, tetralin, and some polycyclic aromatic hydrocarbons. This limited list of substrate types is certainly an underestimate of the true metabolic diversity inherent in this taxon of microorganisms (see Sect. 5). Unlike their sulfate-reducing counterparts, the known iron-reducing *Deltaproteobacteria* metabolize only monoaromatic substrates. The methanogenic biodegradation of hydrocarbons involves syntrophic interactions between bacterial and archaeal microorganisms. The important implication of such interactions is the conversion of large molecular weight hydrocarbons to the smallest hydrocarbon – methane. The involvement of *Deltaproteobacteria* as important participants in such syntrophic consortia will be briefly considered in Sect. 3.

Hydrocarbon-degrading *Deltaproteobacteria* are often detected in diverse environments based on characteristic genes encoding the catalytic subunit of the requisite activating enzymes (Winderl et al. 2007; Callaghan et al. 2010; von Netzer et al. 2013, 2016; Stagars et al. 2016, 2017). Yet the majority of isolates are mesophilic bacteria, and only a single thermophile is in culture. Given the importance of anaerobic hydrocarbon biodegradation as a major biogeochemical process, the potential to obtain other isolates from a multitude of environments is likely to be a rewarding scientific endeavor (see Sect. 5). One feature of hydrocarbon-degrading *Deltaproteobacteria* is the specificity of substrates that support the growth of these organisms. However, it is also known that some *n*-alkane-degrading bacteria can cometabolize nongrowth substrates including aromatic compounds and form partially oxidized intermediates (Rabus et al. 2011; Jarling et al. 2015; Wilkes et al. 2016). Extrapolation of such generalizations to iron-reducing microbes is not yet possible as *n*-alkane-utilizing ferric iron-metabolizing *Deltaproteobacteria* are not known.

2 Anaerobic Metabolism of Hydrocarbons

Hydrocarbons, by definition, are only composed of carbon and hydrogen. These elements are connected by strong sigma bonds that help account for the relatively low degree of chemical reactivity of the resulting structures. Despite the relative stability of hydrocarbons, there is no doubt that these compounds can be metabolized by microorganisms in the absence of molecular oxygen. Under anaerobic conditions, hydrocarbon biodegradation is usually coupled with the utilization of a variety of external electron acceptors. Moreover, the catabolism of hydrocarbons has been demonstrated with an array of strictly anaerobic taxa (see below and elsewhere in this series). Hydrocarbons that are gases, liquids, and solids can be activated through oxygen-independent mechanisms including radical-mediated addition to fumarate, hydroxylation, carboxylation, and hydration. The genetics, enzymology, and distribution of the hydrocarbon activation processes have been reviewed with great frequency over the years, and the reader is encouraged to consult other resources for a perspective on these matters (Widdel and Rabus 2001; Widdel et al. 2006; Meckenstock and Mouttaki 2011; Callaghan 2013; Heider et al. 2016b; Rabus et al. 2016). This review will focus more specifically on the *Deltaproteobacteria* that catalyze these reactions.

2.1 Organisms That Can Metabolize Aliphatic Hydrocarbons

"*Desulfococcus oleovorans*" strain Hxd3 was the first described organism able to biodegrade *n*-alkanes under strictly anaerobic sulfate-reducing conditions (Aeckersberg et al. 1991). The organism was enriched and isolated from an oil-water separator in Germany. The cells were tiny short rods transitioning to ovals. In addition to *n*-hexadecane, the bacterium could use *n*-dodecane, *n*-tetradecane, *n*-pentadecane, *n*-heptadecane, *n*-octadecane, and *n*-eicosane as substrates for growth. "*D. oleovorans*" strain Hxd3 can also utilize alkenes, 1-hexadecanol, 2-hexadecanol, as well as varying chain length fatty acids ranging from butyrate to stearate. The organism is capable of the complete oxidation of the parent alkane to CO_2. The organism grows best at temperatures of 28–30 °C and requires seawater conditions (NaCl 20 g/l and $MgCl_2 \times 6H_2O$ 3 g/l). Remarkably, this is the only organism known so far that activates alkanes through anaerobic hydroxylation reactions followed by carboxylation (for review, see Callaghan 2013).

The genus *Desulfatibacillum* contains the greatest number of species that are capable of metabolizing *n*-alkanes and *n*-alkenes. Cravo-Laureau et al. (2004a, b) proposed the taxon to accommodate hydrocarbon-degrading sulfate-reducing bacteria isolated from oil-polluted marine sediments along the coast of France. The same authors described the type strain *Desulfatibacillum aliphaticivorans* CV2803^T (Cravo-Laureau et al. 2004a) and *Desulfatibacillum alkenivorans* PF2803 (Cravo-Laureau et al. 2004b). Based on 16S rRNA gene phylogeny, other alkane-degrading sulfate-reducing bacteria previously obtained were placed in this genus (Aeckersberg et al. 1998; So and Young 1999).

The cells of all species affiliated with this genus are slightly curved, non-motile, nonspore-forming mesophilic rods that completely oxidize hydrocarbons. The strains able to utilize *n*-alkanes activate them by fumarate addition reactions. Though all members form a coherent taxon at the genus level, several questions of species delineation remain.

D. aliphaticivorans and *D. alkenivorans* are clearly separate species as their G + C content is vastly different (41.4 and 57.8 mol%, respectively). This difference is also reflected in DNA-DNA hybridization relatedness of 48.8%, well below the conventional threshold value of 70% for species differentiation (Wayne et al. 1987). Phenotypically *D. aliphaticivorans* is able to biodegrade C_{13}–C_{18} *n*-alkanes as well as C_7–C_{23} *n*-alkenes. However, *D. alkenivorans* is restricted to C_8–C_{23} *n*-alkene utilization. The same two isolates also have different salinity preferences, with *D. aliphaticivorans* and *D. alkenivorans* exhibiting optima at 24 g/L and 10 g/L NaCl, respectively.

The alkane-degrading strain AK-01 is also affiliated with the genus *Desulfatibacillum.* This organism was isolated from an active consortium enriched from the petroleum-contaminated sediments of Arthur Kill, an intertidal waterway between Staten Island and New Jersey (So and Young 1999). Phenotypically it is similar to other representatives of the genus, and it degrades C_{13}–C_{18} *n*-alkanes and C_{15}–C_{16} *n*-alkenes. Delineation of strain AK-01 as an individual species is uncertain. When the organism was isolated, only two other alkane-degrading sulfate-reducing

bacteria had been described. The development of new sequencing approaches for comparison of bacterial genomes has become the technique of choice for delineation of species with a high degree of precision (Rosselló-Móra and Amann 2015; Whitman 2015). Sequencing of the genomes of strain AK-01 (Callaghan et al. 2012) and *D. aliphaticivorans* CV2803 (IMG genome ID 2522572160) allowed for a pairwise comparison of the two genomes based on average nucleotide identity (ANI) and alignment fraction (AF). The analysis revealed values of 97.5 ANI and 0.87 AF (analysis by CRM), well above the intraspecies threshold proposed of 96.5 and 0.6, respectively (Varghese et al. 2015). Phenotypically, strains AK-01 and CV2803 both utilize alkanes and alkenes; they have about the same temperature optima and use the same range of electron acceptors. Collectively, these features argue that correct taxonomic position for strain AK-01 is as a strain of *D. aliphaticivorans*.

Another *Desulfatibacillum* strain Pnd3 obtained from marine sediments (Aeckersberg et al. 1998), represents a separate species. This conclusion is based on its phenotypic distinctiveness from *D. aliphaticivorans* and *D. alkenivorans*. Strain Pnd3 utilizes a more restricted range of *n*-alkanes for growth, (C_{14}–C_{17}) as well as 1-hexadecene and 1-hexadecanol. The cell could also grow using formate in addition to a wide range of fatty acids (C_3–C_{18}), but not acetate. Salinity preferences for the organism are not known for certain, but based on the seawater medium used for cultivation (the same as that used for "*D. oleovorans*" strain Hxd3), and the habitat from which it was isolated, the cell likely requires marine conditions.

The *Desulfosarcina/Desulfococcus* cluster is a diverse group of marine sulfate-reducing bacteria. It has been shown that the organisms of this clade are the primary alkane-degrading bacteria in marine hydrocarbon seeps (Kleindienst et al. 2014). Organisms affiliated with *Desulfosarcina/Desulfococcus* (DSS) cluster are discussed in more detail in the anaerobic methane oxidation chapter of this series (Knittel et al. 2018) as they participate in syntrophic consortia exhibiting this activity. Of course, methane is not the only gaseous hydrocarbon in petroliferous reservoirs and marine seeps. Sulfate-reducing bacteria able to biodegrade gaseous short-chain alkanes such as propane and butane have been isolated and are phylogenetically ascribed to the *Desulfosarcina/Desulfococcus* cluster of the *Deltaproteobacteria*. Specifically, the butane-degrading strain BuS5 has been isolated from a mesophilic enrichment culture obtained from a marine hydrocarbon seep (Kneimeyer et al. 2007). Morphologically, the cells are very short ovals, often occurring in pairs, and have a DNA G + C content of 40.9 mol%. Strain BuS5 exhibited a restricted range of alkane metabolism with only propane and butane activated by fumarate addition reactions. Unlike other hydrocarbons, propane could be attacked at both a terminal and subterminal carbon atom to form the corresponding isomeric succinate metabolites (Kneimeyer et al. 2007).

In addition to sulfate-reducing bacteria that can metabolize gaseous substrates, the *Desulfosarcina/Desulfococcus* cluster contains other hydrocarbon-utilizing organisms such as strain PL12 (Higashioka et al. 2009). This strain has been classified as a novel species called *Desulfosarcina alkanivorans* sp. nov. $PL12^T$ (Watanabe et al. 2017). This bacterium biodegrades *n*-hexane and *n*-decane along with a wide range of other non-hydrocarbon substrates. Interestingly, strain PL12

cells were mesophilic short motile rods isolated as a minor member of a *p*-xylene-degrading enrichment (Nakagawa et al. 2008). Alkanes were oxidized completely by the organism, but the mechanism of substrate activation is not yet known.

Another validated sulfate-reducing *n*-alkane-degrading genus is *Desulfoglaeba.* Thus far, the genus has two strains *Desulfoglaeba alkanexedens* strain ALDC and strain Lake (Davidova et al. 2006). They are phylogenetically close relatives but have been isolated from distant locations and have slightly different *n*-alkane ranges that each can utilize for growth (Table 1). The type strain *Desulfoglaeba alkanexedens ALDC*^{T} was isolated from an alkane-degrading consortium enriched from sludge collected from a facility processing shipboard oily wastewater. Strain Lake was isolated from oil field production waters. The source consortium enriched from the oily sludge was used in early experiments to demonstrate that a series of *n*-alkanes were activated by fumarate addition under sulfate-reducing conditions (Kropp et al. 2000). The same mechanism was confirmed in pure cultures for the two strains of *Desulfoglaeba*.

Only the type strain of *Desulfoglaeba* has been studied in detail. Cells of this mesophilic organism are slightly curved short rods with oval ends that tend to form large clusters. Sodium chloride is not required for growth, but the organism can tolerate up to 55 g/L of salt. The DNA G + C content of this organism is 53.6 mol%. *Desulfoglaeba* strains utilize a relatively narrow range of substrates to support growth. The type strain can metabolize C_6–C_{12} *n*-alkanes and a few non-hydrocarbon substrates. The strains oxidize *n*-alkanes completely using fumarate addition reactions to activate the hydrocarbon substrates.

Desulfatiferula is another genus that consists of two strains of alkene-degrading bacteria, *Desulfatiferula olefinivorans* LM2801 (Cravo-Laureau et al. 2007; Grossi et al. 2011) and *Desulfatiferula berrensis* BE2801 (Hakil et al. 2014). The organisms were isolated from brackish sediments polluted by the wastewater treatment plant of a petrochemical factory. The cells are mesophilic slightly curved small rods that are motile due to polar flagella. They don't require NaCl but can tolerate salt concentrations up to 50 g/L. The *Desulfatiferula* strains are unable to utilize *n*-alkanes but can oxidize *n*-alkenes. *D. olefinivorans* LM2801 utilizes C_{14}–C_{23} alkenes as electron donors with sulfate as an electron acceptor. *D. berrensis* BE2801 uses a slightly different range of alkenes (C_{12}–C_{23}) with sulfate, sulfite, elemental sulfur, and fumarate as electron acceptors. Unlike most described hydrocarbon-degrading organisms, the two species of *Desulfatiferula* oxidize *n*-alkenes incompletely. That is, mass balance calculations revealed that strain LM2801 oxidized 1-eicosene to acetate, while strain BE2801 produced both acetate and CO_2. *D. olefinivorans* LM2801 and *D. berrensis* BE2801 have G + C content of 45.5 mol% and 50.2 mol%, respectively. DNA-DNA hybridization between the two strains was only 14.8% similar, confirming their placement as individual species within the *Desulfatiferula* genus.

The genus *Desulfothermus* contains the only thermophilic *n*-alkane-degrading sulfate-reducing bacterium in the *Deltaproteobacteria*. Strain TD3 was isolated from sediments of the Guaymas Basin, part of a tectonic spreading zone where hydrothermal activity led to the formation of various hydrocarbons (Rueter et al. 1994).

Table 1 *Deltaproteobacteria* isolates that biodegrade hydrocarbons

Organism	Hydrocarbons utilized	Mechanism of activation	Electron acceptors	T(°C)	pH	Salinity (g/liter)	References
Aliphatic hydrocarbons							
"*Desulfococcus oleovorans*" strain Hxd3	*n*-alkanes C_{12}–C_{20} *n*-alkenes C_{14}, C_{16}, C_{17}	Hydroxylation followed by carboxylation	SO_4^{2-}	28–30	NR	32	Aeckersberg et al. 1991, 1998; Callaghan 2013
Desulfatibacillum strain Pnd3	*n*-alkanes C_{14}–C_{17} *n*-alkenes C_{16}	NR	SO_4^{2-}	30		32	Aeckersberg et al. 1998
Desulfatibacillum alkenivorans strain AK-01	*n*-alkanes C_{13}–C_{18} alkenes C_{15}, C_{16}	Addition to fumarate	SO_4^{2-} SO_3^{2-} $S_2O_3^{2-}$	33–35	6.9–7.0	10	So and Young 1999; Callaghan et al. 2006
Desulfatibacillum alkenivorans, PF2803^T	*n*-alkenes C_8–C_{23}	NR	SO_4^{2-} SO_3^{2-} $S_2O_3^{2-}$	28–30	6.8	10	Cravo-Laureau et al. 2004b
Desulfatibacillum aliphaticivorans CV 2803^T	*n*-alkanes C_{13}–C_{18} *n*-alkenes C_7–C_{23}	*n*-alkanes: addition to fumarate *n*-alkenes: hydration at C1	SO_4^{2-} SO_3^{2-} $S_2O_3^{2-}$	28–35	7.5	24	Cravo-Laureau et al. 2004a, 2005; Grossi et al. 2007
Desulfosarcina/Desulfococcus group strain BuS5	*n*- alkanes C_3–C_4	C1 addition to fumarate	SO_4^{2-}	28	6.9	32	Kniemeyer et al. 2007
Desulfosarcina alkanivorans strain PL12^T	*n*- alkanes C_6; C_{10}	NR	SO_4^{2-} SO_3^{2-} $S_2O_3^{2-}$ Fe (III)	30–34	7.0–7.3	32	Higashioka et al. 2009; Watanabe et al. 2017
Desulfoglaeba alkanexedens ALDC^T	*n*- alkanes C_6–C_{12}	Addition to fumarate	SO_4^{2-} $S_2O_3^{2-}$	31–37	6.5–7.2	32 Not required	Davidova et al. 2006

(*continued*)

Table 1 (continued)

Organism	Hydrocarbons utilized	Mechanism of activation	Electron acceptors	T(°C)	pH	Salinity (g/liter)	References
Desulfoglaeba strain Lake	*n*-alkanes C_6–C_{10}	Addition to fumarate	SO_4^{2-}	31–37	6.8–8.2	1–25	Davidova et al. 2006; Davidova and Suflita 2005
Desulfatiferula olefinivorans LM2801^T	*n*-alkenes C_{14}–C_{23}	NR	SO_4^{2-}	30–36	7.5	6–10	Cravo-Laureau et al. 2007
Desulfatiferula berrensis BE2801^T	*n*-alkenes C_{12}–C_{20}	NR	SO_4^{2-} SO_3^{2-} S^o Slight growth $S_2O_3^{2-}$; NO_3^-	30–32	7.2–7.4	5–15	Hakil et al. 2014
Desulfothermus naphthae TD3^T	*n*-alkanes C_6–C_{14}	NR	SO_4^{2-} $S_2O_3^{2-}$	60–65	6.5–6.8	26	Reuter et al. 1994; Kuever et al. 2005a
Mono- and polyaromatic hydrocarbons							
Desulfobacula toluolica Tol2	Toluene	Addition to fumarate	SO_4^{2-}	28	7.0–7.1	23	Rabus et al. 1993; Rabus and Heider 1998
Desulfotignum toluenicum H3^T	Toluene	NR	SO_4^{2-} SO_3^{2-}	34	7.2	15	Ommedal and Torsvik 2007
Desulfosarcina cetonica str. 480	Toluene	NR	SO_4^{2-} S^o $S_2O_3^{2-}$	30	NR	NaCl Not required	Harms et al. 1999; Kuever et al. 2005b
Strain PRTOL1	Toluene	Addition to fumarate	SO_4^{2-} $S_2O_3^{2-}$ SO_3^{2-}	35[a]	NR	NaCl Not required	Beller et al. 1996; Beller and Spormann 1997
Strain EbS7	Ethylbenzene	Addition to fumarate	SO_4^{2-}	31–32	7.5	32	Kniemeyer et al. 2003
Desulfosarcina ovata, str. oXyS1	*o*-xylene toluene *o*-alkyl toluenes	NR	SO_4^{2-}	32	7.5	32	Harms et al. 1999; Kuever et al. 2005b

Desulfosarcina widdelii PP31^T	*p*-xylene	NR	SO_4^{2-}	28	7.3–7.8	32	Nakagawa et al. 2008; Watanabe et al. 2017
Strain mXyS1	*m*-xylene toluene *m*-alkyl toluenes	NR	SO_4^{2-}	30	7.2	32	Harms et al. 1999
Geobacter metallireducens GS-15	Toluene benzene	NR	Fe (III) Mn(IV) NO_3^-	33	6.7[a]	NaCl Not required	Lovley and Lonergan 1990; Lovley and Phillips 1988; Zhang et al. 2012
Geobacter grbiciae TACP-2^T and TACP-5^T	Toluene	NR	Fe (III) AQDS	30[a]	NR	NaCl Not required	Coates et al. 2001
Geobacter toluenoxydans sp. TMJ1^T	Toluene	Addition to fumarate	Fe (III)	25–32	6.6–7.0	NaCl Not required	Kunapuli et al. 2010
Geobacter strain Ben	Benzene Toluene	NR	Fe (III) AQDS	30	NR	NR	Zhang et al. 2012
Strain TRM1	Toluene	NR	SO_4^{2-}	30	7.4	NR	Meckenstock 1999
Geobacter daltonii FRC-32	Toluene	Addition to fumarate (based on genome)	Fumarate (used with toluene)	30	6.7–7.3	NaCl not required	Prakash et al. 2010
Strain NaphS2 Strain NaphS3 Strain NaphS6	Naphthalene 2-methyl-naphthalene	Carboxylation (naphthalene) addition to fumarate (2-MN)	SO_4^{2-}	28–30	7.2[a]	32	Galushko et al. 1999; Musat et al. 2009

[a]When optimum is unknown cultivation condition is provided
NR – not reported

Strain TD3 was later named and validated as *Desulfothermus naphthae* TD3 (Kuever et al. 2005a). The cells were slightly curved motile rods that grew optimally at 55–60 °C under marine conditions. It grew with oil and completely oxidized C_6–C_{14} *n*-alkanes. The G + C content for the organism is much lower (37.4 mol%) than any other mesophilic hydrocarbon-degrading *Deltaproteobacteria*.

2.2 Organisms That Metabolize Aromatic Hydrocarbons

Unlike the aliphatic hydrocarbon-degrading relations, relatively few fully described and verified *Deltaproteobacteria* are capable of metabolizing aromatic ring structures. Most isolates are those that can anaerobically biodegrade toluene.

Desulfobacula toluolica Tol2 is a sulfate-reducing bacterium isolated from marine sediments and described by Rabus and colleagues (Rabus et al. 1993). It was the first pure culture of a sulfate-reducing bacterium able to biodegrade toluene. The cells exhibit an oval to coccoid morphology and grow well at 28 °C and require marine conditions (NaCl, 20 g/L and $MgCl_2$, 2.7 g/L). The G + C content of *D. toluolica* Tol2 is 42 mol%. The organism is metabolically diverse and can completely oxidize a wide range of organic compounds, but toluene was the only hydrocarbon substrate utilized. However, the cell could cometabolize *p*-xylene (Rabus and Widdel 1995). It was subsequently determined that the initial attack is by fumarate addition to the methyl carbon of toluene catalyzed by a benzylsuccinate synthase (Rabus and Heider 1998; Wöhlbrand et al. 2013). This metabolic pathway was further supported by genomic studies that identified gene clusters for benzylsuccinate synthase *bssA-F* and for β-oxidation of benzylsuccinate to benzoyl-CoA (*bbsA-H*). The protein gene products had high similarities to the analogous proteins in toluene-degrading denitrifying bacteria (Wöhlbrand et al. 2013).

Strain PRTOL1 is another sulfate-reducing bacterium capable of toluene degradation, isolated soon after *D. toluolica* Tol2. Strain PRTOL1 was isolated from a soil contaminated with aviation fuel and described as an oval-shaped non-motile mesophilic bacterium (Beller et al. 1996). Phylogenetic analysis based on 16S rRNA gene sequence showed only a distant relatedness to *D. toluolica* Tol2. The closest relative of the organism is *Desulforhabdus amnigenus* (96% similarity). Strain PRTOL1 mineralized toluene completely with the stoichiometrically expected amount of sulfate utilized. The conversion of deuterium-labeled toluene to deuterated benzylsuccinic acid by a growing culture as well as a permeabilized cell preparation confirmed that toluene was also activated by addition to fumarate (Beller et al. 1996; Beller and Spormann 1997). A stable isotope study also revealed that strain PRTOL1 cometabolized *o*-xylene-d_{10} with the concomitant formation of labeled fumarate addition metabolites.

Desulfotignum toluenicum strain H3 is the only species within the genus that can oxidize toluene with sulfate as an electron acceptor (Ommedal and Torsvik 2007). The isolate was obtained from an anaerobic enrichment culture inoculated with microorganisms from an oil reservoir model column supplied with crude oil as a carbon source (Myhr et al. 2002). The cells were mesophilic straight to slightly

curved non-motile rods. Strain H3 could oxidize toluene completely and grow with crude oil, presumably at the expense of toluene. The DNA G + C content for this organism was 52.0 mol%. Phylogenetically, *D. toluenicum* strain H3 was 98.7–99.9% similar to *Desulfotignum balticum*, but the inability of *D. balticum* to utilize toluene and a DNA-DNA hybridization value of only 56.1% firmly identified these organisms as separate species.

The aforementioned genus *Desulfosarcina* is part of the *Desulfosarcina/Desulfococcus* cluster. Apart from the involvement of *Desulfosarcina* in anaerobic methane and higher *n*-alkane oxidation, some of these *Deltaproteobacteria* are also capable of aromatic hydrocarbon biodegradation. *Desulfosarcina ovata* strain oXyS1 was enriched in a sulfate-reducing culture given crude oil as a source of organic carbon and eventually isolated using *o*-xylene as a sole substrate (Rueter et al. 1994; Harms et al. 1999). The cells of the mesophilic strain oXyS1 were elongated slightly curved rods with a growth optimum at 32 °C. Strain oXyS1 could grow on and completely oxidize toluene, *o*-xylene, and *o*-ethyltoluene. It was unable to use *meta*-substituted alkyltoluenes. The initial phylogenetic analysis revealed that strain oXyS1 closest relatives included *Desulfosarcina variabilis* (98.7%) and *Desulfobacterium cetonica* strain 480 (98.4%). However, *D. variabilis* could not utilize toluene or alkylbenzenes. *Desulfobacterium cetonicum*, str. 480, isolated from an oil field on butyrate (Galushko and Rozanova 1995), could utilize benzoate. Further studies demonstrated that *D. cetonica* could grow with toluene but not *o*-xylene (Harms et al. 1999). Subsequent studies found that *Desulfobacterium cetonicum* strain 480 should be reclassified as *Desulfosarcina cetonica* (Kuever et al. 2005b;). Strain oXyS1 was firmly placed into the genus *Desulfosarcina* as *Desulfosarcina ovata* oXyS1 (Kuever et al. 2005b)

Another *Desulfosarcina* strain mXyS1 was also characterized in the same study (Harms et al. 1999). It specifically utilized *meta*-substituted alkylbenzenes and could not oxidize *ortho*-substituted hydrocarbon counterparts. However, both strains mXyS1 and oXyS1 could grow on toluene. Phylogenetic analysis showed that strain mXyS1 branched deeply in the *Deltaproteobacteria*. It was just 86.9% similar to its closest relative *Desulfococcus multivorans*. So far, it is loosely affiliated with the Family *Desulfobacteriaceae*, but a more specific taxonomic characterization has not been completed.

A rod-shaped bacterial culture, strain PP31, that metabolized *p*-xylene with the stoichiometric reduction of sulfate was obtained from petroleum-contaminated sediments collected in Kuwait (Nakagawa et al. 2008). Subsequent studies (Higashioka et al. 2012) revealed that strain PP31 was rather specific for *p*-xylene and incapable of growth on the *ortho*- or *meta*-xylene isomers, a variety of other aromatic hydrocarbons or *n*-hexane. This organism was placed into genus *Desulfosarcina* as a novel species, *Desulfosarcina widdelii* (Watanabe et al. 2017). An isolate capable of toluene utilization has been obtained from *p*-xylene-degrading sulfate-reducing enrichments. Strain 28bB2T was closely (99%) related to *Desulfosarcina ovata*, but unlike *D. ovata*, the organism could not grow on xylene isomers (Higashioka et al. 2010)

A still unclassified sulfate-reducing bacterium capable of toluene biodegradation is strain TRM1. This organism was isolated from enrichments originally obtained

from a BTEX-contaminated (benzene, toluene, ethylbenzene, and xylene isomers) aquifer near Stuttgart (Meckenstock 1999). Strain TRM1 is a mesophilic bacterium that oxidizes toluene completely. It also can metabolize toluene in syntrophic co-culture with *Wolinella succinogenes* provided that either nitrate or fumarate is available as a terminal electron acceptor. Another isolate obtained from the same enrichment as strain TRM1 is strain OX39. Unlike strain TRM1, it did not metabolize toluene but grew on o-xylene and m-xylene. Toluene and its biodegradation products inhibited the growth of strain OX39 (Meckenstock et al. 2004).

The only known pure culture sulfate-reducing bacterium able to biodegrade ethylbenzene is strain EbS7 (Kniemeyer et al. 2003). This non-motile mesophilic isolate was obtained from Guaymas Basin sediments and forms oval-shaped rods with gas vesicle inclusions. Strain EbS7 is remarkably substrate-specific as only ethylbenzene is used as a hydrocarbon substrate. Unlike ethylbenzene-degrading denitrifying bacteria that activate the substrate by dehydrogenation, the primary reaction of strain EbS7 was to add fumarate to the methylene bridge of the molecule. The latter reaction pathway confirmed the earlier suggestion that ethylbenzene could also be transformed by a fumarate addition reaction (Elshahed et al. 2001). Phylogenetically, strain EbS7 is closely related to strain mXyS1 (97.6%) and strain NaphS2 (96.2%), organisms that could grow with sulfate as an electron acceptor on *m*-xylene or naphthalene, respectively. These three strains form a phylogenetically coherent deltaproteobacterial clade but have very different substrate preferences, justifying their placement as individual species.

As indicated above, members of the iron-reducing deltaproteobacterial Family *Geobacteraceae* are well known for their ability to oxidize aromatic compounds, but there are no known representatives able to oxidize other hydrocarbons. *Geobacter metallireducens* GS-15 was the first pure culture known to oxidize an aromatic hydrocarbon (toluene) with ferric iron as an electron acceptor. The organism was isolated from an enrichment obtained from Potomac River sediments that were amended with amorphous Fe(III)-oxyhydroxide as an electron acceptor and acetate as an electron donor. The cells of the isolated strain GS-15 were mesophilic non-motile small rods that oxidized acetate completely (Lovely and Phillips 1988). Later studies demonstrated that strain GS-15 could also completely mineralize toluene with the stoichiometric reduction of ferric to ferrous iron, a finding later confirmed with ring-labeled ^{14}C-toluene (Lovley and Lonergan 1990). The ability to mineralize toluene is not ubiquitously distributed in among *Geobacter* species, but toluene biodegradation has been demonstrated with *Geobacter grbiciae* TACP-2 (Coates et al. 2001) and *Geobacter toluenoxydans* sp. TMJI (Kunapuli et al. 2010). Further, *Geobacter daltonii* FRC-32 can oxidize toluene with fumarate as an electron acceptor (Prakash et al. 2010). For a period of time, it seemed that *Geobacter* species could only oxidize toluene. However, molecular studies of iron-reducing benzene-contaminated environments indicated that *Geobacter*-like species were enriched in zones of benzene oxidation (Anderson et al. 1998; Rooney-Varga et al. 1999). After a decade of effort, a benzene-degrading *Geobacter* strain Ben was isolated (Zhang et al. 2012) from a petroleum-contaminated aquifer in Bemidji, MN, USA. Benzene oxidation was in stoichiometric agreement with ferric iron reduction and

confirmed by radioisotopic experiments with ^{14}C-benzene. The cells of the mesophilic *Geobacter* strain Ben are slightly curved rods with round ends, and they prefer freshwater conditions. Phylogenetic analysis demonstrated that strain Ben was closely related to *Geobacter daltonii* (99%). Studies of strain Ben prompted a reevaluation of the metabolic capabilities of *Geobacter metallireducens* GS-15, and it was found that the latter was also capable of benzene metabolism when the substrate was provided at non-inhibitory concentrations (Zhang et al. 2012). Other findings implicate the involvement of *Deltaproteobacteria* in benzene metabolism. For instance, molecular studies identified sequence clones, SB-9, SB-29, SB-30, and SB-21, affiliated with the Family *Desulfobacteriaceae* in a benzene-degrading sulfate-reducing consortium (Phelps et al. 1998). Stable isotope probing terminal restriction fragment length polymorphism analysis helped detect the enrichment of clone SB-21 during the growth of the consortium on ^{13}C-labeled benzene (Oka et al. 2008). However, the direct involvement of these organisms in benzene degradation is not fully established.

Compared to monoaromatic substrates, much less progress has been made on the isolation of pure cultures able to anaerobically biodegrade polycyclic aromatic hydrocarbons (PAH). So far, only three deltaproteobacterial strains have been isolated that can couple sulfate reduction with naphthalene or 2-methylnaphthalene metabolism.

Strain NaphS2 has been isolated from North Sea sediments with naphthalene as a sole organic electron donor (Galushko et al. 1999). Mass balance determinations verified that naphthalene was completely oxidized with the stoichiometrically expected amount of sulfate reduced. Two other strains, NaphS3 and NaphS6, have also been isolated from Mediterranean sediments (Musat et al. 2009). These strains could also biodegrade naphthalene with the stoichiometric reduction of sulfate. All three of these naphthalene-degrading stains could also oxidize 2-methylnaphthalene. Phylogenetic analysis based on 16S rRNA genes revealed that the strains were closely related (Musat et al. 2009). In particular, strains NaphS2 and NaphS3 were 99.8% similar indicating that they were likely the same species, although they were obtained from different geographic locales.

3 Anaerobic Hydrocarbon Biodegradation by Mixed Microbial Cultures

Anaerobic biodegradation is fundamentally different from aerobic metabolism in that the catalytic entity is often not a single organism. Syntrophism plays a crucial role in metabolism of many forms of organic matter, including hydrocarbons. In anoxic environments, particularly when sulfate is depleted (but sometimes even in the presence of alternate electron acceptors (Gieg et al. 2008; Struchtemeyer et al. 2011), substrate decay can be coupled with the formation of methane. Syntrophism is a tightly coupled metabolic cooperation between partner organisms that allows them to collectively overcome energetic barriers that would normally preclude substrate transformation. To make some bioconversions thermodynamically

feasible, metabolites produced by the organism initiating substrate attack are kept at very low steady-state concentrations by the partner organism (Stams 1994; Schink 1997; McInerney et al. 2008). The basis for such syntrophic interactions is often a function of critical metabolites like H_2, formate, or acetate. *Deltaproteobacteria* affiliated with the Family *Syntrophacea* often play a central role in syntrophic consortia by acting as initiating partners (Table 2). These organisms often cooperate with hydrogenotrophic or/and acetoclastic methanogenic *Archaea* to facilitate the degradation of hydrocarbons with the production of stoichiometric quantities of methane and carbon dioxide. If initiating *Deltaproteobacteria* are coupled to hydrogenotrophic sulfate-reducing bacteria, the consortium will form sulfide instead of methane. In some instances, like anaerobic methane oxidation and the recently described syntrophic butane metabolism, specific thermophilic *Archaea* initiate biodegradation, and sulfate-reducing *Deltaproteobacteria* are the recipients of the interspecies electron transfer process (Laso-Perez et al. 2016).

A broad range of compounds can be completely mineralized by the syntrophic association of *Bacteria* and methanogenic *Archaea*. One of the most intriguing examples of such cooperation is the methanogenic degradation of crude oil (Townsend et al. 2003; Gieg et al. 2008; Berdugo-Clavijo and Gieg 2014) and individual oil constituents, such as the BTEX hydrocarbons (Grbić-Galić and Vogel 1987; Beller et al. 2002; Ulrich and Edwards 2003) and *n*-alkanes. However, there is no reason to presume that such associations necessarily involve *Deltaproteobacteria*. For example, the methanogenic biodegradation of isoparaffins proceeds through fumarate addition reactions that are catalyzed by a novel member of the Family *Peptococcaceae* (Order Clostridiales) and *Archaea* (Abu Laban et al. 2015a). That point notwithstanding, we will focus on hydrocarbon-degrading metabolic consortia with a high likelihood of deltaproteobacterial involvement.

The group of short-chain light *n*-alkanes occupies a special position in the spectrum of substrates that are degraded by enrichment cultures. With the exception of *n*-ethane, about which we know relatively little, the gaseous hydrocarbons *n*-propane and *n*-butane as well as *n*-pentane (liquid) are metabolized with the involvement of the Desulfosarcina/Desulfococcus group or Desulfococcus-Desulfonema-Desulfosarcina cluster (Kneimeyer et al. 2007; Savage et al. 2010; Jaekel et al. 2013). Within this group, there are tightly coupled consortia that are mechanistically comparable to the anaerobic methane oxidation syntrophic associations. At the same time, these associations have the potential to eventually yield axenic cultures capable of growing on short-chain *n*-alkanes. Interestingly, enrichments from the same environment yielded a pure mesophilic butane-degrading strain (BuS5), as well as an obligatory syntrophic culture (Butane50) containing a thermophilic *Archaea* that activated butane by alkyl-coenzyme M formation (Laso-Perez et al. 2016). That is, "*Candidatus Syntrophoarchaeum*" acted as a "methanogen" equivalent and activated butane by converting it to 1-butyl-CoM and 2-butyl-CoM by methyl coenzyme M reductase. Gene expression data suggested that 2-butyl-CoM was converted to butyryl-CoA by an unknown mechanism and the latter further oxidized by ß-oxidation. Ca. *Syntrophoarchaeum* is affiliated with the deltaproteobacterial phylotype HotSeep-1 retrieved from the

Table 2 Hydrocarbon-degrading enrichment cultures with implicated involvement of *Deltaproteobacteria*

Enrichment culture	Hydrocarbons utilized	Electron-accepting conditions	Implicated *Deltaproteobacteria*	Origin and T° preferences	References
Aliphatic hydrocarbons					
Enrichment (Butane 12-GMe)	*n*-butane	Sulfate-reducing	*Desulfosarcina/Desulfococcus* group	Marine, psychotrophic	Kneimeyer et al. 2007; Jaekel et al. 2013
Enrichments Prop12-GMe and But12-HyR	*n*-propane *n*-butane	Sulfate-reducing	*Desulfosarcina/Desulfococcus* group	Marine, psychotrophic	Jaekel et al. 2013
Propane-degrading enrichment	*n*-propane	Sulfate-reducing	*Desulfococcus-Desulfonema-Desulfosarcina* cluster Lineage gaseous alkane-associated group[a]	Terrestrial natural gas seep mesophilic	Savage et al. 2010
Pentane-degrading enrichment	*n*-pentane	Sulfate-reducing	*Desulfococcus-Desulfonema-Desulfosarcina* cluster Lineage liquid alkane-associated group[a]	Terrestrial natural gas seep; mesophilic	Savage et al. 2010
Enrichment culture	*n*-C_{16}	Methanogenic	Family Syntrophaceae *Smithella*	Ditch sediments; mesophilic	Zengler et al. 1999; Embree et al. 2013; Tan et al. 2014a, b
Short-chain consortia	*n*-C_6 to *n*-C_{10}	Methanogenic	No data on bacteria involved	Oil sand tailings	Siddique et al. 2006
Long chain consortia	*n*-C_{14} to *n*-C_{18}	Methanogenic	*Syntrophus*[a] is predominant but its role not proved	Oil sand tailings	Siddique et al. 2011
Iso-alkanes consortia	C_7-C_8 iso-alkanes	Methanogenic			Abu Laban et al. 2015a
SCADC	mixture *n*-C_6 to *n*-C_{10} and iso-alkanes	Sulfate-reducing	*Desulfobacteraceae, Smithella* sp. SCADC, *Desulfoglaeba*	Oil sands tailings ponds	Tan et al. 2015

(*continued*)

Table 2 (continued)

Enrichment culture	Hydrocarbons utilized	Electron-accepting conditions	Implicated *Deltaproteobacteria*	Origin and T° preferences	References
Enrichment culture	Light crude oil and heavy crude oil	Methanogenic	*Smithella*[a] detected but the involvement in activation is not proved	Production waters of heavy oil reservoir mesophilic	Berdugo-Clavijo and Gieg 2014
Enrichment culture	*n*-C_{16}	Methanogenic	*Syntrophaceae*[a] Involvement is not proved	Disposal water treatment field; mesophilic	Cheng et al. 2013
Enrichment culture RODC	Residual oil *n*-alkanes	Methanogenic	*Desulfobacterales*[a] *Syntrophpobacterales*[a]	Gas condensate-contaminated subsurface sediments; mesophilic	Gieg et al. 2008
Enrichment culture SDB	C_{18}–C_{50} C_{28}(opt)	Methanogenic	*Smithella*	Contaminated marine sediments; mesophilic	Wawrik et al. 2016
Mono- and polyaromatic hydrocarbons					
Enrichment cultures	Benzene toluene *m*-and *p*-xylenes	Iron-reducing	*Geobacter* spp. as terminal syntrophic partners of *Desulfomonile* sp.	Polluted sediments and groundwater; mesophilic	Botton et al. 2007
Enrichment culture	*p*-xylene	Sulfate-reducing	A sulfate-reducing bacteria close to oXyS1	Contaminated marine harbor sediments; mesophilic	Nakagawa et al. 2008
Enrichment culture	Benzene	Sulfate-reducing	*Deltaproteobacteria* clone BznS295	Marine, mesophilic	Musat and Widdel 2008
Enrichment culture	Benzene	Iron-reducing	*Geobacter* spp.	Fresh water; mesophilic	Rooney-Varga et al. 1999
Enrichment culture	Benzene	Methanogenic	*Deltaproteobacteria* ORM2	Soil from oil refinery and gas station; mesophilic	Ulrich and Edwards 2003; Luo et al. 2016
Enrichment culture N47	Naphthalene	Sulfate-reducing	*Deltaproteobacterium* N47	Fresh water; mesophilic	Meckenstock et al. 2000; Bergmann et al. 2011
Enrichment culture	Phenanthrene	Sulfate-reducing	*Deltaproteobacteria*	Marine; mesophilic	Davidova et al. 2007

[a]The role of bacteria is likely but not confirmed

metagenome. HotSeep-1 contained *Ca.* "*Desulfofervidus auxilii*" that was shown to be an electron-consuming partner during high-temperature anaerobic methane oxidation (Krukenberg et al. 2016). *Candidatus Syntrophoarchaeum* lacked functional genes for sulfate reduction and thus relied on its syntrophic partner for electron utilization. Most likely, the deltaproteobacterial HotSeep-1 functioned as an electron acceptor growing on reducing equivalents supplied by the butane-degrading archaeon.

In most known cases, methanogenic *Archaea* are on the receiving end of interspecies electron transfer processes. Methanogenic transformation of *n*-alkanes has been reported for short- (C_6–C_{10}) (Siddique et al. 2006; Cheng et al. 2013; Tan et al. 2013; Abu Laban et al. 2015a), medium- (C_{14}–C_{18}) (Zengler et al. 1999; Anderson and Lovley 2000; Tan et al. 2014a, b; Siddique et al. 2011; Mbadinga et al. 2012; Liang et al. 2016), and long-chain paraffins (C_{25}–C_{50}) (Wawrik et al. 2016), in addition to some PAHs (Berdugo-Clavijo et al. 2012) and various crude oils (Jones et al. 2008; Gieg et al. 2008; Berdugo-Clavijo and Gieg 2014). Gray et al. (2010) and Gieg et al. (2014) have reviewed the available information on methanogenic degradation of hydrocarbons. Unfortunately, isolation of the requisite microorganisms and clarification of the specific roles they play have remained somewhat enigmatic. However, progress in molecular microbial ecology has allowed for some powerful insights into the nature of syntrophic alkane mineralization.

The importance of syntrophism for anaerobic alkane biodegradation was clear ever since the first report of hexadecane conversion to methane (Zengler et al. 1999). The authors suggested that hexadecane was degraded by syntrophic association between the genus *Syntrophus* with a hydrogenotrophic *Methanospirillum* and an acetoclastic *Methanosaeta* that consumed H_2 and acetate, respectively. Subsequent sequencing analysis of the culture verified the predominance of *Smithella* (phylogenetically closely related to *Syntrophus*) as well as several *Archaea* including *Methanosaeta*, *Methanocalculus*, and *Methanoculleus* (Embree et al. 2014, 2015). The latter are clearly dependent on hydrogen and acetate produced during the course of substrate metabolism; thus the organism(s) that activate the parent alkane plays the rate-limiting role in the overall biodegradation process. In several studies, *Bacteria* affiliated with the Family *Syntrophaceae* and particularly the genera *Smithella* and *Syntrophus* were identified as predominant in hydrocarbon-degrading microbial assemblages (Jones et al. 2008; Gray et al. 2011; Siddique et al. 2011; Cheng et al. 2013; Berdugo-Clavijo and Gieg 2014; Embree et al. 2014; Wawrik et al. 2016). Thus far, members of these genera that are directly involved in anaerobic alkane biodegradation have not been isolated. However, progress in studying anaerobic alkane metabolism allowed for the identification of the genes coding for an alkylsuccinate synthase, the enzyme that catalyzes fumarate addition reactions (Callaghan et al. 2008a; Grundmann et al. 2008). The large catalytic subunit (*assA*/*masD*) has been recognized as marker for anaerobic alkane metabolism (Callaghan et al. 2010).

Using metagenomic and metatranscriptomic analysis and single-cell genome sequencing, several *n*-alkane-degrading methanogenic cultures were interrogated for this marker gene. When the *n*-alkane-degrading culture reported by Zengler

et al. (1999) was investigated, no *ass* genes were initially detected based only on sequence alignment for annotation of gene sequences (Embree et al. 2014). However, reanalysis of the same data by Tan and colleagues (2014a, b) revealed that the *assA* gene was indeed part of the requisite "*Smithella*" sp. ME-1 draft genome. The same group analyzed draft genome sequences affiliated with *Smithella* spp. obtained from a methanogenic alkane-degrading culture SCADC (Tan et al. 2013) and from an oil field produced water and detected putative *assABC* genes indicative of the genetic potential for alkane activation by fumarate addition (Tan et al. 2014b). Later, five contigs encoding homologs of the catalytic subunit *assA* and the corresponding mRNA transcripts associated with the assembled draft genome for a "*Smithella*" spp., a dominant organism in an octacosane (C28)-degrading methanogenic consortium (Wawrik et al. 2016), were identified. This type of data supported the critical role of syntrophic *Deltaproteobacteria* in methanogenic alkane degradation and suggests that the parent hydrocarbons were initially activated by fumarate addition reactions.

Members of *Syntrophaceae* are not the only organisms capable of activating alkanes under anaerobic conditions. In the methanogenic hexadecane enrichments, multiple clones were related to *D. alkenivorans* AK-01, an organism known for its ability to biodegrade the parent hydrocarbon in co-culture with the hydrogenotrophic *Methanospirillum hungatei* JF-1 (Callaghan et al. 2012). This ability is likely reasonably common for alkane-degrading *Deltaproteobacteria* as a co-culture of *Desulfoglaeba alkanexedens* ALDC and *Methanospirillum hungatei* JF-1 could biodegrade dodecane with the production of stoichiometrically expected quantities of methane (unpublished results). In the methanogenic short-chain alkane-degrading culture SCADC, the most likely candidate for initial alkane attack turned out to be an organism that was most closely related to *Peptococcaceae*, a *Clostridia* and not a *Deltaproteobacteria* (Tan et al. 2015).

4 Genomics of Hydrocarbon-Degrading *Deltaproteobacteria*

4.1 Genome Availability and Features

There are a relatively limited number of hydrocarbon-degrading *Deltaproteobacteria* currently available in axenic culture. Despite the dearth of available cultivars, sequence information on this small subset have yielded important findings on the physiology and ecology of hydrocarbon activation mechanisms, as well as the identification of conserved markers useful for genomic, transcriptomic, and proteomic investigations of anaerobic hydrocarbon biodegradation.

There are a total of 14 genomes from pure culture hydrocarbon-degrading *Deltaproteobacteria* (Table 3). These genomes are publicly available in the Integrated Microbial Genomics database from the Joint Genome Institute (http://genome.jgi.doe.gov). The genomes are moderate to large in size ranging from ~3 to 7 Mb, with the largest genomes belonging to members of the *Desulfobacterales*. Each genome has a high coding density with >85% of all

Table 3 Select statistics and features for available genomes of hydrocarbon-degrading *Deltaproteobacteria*

Organism	Genome size (Mbp)	Total gene count	Coding bases (%)	16S rRNA gene count	GC content (%)
Desulfobacterales; Desulfobacteraceae					
"*Desulfococcus oleovorans*" Hxd3	3.94	3320	88	1	56
"*Desulfatibacillum alkenivorans*" AK-01	6.52	5361	88	2	54
Desulfatibacillum alkenivorans PF2803	6.47	5303	87	1	55
Desulfatibacillum aliphaticivorans CV 2803	6.47	5375	88	2	54
Desulfosarcina sp. BuS5	3.60	3636	86	1	41
Desulfosarcina cetonica str. 480	7.04	7962	86	1	56
Desulfobacula toluolica Tol2	5.20	4435	88	4	41
Strain NaphS2	6.55	7278	87	2	50
Desulfuromonadales; Geobacteraceae					
Geobacter metallireducens GS-15	4.01	3663	92	3	59
Geobacter toluenoxydans TMJ1	4.20	5484	88	1	54
Desulfuromonadales; Pelobacteraceae					
Pelobacter acetylenicus DSM 3246	3.21	3082	88	4	57
Pelobacter acetylenicus DSM 3247	3.18	2969	88	3	57
Pelobacter sp. SFB93	3.22	3033	87	3	53
Syntrophobacteraceae					
Desulfoglaeba alkanexedens ALDC	3.37	3082	84	2	59

Data for each genome obtained from Integrated Microbial Genomes database

bases coding. The *Desulfuromonadales* genomes tend to contain more 16S rRNA gene copies per genome than the *Desulfobacterales*. Previous work has showed a correlation between increased 16S rRNA gene copy number and higher growth rate (Klappenbach et al. 2000; Stevenson and Schmidt 2004). This correlation is supported by the longer doubling times of 3 days and 5.3 days reported for strain AK-01 (So and Young 1999) and *D. alkanexedens* (Davidova et al. 2006), respectively, compared to 5 h for *Pelobacter acetylenicus* (Schink 1985) and 27 h for *D. toluolica* Tol2 (Rabus et al. 1993) as determined on their respective hydrocarbon substrates.

The advancement of the "omics" research techniques has allowed for the recovery of draft genomes from uncultivated putative hydrocarbon-metabolizing taxa. Draft genomes for "*Smithella*" spp. ME-1, SCADC, D17, and SDB have

been recovered from methanogenic enrichment cultures and linked to *n*-alkane metabolism via fumarate addition (see Sect. 4.2.1) (Embree et al. 2014; Tan et al. 2014a, b; Wawrik et al. 2016). Stable isotope probing coupled with metagenomic sequencing yielded a draft genome "*Desulfobulbaceae* Tol-SR" encoding a *bss* gene cluster from a sulfidogenic toluene-degrading enrichment (Abu Laban et al. 2015b), and the draft genome for the PAH-degrading deltaproteobacterium N47 was obtained through genomic binning of the highly enriched sulfidogenic culture (Bergmann et al. 2011).

4.2 Genomics of Oxygen-Independent Hydrocarbon Activation

The catabolism of a variety of hydrocarbons has been demonstrated in a wide array of strictly anaerobic taxa, as discussed in this chapter. It will likely be of interest to readers to know that there is a database of anoxic hydrocarbon degradation genes "AnHyDeg" that is a freely available curated resource for investigators (Callaghan and Wawrik 2016). The salient aspects of anaerobic hydrocarbon biodegradation are briefly detailed below.

4.2.1 Addition to Fumarate

Alkanes, iso-alkanes, cyclic alkanes, and alkyl-substituted mono- and polycyclic aromatic hydrocarbons can all be initially activated through the radical-mediated addition to fumarate by the alkyl- and arylalkylsuccinate synthases of the glycyl radical enzyme superfamily. These enzymes and their mechanisms have been explored (Callaghan 2013; Heider et al. 2016b). Benzylsuccinate synthase (*bss*) is the model for the alkyl-/arylalkylsuccinate synthases and is a heterohexameric enzyme, a dimer of a heterotrimer (αβγ) subunits (Leuthner et al. 1998). The α-subunit contains the conserved catalytic glycine and cysteine residues responsible for radical hydrogen abstraction from the methyl group of the arylalkyl substrate (e.g., toluene, xylene, ethylbenzene) and is encoded by *tutD/bssA*. The β- and γ-subunits encoded by *tutG/bssB* and *tutF/bssC* were found to contain [4Fe-4S] clusters that were integral to subunit binding and proper enzyme confirmation (Li et al. 2009; Funk et al. 2014). The catalytic glycyl radical is formed via post-translational modification by the activase enzyme, a S-adenosyl-L-methionine superfamily protein encoded by *tutE/bssD*. Thus, the fully functional Bss requires the translated products of the *bssABCD* genes. The other enzymes of Sthe alkyl-/arylalkylsuccinate synthases, alkylsuccinate synthase (*ass/mas*) (Callaghan et al. 2008a; Grundmann et al. 2008), naphthylmethylsuccinate synthase (*nms*) (Annweiler et al. 2000, 2002), hydroxybenzylsuccinate synthase (*hbs)* (Strijkstra et al. 2014), and (4-isopropyl)benzylsuccinate synthase (*ibs*) (Strijkstra et al. 2014) all utilize a similar multimeric structure to the model Bss and are encoded by the respective homologous genes.

As members of the glycyl radical enzyme superfamily, the catalytic subunit of the alkyl and arylalkylsuccinate synthases is related to several other ubiquitous enzymes such as pyruvate formate lyases; however, the succinate synthase α-subunits can be distinguished through comparison of conserved cysteine residues through sequence alignment (Callaghan et al. 2008a). Furthermore, the various

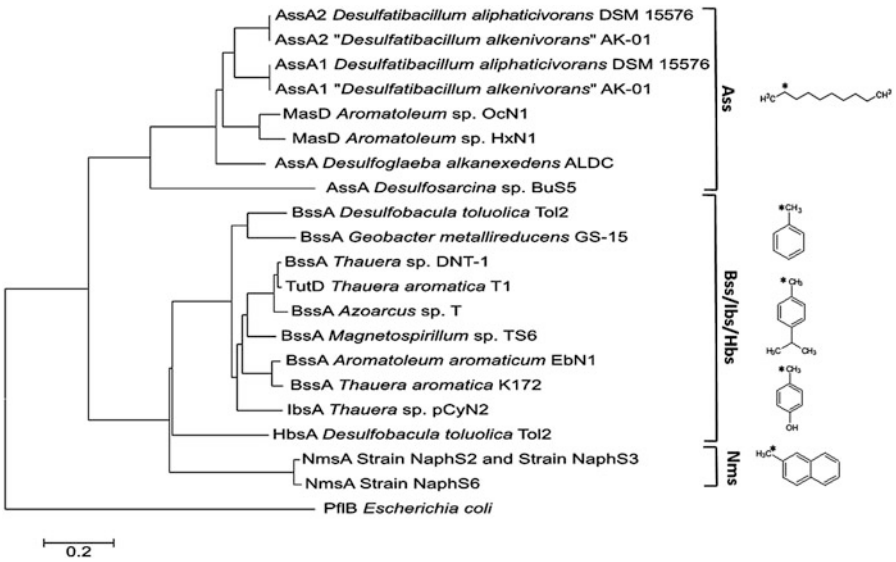

Fig. 1 Phylogeny of succinate synthase active subunits and associated substrates. Neighbor-joinning dendrogram showing protein phylogeny of succinate synthase catalytic subunits from cultivated anaerobic hydrocarbon-degrading taxa (not all belong to the *Deltaproteobacteria*). Examples of substrates for each clade of succinate synthases are shown with the asterisk (*) indicating the site of fumarate addition

succinate synthases generally cluster by substrate class (e.g., alkyl, polyaromatic, etc.) and form subclades when phylogenetically compared (Fig. 1). This would therefore seem like an essential analysis for verifying the identity of putative succinate synthase α-subunit genes recovered from isolates and environmental sequence studies. Strain AK-01 is the model organism for alkane metabolism via addition to fumarate (Callaghan et al. 2012) and like *D. aliphaticivorans*, *Desulfoglaeba alkanexedens* (unpublished result), "*Smithella* sp. SCADC" (Tan et al. 2014b), and the denitrifying betaproteobacterial strain HxN1 (Webner 2012) contains multiple Ass gene clusters. A transcriptomic study of strain AK-01 showed differential transcription of the Ass1 (and not Ass2) gene clusters under alkane-catabolizing conditions, regardless of substrate chain length of the tested *n*-alkane (Herath et al. 2016). The exact role of the Ass2 gene cluster in this organism is as yet unknown (Herath et al. 2016).

4.2.2 Carboxylation

In contrast to the alkylbenzenes and alkylnaphthalenes, benzene and naphthalene do not have alkyl substituent groups to act as sites for initial biological attack. Stable isotope evidence in enrichment cultures has documented either the incorporation of a methyl group (followed by subsequent transformation via the fumarate addition pathway) or the direct addition of a carbonate moiety (see references within Meckenstock and Mouttaki 2011; Meckenstock et al. 2016). Mouttaki and

colleagues demonstrated the direct carboxylation of naphthalene to 2-naphthoic acid in cell extracts of the marine sulfate-reducing deltaproteobacterial enrichment culture N47 (Mouttaki et al. 2012). Several candidate naphthalene carboxylase genes were identified through the analysis of the genome of naphthalene-degrading isolate NaphS2 and genome bin of the highly enriched N47 (DiDonato et al. 2010; Bergmann et al. 2011). Several candidate genes related to phenolic carboxylate decarboxylase (UbiD family) were upregulated in NaphS2 during growth on naphthalene and were postulated to encode the putative naphthalene carboxylase (DiDonato et al. 2010). Interestingly, the naphthalene carboxylation activity of the N47 cell extracts was not dependent on ATP hydrolysis (Mouttaki et al. 2012) in contrast to this requirement for the UbiD family decarboxylases. Stable isotope probing and metaproteomic analysis of sulfidogenic naphthalene-degrading groundwater enrichment cultures showed evidence of direct carboxylation by *Desulfobacteraceae*-affiliated taxa (Kümmel et al. 2015). Similarly, carboxylase genes have been identified in a gram-positive enrichment culture actively degrading benzene under iron-reducing conditions (Abu Laban et al. 2010). Clearly direct carboxylation is an important activation mechanism for the degradation of unsubstituted aromatic hydrocarbons, but further research is needed to identify the physiological requirements for these activities and their ecological distribution within impacted environments.

4.2.3 Hydroxylation

The oxygen-independent hydroxylation pathway is best studied in the denitrifier *Aromatoleum aromaticum* EbN1 wherein the alkyl side chain of ethylbenzene is initially dehydrogenated and subsequently hydroxylated through hydration of the resulting unsaturation as reviewed in (Heider et al. 2016a). This process is catalyzed by ethylbenzene dehydrogenase, a molybdenum heterotrimeric enzyme encoded by *ebdABC* (Johnson et al. 2001; Rabus et al. 2002). Related enzymes have been identified catalyzing similar oxygen-independent hydroxylations of alkylated aromatic substrates (e.g., p-cymene by *A. aromatoleum* pCyN1), as well as a putative C2-methylene hydroxylase acting on *n*-alkanes in "*D. oleovorans*" strain Hxd3 (Callaghan et al. 2008b; Strijkstra et al. 2014). Initial studies with "*D. oleovorans*" strain Hxd3 hypothesized a direct carboxylation mechanism for the activation of the alkane substrate based upon stable isotope incorporation studies (So et al. 2003). However, subsequent proteogenomic and metabolite-based investigations now indicate that strain Hxd3 initially hydroxylates the alkane via ethylbenzene dehydrogenase-like putative C2-methylene hydroxylase to the corresponding alkan-2-ol (Rabus et al. 2016). The putative C2-methylene hydroxylase is encoded by *ahyABC*, with the *ahyA* being the proposed catalytic subunit as in the orthologous ethylbenzene dehydrogenase (Callaghan et al. 2008b). No potential Ass/Mas genes were detected within the genome of this organism, further supporting the contention that the alkane activation mechanism utilized by "*D. oleovorans*" strain Hxd3 (Callaghan et al. 2008b) must be fundamentally different from the better known fumarate addition mechanism.

4.2.4 Hydration

The oxygen-independent catabolism of alkynes has received relatively little study compared to other hydrocarbon chemical classes, no doubt reflecting the fact that these hydrocarbons are less quantitatively important in oils and refined products. Acetylene is the sole currently known alkyne catabolized under anaerobic conditions. *Pelobacter acetylenicus* ferments acetylene to acetate and ethanol after an initial activation to acetaldehyde (Schink 1985). The initial activation of the alkyne is a hydration reaction catalyzed by acetylene hydratase, a tungsten iron-sulfur enzyme that has been purified and studied extensively (Rosner and Schink 1995; Meckenstock et al. 1999; Seiffert et al. 2007; tenBrink et al. 2011). Currently, acetylene fermentation has only been identified within the genus *Pelobacter*, including *P. acetylenicus* DSM3246 and DSM3247 (Schink 1985) and *Pelobacter* sp. SFB93 (Miller et al. 2013). Acetylene hydratase is a soluble monomer encoded by the gene *ahy* (Rosner and Schink 1995) and has been identified within the genomes of all three of these organisms (Akob et al. 2017). A recent cultivation study showed acetylene inhibition of reductive dehalogenation by *Dehalococcoides mccartyi* 195 could be alleviated through co-culture with *Pelobacter* sp. SFB93 (Mao et al. 2017). Acetylene is produced in groundwaters contaminated with chlorinated solvents through the chemical reduction of the contaminants and has been postulated as an important component of the anoxic atmosphere of ancient Earth (Oremland and Voytek 2008). The observation of acetylene fermentation activity in previous environmental surveys (Culbertson et al. 1981) and numerous potential *ahy* gene sequences currently present within the public sequence repositories (Akob et al. 2017) suggests that acetylene fermentation may be an important biogeochemical process in anoxic habitats and that oxygen-independent alkyne metabolism may be a relatively widespread phenomena.

4.3 Central Metabolism and Energy Conservation Processes

4.3.1 Energy Conservation

Deltaproteobacteria capable of the anoxic catabolism of hydrocarbons can couple these oxidative reactions to several different electron accepting processes, both respiratory and fermentative. The *Desulfobacterales* members are most commonly associated with sulfate reduction in natural and engineered petroleum-associated habitats but can utilize other sulfoxyanion electron acceptors as well (see Sect. 4.3.2).

Under respiratory conditions, the acetyl-CoA generated by β-oxidation of aliphatic or aromatic hydrocarbon substrates gets completely oxidized to yield further reducing equivalents for energy conservation. The genomes of each of the cultivated hydrocarbon-degrading *Desulfobacterales* and *Geobacteraceae* contain at least a single copy of *cooS* encoding the catalytic subunit of the bifunctional carbon monoxide dehydrogenase/acetyl-CoA synthase, the essential enzyme of the Wood-Ljungdahl pathway (Ragsdale 2008). In addition, these taxa, except for *G. toluenoxydans*, possess a putative AMP-forming acetyl-CoA synthetase (Acs). During the anaerobic biodegradation of aromatic hydrocarbons, benzoate is often

a critical intermediate. Synthesis of ATP during benzoate fermentation by Acs1 was recently demonstrated in the deltaproteobacterium *Syntrophus aciditrophicus* (James et al. 2016).

In contrast, *Pelobacter acetylenicus* and *Pelobacter* sp. SFB93 are the only known obligately fermentative anaerobic hydrocarbon-degrading pure cultures. None of the genomes of these organisms encode a respiratory pathway (Akob et al. 2017; Sutton et al. 2017a, b). However, these organisms possess a phosphotransacetylase/acetate kinase (Pta/Ack) system, as well as a single copy of the putative AMP-forming acetyl-CoA synthetase (Acs) (Sutton et al. 2017a, b). Genes for the enzymes of the reverse TCA cycle are absent. A single copy of *cooS* was detected in genome of *P.* sp. SFB93, but not in *P. acetylenicus*. Perhaps these organisms utilize the two-step Pta/Ack pathway for energy conservation during acetylene fermentation, but this remains to be demonstrated. The draft genomes of the uncultivated alkane-degrading *Smithella* are similarly devoid of respiratory pathway genes and possess an AMP-forming Acs (Tan et al. 2014b; Wawrik et al. 2016). Unlike the fermentative *Pelobacter* spp., no Pta/Ack system was observed in these draft genomes, lending credence to the suggestion that the Acs may represent an important energy conservation mechanism during anaerobic hydrocarbon metabolism under fermentative conditions in *Deltaproteobacteria*.

4.3.2 Sulfur Metabolism

All of the available genomes from this group excluding those of the *Desulfuromonadales* possess the genes for dissimilatory sulfate reduction via APS reductase (*aprAB*) and catalytic dissimilatory sulfite reductase subunits (*dsrAB*) (Table 4). In addition to sulfate, thiosulfate and sulfite have been shown to support growth of many sulfate-reducing species (Table 4). Thiosulfate reductase (*phs*A catalytic subunit) was found in each of the genomes available for these taxa. The activity of the thiosulfate reductase can also be linked to sulfite reduction by the Dsr system to yield a stoichiometric balance of two sulfides per thiosulfate reduced. Previous work on *Desulfovibrio vulgaris* growth yields under sulfate- versus thiosulfate-reducing conditions showed net ATP yields of 1 and 3 mol per mol substrate, respectively (Badziong and Thauer 1978). This physiological advantage suggests that thiosulfate is preferred when both electron acceptors are available in oxygen-limited petroleum-contaminated environments. The *Geobacter* and *Pelobacter* spp. do not possess the genes for either dissimilatory sulfate or thiosulfate reduction (Table 3). None of the organisms in this group possess the sulfur-oxidizing (SOX) genes nor have any been reported to oxidize reduced sulfur species.

4.3.3 Nitrogen Metabolism

The hydrocarbon-degrading *Deltaproteobacteria* generally do not possess denitrification genes or genes for the dissimilatory nitrate reduction to ammonium (DNRA). *G. metallireducens* GS-15 is the only organism within this group of cultivars to possess genes for the dissimilatory reduction of nitrate (*narGHIJ*), as well as the genes encoding the cytochrome c nitrite reductase (*nrfAH*) catalyzing the reduction of nitrite to ammonium (Table 4). Genes encoding nitrification enzymes are

Table 4 Catabolic and dissimilatory genes in available genomes from hydrocarbon-degrading *Deltaproteobacteria*

	Catabolic acetyl-CoA pathways			Sulfur metabolism			Nitrogen metabolism			
Organism	Pta/Ack	W-L	ACS	*aprAB*	*dsrAB*	*phsA*	*narG*	*nosZ*	*nrfA*	*nifHDK*
Desulfobacterales; *Desulfobacteraceae*										
"*Desulfococcus oleovorans*" Hxd3		●	●	●	●					
"*Desulfatibacillum alkenivorans*" AK-01		●	●	●	●	●				●
Desulfatibacillum alkenivorans PF2803		●	●	●	●	●				●
Desulfatibacillum aliphaticivorans CV 2803		●	●	●	●	●				●
Desulfosarcina sp. BuS5		●	●	●	●					
Desulfosarcina cetonica str. 480		●	●	●	●	●				●
Desulfobacula toluolica Tol2		●	●	●	●	●				
Strain NaphS2		●	●	●	●	●	●			●
Desulfuromonadales; *Geobacteraceae*										
Geobacter metallireducens GS-15	●	●	●				●		●	●
Geobacter toluenoxydans TMJ1	●	●								●
Desulfuromonadales; *Pelobacteraceae*										
Pelobacter acetylenicus DSM 3246	●		●							●
Pelobacter acetylenicus DSM 3247	●		●							●
Pelobacter sp. SFB93	●	●	●							●
Syntrophobacteraceae										
Desulfoglaeba alkanexedens ALDC		●	●	●	●	●				●

Data from Integrated Microbial Genomes database

Abbreviations: *Pta/Ack* Phosphotransacetylase/acetate kinase, *W-L* Wood-Ljungdahl pathway, *ACS* Acetyl-CoA synthetase (putative AMP-forming)

similarly not detected. Interestingly, "Deltaproteobacterium N47" draft genome contains the complete DNRA pathway, but growth has not been documented with nitrate as an electron acceptor (Bergmann et al. 2011). These findings suggest that inorganic nitrogen compounds generally do not serve as important energy conservation compounds for most hydrocarbon-degrading *Deltaproteobacteria*. These organisms often have a marine origin and/or ancestry, and such findings are consistent with the scarce quantity of nitrate relative to far more plentiful electron acceptors (e.g., sulfate) in such habitats. This is in contrast to the finding that nitrogen cycle pathways (denitrification and/or DNRA) are widely distributed across multiple genera within the *Deltaproteobacteria*, particularly in soil metagenomes, with DNRA being much more prevalent (Nelson et al. 2016).

Nitrogenase genes (*nifHDK*) were found in the genomes of most organisms (Table 3), in agreement with the wide distribution of Cluster III *nifH* genes across anaerobic taxonomic groups, including the *Deltaproteobacteria* (Gaby and Buckley 2011). At the time of writing, diazotrophy during anaerobic hydrocarbon metabolism has only been demonstrated in *Pelobacter* sp. SFB93 (Akob et al. 2017). Interestingly, despite *Pelobacter acetylenicus* DSM3246 possessing both *ahy* and all necessary *nif* genes, nitrogenase activity was absent in cells cultivated in the absence and presence of ammonium (Akob et al. 2017). Additionally, *nifD* transcripts were previously detected in petroleum-contaminated sediments exhibiting active hydrocarbon biodegradation (Holmes et al. 2004). The introduction of petroleum hydrocarbons to a habitat would dramatically raise the C:N ratio and impart a limitation for reduced nitrogen species on the ecosystem. The work by Akob and colleagues demonstrated that an obligate anaerobe is capable of nitrogen fixation during hydrocarbon degradation (Akob et al. 2017). This finding and the widespread distribution of the diazotrophy pathway in hydrocarbon-degrading *Deltaproteobacteria* suggest this may represent an important ecological activity within hydrocarbon-laden anoxic habitats.

4.3.4 Metal Reduction

As mentioned earlier, *Geobacter metallireducens* can utilize a variety of metals (Fe, Mn, U(VI)) as terminal electron acceptors including insoluble iron and manganese oxides and metal electrodes. Functional genetic studies of *G. metallireducens* and *G. sulfurreducens* have shown two mechanisms utilized by members of this genus for the extracellular transfer of electrons: production of electrically conductive pili and cell surface localized *c*-type cytochromes (Reguera et al. 2005; Sydow et al. 2014; Estevez-Canales et al. 2015). With the development of a genetic system for *G. metallireducens*, Tremblay et al. (2012) demonstrated the importance of pili and flagellin biosynthesis for insoluble iron oxide reduction. Deletion of *pilA* (encoding the structural monomer of the type IV pilus) eliminated iron oxide reduction activity in *G. metallireducens* but had no effect on soluble Fe(III) reduction (Tremblay et al. 2012). Similarly, *G. sulfurreducens* Δ*pilA* mutants showed no electron transfer to iron oxides or electrodes without any deficiency in soluble iron reduction (Reguera et al. 2005, 2006; Nevin et al. 2009). Recent studies of conductive pili from *Geobacter* spp. have shown the conductive property of these nanowires arises

from closely spaced aromatic amino acids that transfer electrons through pi-pi orbital interactions (Malvankar et al. 2012; Vargas et al. 2013; Malvankar et al. 2015). The PilA of *G. metallireducens* and *sulfurreducens* is truncated relative to non-electrically active pili-producing organisms such as *Pseudomonas aeruginosa*, and this particular feature may be responsible for the interactive orientation of aromatic side chain residues within the protein (Reguera et al. 2005; Lovley and Malvankar 2015; Holmes et al. 2016). The conductivity of nanowires has been shown to differ significantly between *Geobacter* spp. (Tan et al. 2017). Studies on the role of flagellar motility in the reduction of insoluble metal oxides by *G. metallireducens* (Δ*fliC* mutants unable to produce flagellin) revealed that non-motile mutant strains had significantly slower Fe(III) reduction rates compared to the wild-type strain in sediment incubations simulating subsurface conditions (Tremblay et al. 2012). *Geobacter metallireducens* does not secrete soluble molecules to shuttle electrons, in contrast to *Shewanella oneidensis* (Nevin and Lovley 2000; Childers et al. 2002). Motility, chemotaxis, and the production of pili and outer membrane *c*-type cytochromes are critical for the extracellular reduction of insoluble oxides by *Geobacter* spp. in the heterogeneous matrices of subsurface habitats (Tremblay et al. 2012; Sydow et al. 2014).

5 Future Research Needs

The biotransformations associated with anaerobic hydrocarbon metabolism are now recognized as biogeochemically consequential for the cycling of carbon in many ecosystems on the planet. However, this insight is based largely on selected molecular surveys and the study of relatively few isolates including some *Deltaproteobacteria*. Such investigations have revealed the fundamental outlines of several novel biochemical mechanisms used by anaerobic microorganisms to activate hydrocarbons as well as equally intriguing subsequent reaction pathway steps. The continued study of hydrocarbon-degrading *Bacteria* in general and *Deltaproteobacteria* in particular is expected to lend additional insight into the fate of these hydrocarbons in underexplored environments. For example, only a single isolate currently represents the hydrocarbon transformation mechanisms inherent in high-temperature environments. No anaerobic hydrocarbon-degrading organisms have been isolated from habitats that exhibit other environmental extremes such as pH or salinity. From this standpoint, it seems somewhat myopic to assume that the full metabolic diversity associated with anaerobic hydrocarbon biodegradation is fully appreciated. Conservatively, there are undoubtedly many other hydrocarbonoclastic *Deltaproteobacteria* (particularly in sediments) and perhaps other novel transformation mechanisms that have yet to be discovered.

This may be particularly true in cases where the fundamental insight on the susceptibility of particular types of hydrocarbons to anaerobic biodegradation is rare or nonexistent. Such is the case for >55,000 naturally occurring terpenoid molecules (Brahmkshatriya and Brahmkshatriya 2013) that include branched chain, cyclic, and multicyclic hydrocarbons that have attracted some attention

as potential biofuel components. The fundamental biosynthetic routes and the outlines of aerobic terpene biodegradation pathways are reasonably well understood (i.e., Marmulla and Harder 2014). The same is far from true when considering the environmental fate of these compounds under anaerobic conditions. A systematic examination of the growth, behavior, physiology, and phylogeny of the anaerobic microorganisms capable of metabolizing naturally occurring terpenoids may allow for the rational integration of these molecules into the existing societal patterns of hydrocarbon use. More specifically, the terpenoids, like other hydrocarbon classes, will exhibit a continuum in their susceptibility to anaerobic decay based on their particular structural and stereochemical features. A priori knowledge of the relative recalcitrance of hydrocarbons will aid in their selection for specific tasks. For instance, a biofuel might be reversed engineered to avoid molecules that are particularly labile and thus more prone to exacerbate the biocorrosion of the fueling infrastructure. On the other hand, selections can also focus on candidate molecules that are not overly persistent in the event they are accidentally spilled in the environment. The diversity of the requisite microbes involved in the transformation of terpenoids as well as other hydrocarbons will provide a biotechnological basis for monitoring and potentially manipulating such bioconversions. The underlying regulatory mechanisms controlling microbial metabolism might allow for desirable bioconversions to be stimulated and undesired ones to be limited.

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The Methane-Oxidizing Bacteria (Methanotrophs)

14

Marina G. Kalyuzhnaya, Oscar A. Gomez, and J. Colin Murrell

Contents

Abstract

Aerobic methane-oxidizing bacteria (methanotrophs) have the unique ability to grow on methane as their sole source of carbon and energy. They are ubiquitous in the environment and play a major role in the removal of the greenhouse gas methane from the biosphere before it is released into the atmosphere. The ability to drive oxygen-dependent methane oxidation was once assumed to be an exceptional property of a very restricted set of microbes belonging to two classes

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of *Proteobacteria*: *Alphaproteobacteria* and *Gammaproteobacteria*. While *Proteobacteria* still form the foundation of the methanotrophic landscape in many ecosystems, the ability to oxidize methane has also been demonstrated in the microbial phyla *Verrucomicrobia* and *Candidatus Methylomirabilis oxyfera* (phylum NC10). Over the years various methanotrophs have also been isolated, including facultative methanotrophs, extremophile species, and anaerobes, thus expanding both the taxonomic diversity and physiological range of methanotrophy. In addition, a number of cross-species interactions that enable efficient methane utilization have been identified, changing the way we view mechanisms of methane utilization. Finally, a thorough revision of core metabolic pathways has been made, and whole-genome metabolic models have been constructed, which facilitate the metabolic engineering of methanotrophic bacteria and expand the potential for their biotechnological applications.

1 Introduction

Methane-oxidizing bacteria or methanotrophs are a group of bacteria that grow on methane as their sole source of carbon and energy. They are a subset of methylotrophic bacteria, which can grow on a number of different one-carbon compounds (Lidstrom 2006). They appear to be ubiquitous in the environment and can be isolated from many different environments, including anoxic zones. They play a major role in the consumption of the atmospheric greenhouse gas methane as well as capturing biologically or geothermally formed methane before it is released into the atmosphere. All described methanotrophic bacteria contain the enzyme methane monooxygenase which oxidizes methane to methanol (reviewed in Lawton and Rosenzweig 2016), which is subsequently oxidized via formaldehyde and formate to carbon dioxide. All methanotrophs use methane to generate reducing power for biosynthesis. Methanotrophs contain specialized metabolic pathways for the assimilation of carbon, which can be incorporated into biomass at the level of formaldehyde (*Gammaproteobacteria*), formate (*Alphaproteobacteria*), or CO_2 (*Alphaproteobacteria*, *Verrucomicrobia*, and NC10). Different metabolic arrangements impact the overall carbon conversion efficiency (summarized in Kalyuzhnaya 2016; Table 1).

There has been considerable interest over the years in the use of methanotrophs or methanotrophic enzymes for the production of single-cell protein (SSP) and polyhydroxybutyrate (PHB), biofuels, and chemicals (reviewed in Strong et al. 2016). Until recently it was thought that methanotrophs could only grow on one-carbon compounds, but now a methanotroph that grows on multicarbon compounds has been characterized (see below). Methanotrophs were first described in the literature over 100 years ago (reviewed in Hanson and Hanson 1996), but it was the pioneering studies of Roger Whittenbury and colleagues that resulted in the isolation and characterization of over 100 methanotrophs from different environments (Whittenbury et al. 1970a). Readers are referred to these original papers for an extensive collection of excellent light micrographs and electron microscopy

photographs which detail the morphological features of a large number of different methanotrophs (Davies and Whittenbury 1970; Whittenbury et al. 1970a, b). The characterization of these methanotrophs provided a classification scheme and taxonomic framework that remained remarkably robust over the following 30 years or so. Bowman and colleagues provided a detailed taxonomic framework for obligate methane-oxidizing bacteria, based largely on the original taxonomy system of Whittenbury and colleagues (Bowman et al. 1993). Since then, many new species of methanotrophic bacteria have been isolated. The taxonomy of the aerobic methane-oxidizing bacteria has recently been summarized in a series of publications in Bergey's Manual of Systematics of Archaea and Bacteria (http://onlinelibrary.wiley.com/book/10.1002/9781118960608) and thus will be only briefly described below.

2 Taxonomy/Phylogeny

The phylogenetic relationship of methane-oxidizing bacteria is shown in Fig. 1. Methanotrophic bacteria can be divided into four main groups: *Gammaproteobacteria* (often referred as Type I or Type X, Fig. 2), *Alphaproteobacteria*

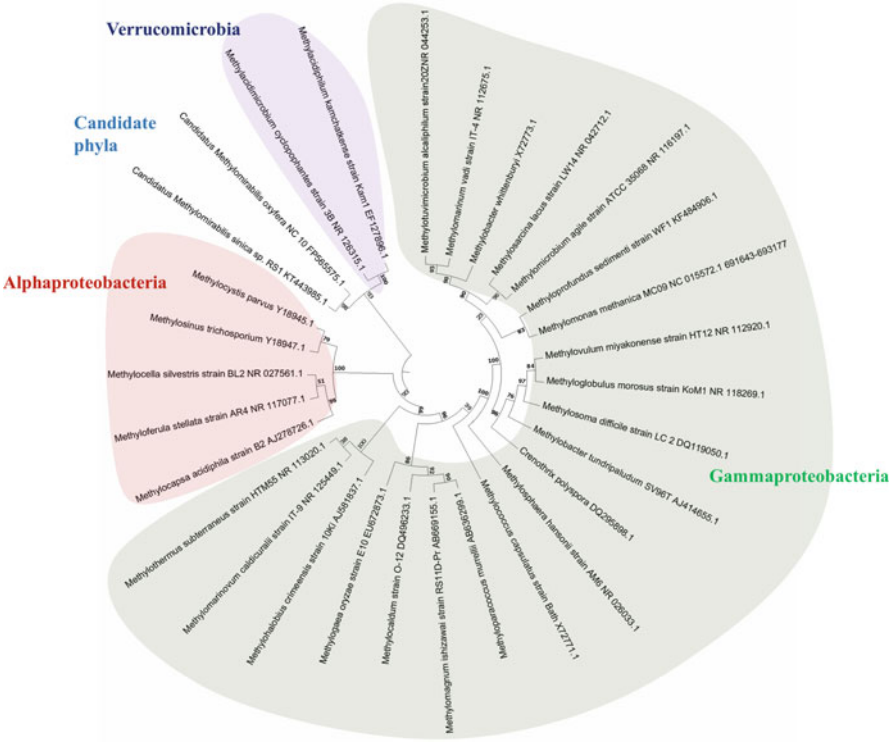

Fig. 1 16S ribosomal RNA gene phylogeny of methane-oxidizing bacteria

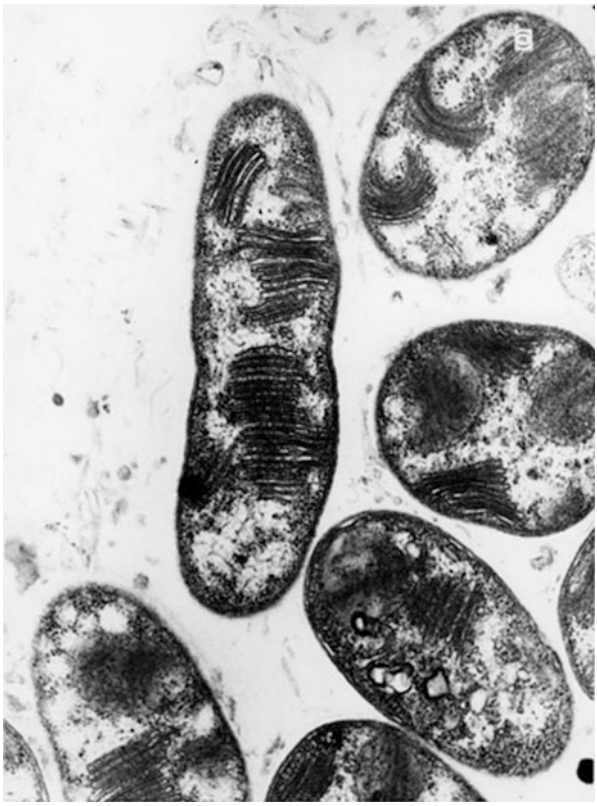

Fig. 2 Electron micrograph of a cross-section of a typical Type I methanotroph *Methylomonas methanica* showing characteristic bundles of intracytoplasmic membranes. (Photograph courtesy of H. Dalton) × 65,000 magnification

(previously known as Type II, Fig. 3), *Verrucomicrobia*, and members of NC10 phylum. Many groups of *Proteobacteria* and *Verrucomicrobia* phyla are represented by axenic cultures. However, many species of methanotrophic bacteria are resistant to cultivation in lab settings. The filamentous bacteria, *Crenothrix polyspora* and *Clonothrix fusca,* are examples of fastidious methanotrophs that cannot be obtained in pure culture (Vigliotta et al. 2007).

The genetic signature of these filamentous bacteria suggests that they are somewhat distant relatives of more conventional members of *Methylococcaceae.* Furthermore, the NC10 phylum is currently represented by a highly enriched consortia of methane-utilizing *Candidatus Methylomirabilis oxyfera* and satellite heterotrophs (Ettwig et al. 2009) and microcolonies of *Candidatus Methylomirabilis sinica* (He et al. 2016).

3 Morphology and Cell Biology

It is well documented that methane oxidation in most *Proteobacteria* and some *Verrucomicrobia* is associated with the formation of dedicated, subcellular compartments (intracytoplasmic membranes, ICMs, Figs. 2 and 3). Typically, gammaproteobacterial methanotrophs contain bundles of intracytoplasmic membranes (Fig. 2),

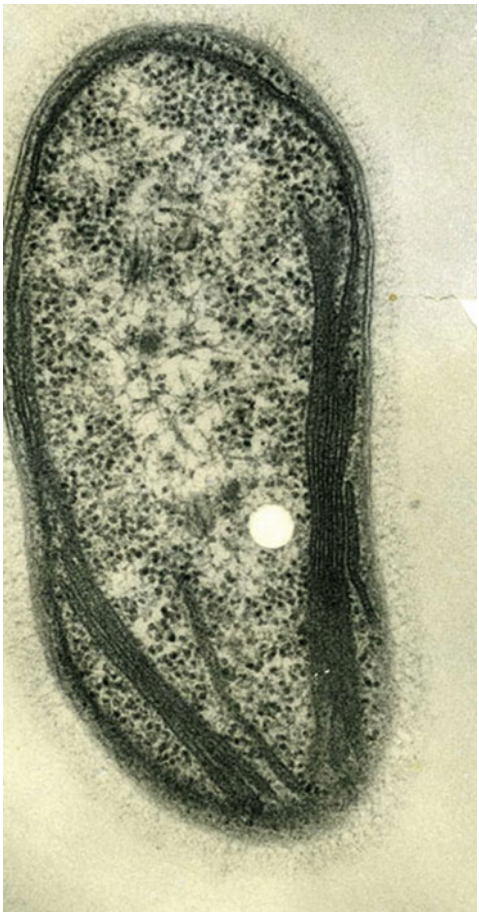

Fig. 3 Electron micrograph of a cross-section of a typical Type II methanotroph *Methylosinus trichosporium* showing characteristic intracytoplasmic membranes arranged around the periphery of the cell. (Photograph courtesy of H. Dalton) × 75,000 magnification

while alphaproteobacterial methanotrophs generally contain intracytoplasmic membranes arranged around the periphery of the cell (Fig. 3). ICMs can readily be visualized by electron microscopy in cells grown on methane in the presence of copper ions. Starvation, methanol growth, or copper-limiting conditions can lead to ICM degradation or reduced formation (Tavormina et al. 2016). The ICMs house the particulate methane monooxygenase (pMMO), the electron transport complexes, and ATP synthase. Some studies suggest that ICMs are formed via invagination of inner membrane, although the mechanisms of biogenesis and regulation of synthesis warrant further study.

S-layers are among other cell structural elements which have been described for some methanotrophic species (e.g., *Methylomicrobium, Methylotuvimicrobium* and *Methylococcus*) (Khmelenina et al. 2013b). While structural elements of S-layers are well documented, the genes and the enzymes involved in their synthesis and their functional roles remain to be discovered. Many gammaproteobacterial methanotrophs, particularly some *Methylobacter* species, form resting stages

as *Azotobacter*-like cysts which develop in stationary-phase cultures. These cyst-like resting stages appear to confer advantages to methanotrophs, including increased survival upon desiccation. Although this is not a unifying feature of all genera of *Gammaproteobacteria* methanotrophs (perhaps the ability to form resting stages has been lost in some strains because of long-term cultivation in the laboratory), it is quite distinctive in some stationary-phase cultures of methanotrophs (see Bowman 2006). Alphaproteobacterial methanotrophs, such as *Methylosinus* and *Methylocystis*, form exospores or lipoidal cysts. The exospores, which bud off from vegetative cells during stationary phase, are resistant to heat (85 °C for 10 min) and can survive periods of drying for 18 months (Bowman 2006). Resting forms, S-layers, and carbohydrate-rich capsules have been discussed as possible targets for biotechnological applications, as possible sources of alginate, nanomaterial, or feedstock (Strong et al. 2016).

4 Physiology and Biochemistry

The main characteristics of aerobic methanotrophs are summarized in Table 1. All methanotrophic bacteria use oxygen for methane activation (Trotsenko and Murrell 2008; Sirajuddin and Rosenzweig 2015) and oxidize methane to methanol via the enzyme methane monooxygenase (MMO) (Anthony 1982; Dalton 2005; Smith and Murrell 2010). Methanotrophs can possess two structurally and biochemically distinct forms of MMO, particulate (pMMO) and soluble methane monooxygenase (sMMO). pMMO is a copper-containing enzyme that is associated with the ICMs. sMMO is a cytoplasmic non-heme iron enzyme complex. Some of the best characterized methanotrophs, *Methylococcus capsulatus* (Bath) and *Methylosinus trichosporium* OB3b, can produce either form of MMO (reviewed in Murrell et al. 2000; Hakemian and Rosenzweig 2007; Ross and Rosenzweig 2017). The single factor that governs expression of the two types of MMO in these organisms is the concentration of available copper. At a high copper-to-biomass ratio, pMMO is produced, whereas sMMO is expressed only when the copper-to-biomass ratio during growth is low. Many methanotrophs such as the *Methylomonas methanica* S-1, *Methylomicrobium alcaliphilum* 20Z (now *Methylotuvimicrobium alcaliphilum* (Orata et al. 2019)), and *Methylomicrobium album* BG8 possess only pMMO. pMMO enzymes, encoded by *pmo* genes (*pmoCAB*), are related to ammonia monooxygenases (AMO, encoded by *amoCAB*); however, the phylogeny of pMMO and AMO enzymes, distributed among diverse bacterial species, is complicated (Tavormina et al. 2011; Coleman et al. 2012; Osborne and Haritos 2018). In addition to *pmoCBA* or *amoCBA* genes with ascribed functions, related sequences are sometimes found in methanotroph genomes in addition to *pmoCAB* gene clusters; however, their function remains undetermined (Tavormina et al. 2011). Furthermore, *pmo*-like gene clusters (*hmoCAB*) in *Actinobacteria* have been linked to oxidation of C_n-alkanes, such as ethane, propane, or butane (Coleman et al. 2012; Wang et al. 2017). Overall, the genetic diversity and distribution of *pmo*-like genes within bacteria is significant (Osborne and Haritos 2018), and the precise roles of

Table 1 General summary of existent methanotrophic cultures

Name	Species	Growth parameters					Culture collection ID [Genome Accession Number (NCBI)]	References
		Growth rate (h^{-1})	Metabolic pathways	pH (opt.)	T °C (opt.)	Others		
Gammaproteobacteria								
Methylococcaceae								
Clonothrix	*C. fusca*	0.01 (when grown with formaldehyde)	pMMO, Mxa	5	(10)	Forms attached or free filaments, surrounded by a yellowish brown distinctive sheath that may be encrusted	**16S rRNA partial sequence** [DQ984190.1]	(Vigliotta et al. 2007)
Crenothrix	*C. polyspora*	NA	BS, dPPP, FDH, H_4F, H_4MTP, pMMO, RuMP, sMMO, Xox	NA	(5)	Can oxidize methane under oxygen-deficient conditions; can reduce nitrate to nitrous oxide	**Not available as a pure culture** [NZ_FUKI00000000.1]	(Cohn 1870; Stoecker et al. 2006; Oswald et al. 2017)
Methylobacter	*M. luteus*	0.12	BS, dPPP, EDD, EMP, FDH, H_4F, H_4MTP, Mxa, pMMO, pSC, RuMP, TCA, Xox	5–9 (6.8)	(37)	Produces yellow water-soluble pigment and bacteriocin-like compounds	**VKM-53B ^{T}*** [NZ_ATYJ00000000.1]	(Starostina et al. 1998; Hamilton et al. 2015)
	M. marinus	0.15	BS, dPPP, EDD, EMP, FDH, H_4F, H_4MTP, Mxa, pMMO, pSC, pXmo RuMP, TCA, Xox	5.5–9 (7)	15–40 (35)	Requires NaCl concs. of 0.1–5%; can form desiccation-resistant cysts	**A45^{T}*** [NZ_ARVS00000000.1]	(Bowman et al. 1993; Flynn et al. 2016)

(*continued*)

Table 1 (continued)

Name	Species	Growth parameters					Culture collection ID [Genome Accession Number (NCBI)]	References
		Growth rate (h^{-1})	Metabolic pathways	pH (opt.)	T °C (opt.)	Others		
	M. psychrophilus	NA	pMMO	6–7.6 (6.8–7.2)	4–20 (10)	Psychrophilic strain	**Z-0021^T* 16S rRNA sequence** [NR_025016.1]	(Omel'chenko et al. 1996)
	M. tundripaludum	0.02	BS, dPPP, EDD, EMP, FDH, H_4F, H_4MTP, Mxa, pMMO, pSC RuMP	5.5–8 (6.8)	5–30 (23)	Poor to no growth on methanol	**SV96^T*** [NZ_AEGW00000000.2]	(Wartiainen et al. 2006b)
	M. whittenburyi	0.1	BS, dPPP, EDD, EMP, FDH, H_4F, H_4MTP, Mxa, pMMO, pSC, RuMP, TCA, Xox	5–8 (6.8)	5–40 (25)	Does not grow on methanol	**UCM-B-3033^T*** [NZ_JQNS00000000.1]	(Whittenbury et al. 1970a; Hamilton et al. 2015)
	Methylobacter sp. BBA5.1	0.06	BS, dPPP, EDD, EMP, FDH, H_4F, H_4MTP, Mxa, pMMO, pSC, pXmo, RuMP, TCA, Xox	NA (7.2)	5–35 (30)	Grows at NaCl concs. of 0.1–5%	**BBA5.1** [NZ_JQKS01000001.1]	(Smith et al. 1997; Flynn et al. 2016)
Methylocaldum	*M. szegediense*	0.03	BS, CBB, dPPP, EDD, EMP, FDH, H_4F, H_4MTP, Mxa, pMMO, pSC, RuMP	NA (6.8)	30–61 (55)	Moderately thermophilic; produces melanin-like pigments and can excrete 4-hydroxyphenyl acetic acid; can produce PHB	**O-12** [NZ_ATXX00000000.1]	Eshinimaev et al. 2004; Medvedkova et al. 2007)

	M. marinum	0.02	BS, CBB, dPP, FDH, H_4F, H_4MTP pMMO, RuMP, SC-EMP, sMMO	6–8 (7)	20–47 (36)	Requires NaCl for growth, optimal growth at 2% NaCl (w/v); growth with methanol at 0.01–2% (v/v)	**S8^T*** [AP017928.1]	(Takeuchi et al. 2014)
Methylococcus	*M. capsulatus* Bath	0.3–0.4	BS, CBB, dPPP, EDD, EMP, FDH, H_4F, H_4MTP, Mxa, pMMO, pSC, RuMP, sMMO, TCA	6–8 (7)	30–55 (42)	Well-established strain for single-cell protein production	**Bath^T*** [NC_002977.6]	(Whittenbury et al. 1970a; Ward et al. 2004)
	M. capsulatus Texas	0.1	BS, CBB, dPPP, EDD, EMP, FDH, H_4F, H_4MTP, Mxa, pMMO, pSC, RuMP, sMMO, TCA	6–8 (7)	30–50 (37)	Thermotolerant; grows well on methanol	**Texas** [NZ_AMCE00000000.1]	(Foster and Davis 1966; Kleiveland et al. 2012)
Methylogaea	*M. oryzae*	0.14	BS, CBB, dPPP, EDD, EMP, FDH, H_4MTP, Mxa, pMMO, pSC, RuMP	5–8 (6.6)	20–37 (30)	Sensitive to NaCl concs. above 0.5% (w/v); grows on methanol at concs. of 0.1–1% (w/v); can produce PHB	**E10^T*** [BBDL00000000.1]	(Geymonat et al. 2011)

(*continued*)

Table 1 (continued)

Name	Species	Growth parameters					Culture collection ID [Genome Accession Number (NCBI)]	References
		Growth rate (h^{-1})	Metabolic pathways	pH (opt.)	T °C (opt.)	Others		
Methyloglobulus	*M. morosus* KoM1	N/A	BS, dPPP, EDD, EMP, FDH, H_4MTP, pMMO, pSC, RuMP, TCA	5–8.5 (6–8)	4–30 (20)	No growth at NaCl concs. above 0.5% (w/v); can fix nitrogen; grows best at reduced oxygen concs. (0.05–0.1 bar)	**KoM1^T*** [NZ_AYLO00000000.1]	(Poehlein et al. 2013; Deutzmann et al. 2014)
Methylomagnum	*M. ishizawai*		BS, CBB, FDH, H_4F, H_4MTP, Mxa, pMMO, PPP, RuMP, sMMO, TCA, Xox	5.5–9 (6.8–7.4)	20–37 (32)	Good growth with 0–0.2% (w/v) NaCl and 0–0.05% methanol (v/v)	**RS11D-Pr^T*** [FXAM00000000.1]	(Khalifa et al. 2015; Frindte et al. 2017)
Methylomarinum	*M. vadi* IT-4	0.33	BS, dPPP, EDD, EMP, FDH, H_4F, H_4MTP, Mxa, pMMO, pSC, PPP, RuMP, TCA, Xox	4.5–7.0 (6.4)	20–44 (37)	Requires NaCl for growth, optimal salinity is of 2–3% NaCl (w/v); produces PHB	**IT-4^T*** [NZ_JPON00000000.1]	(Hirayama et al. 2013; Flynn et al. 2016)
Methylomicrobium	*M. agile* A30	0.06	BS, dPPP, EDD, EMP, FDH, H_4F, H_4MTP, Mxa, pMMO, pSC, RuMP	6–9 (7)	10–37 (27)		**A30^T*** [NZ_JPOJ00000000.1]	(Whittenbury et al. 1970a; Foster and Davis 1966)
	M. album BG8	0.11	BS, dPPP, EDD, EMP, FDH, H_4F, H_4MTP, Mxa, pMMO, pSC, RuMP, TCA, Xox	6–9 (7)	10–37 (27)		**BG8^T*** [NZ_AFJF00000000.2]	(Whittenbury et al. 1970a; Kits et al. 2013)

	M. lacus LW14	0.05	BS, dPPP, FDH, H_4F, H_4MTP, pMMO, pSC, RuMP	NA (6.8)	NA (6.8)		**LW14^T*** [NZ_AZUN00000000.1]	(Kalyuzhnaya et al. 2015a)
Methylotuvimicrobium	*M. alcaliphilum* 20Z	0.12	BS, EDD, EMP, FDH, H_4F, H_4MTP, Mxa, pMMO, PPP pSC, RuMP, TCA	6.5–10 (9)	8–37 (30)	Optimal growth at NaCl concs. of 3% (w/v); accumulates ectoine and sucrose to promote osmotolerance resistant to desiccation	**20Z^T*** [NC_016112.1]	(Khmelenina et al. 1997; Kalyuzhnaya et al. 2008; Orata et al., 2019; Vuilleumier et al. 2012)
	M. buryatense 5G	0.23	BS, EDD, EMP, FDH, H_4F, H_4MTP, Mxa, pMMO, pSC, PPP, RuMP, sMMO, TCA	6–11 (9)	4–45 (28–30)	Optimal growth at 0.5–0.75% NaCl (w/v); resistant to desiccation	**5B^T*** [NZ_AOTL00000000.1]	(Kalyuzhnaya et al. 1999; Khmelenina et al. 2013)
Methylomonas	*M. methanica* MC09	NA	BS, dPPP, EDD, EMP, FDH, H_4F, H_4MTP, Mxa, pMMO, pSC, RuMP, sMMO, TCA	6.6–8	(30)	Strain was isolated from seawater, grows at 5% NaCl (w/v)	**MC09** [NC_015572.1]	(Dworkin and Foster 1956; Boden et al. 2011)
	Methylomonas LW13	0.16	BS, dPPP, EDD, EMP, FDH, H_4F, H_4MTP, Mxa, pMMO, RuMP, SC, sMMO	NA (6.8)	NA (30)	Does not require NaCl for growth	**LW13** [JNLB00000000.1]	(Auman et al. 2000; Kalyuzhnaya et al. 2015a)

(*continued*)

Table 1 (continued)

Name	Species	Growth parameters					Culture collection ID [Genome Accession Number (NCBI)]	References
		Growth rate (h^{-1})	Metabolic pathways	pH (opt.)	T °C (opt.)	Others		
	M. paludis	NA	FDH, Mxa, pMMO, RuMP	3.8–7.3 (5.8–6.4)	8–30 (22)	Will not grow at NaCl concs. above 0.1% (w/v); optimal methanol concs. of 0.25% (v/v)	**MG30^{T}* 16S partial rRNA sequence** [NR_108887.1]	(Danilova et al. 2013)
	M. lenta	NA	BS, dPPP FDH, pMMO	6.3–7.8 (7.0)	15–28 (20)	Slow-growing strain; sensitive to methanol concs. ≥0.5% (v/v)	**R-45377^{T}*** [NZ_LUUI00000000.1]	(Hoefman et al. 2014a)
Methyloparacoccus	*M. murrellii*	NA	Mxa, pMMO	5.8–9 (6.5)	20–37 (25–33)	Sensitive to addition of 1% NaCl (w/v)	**LMG 27482^{T}* 16S partial rRNA sequence** [AB636299.1]	(Hoefman et al. 2014b)
Methyloprofundus	*M. sedimenti*	0.08	BS, dPPP, FDH, H_4F, H_4MTP, pMMO, pSC, RuMP	6–8 (7.0)	4–26 (23)	Requires NaCl for growth, optimal concs. being 2% (w/v)	**WF1^{T}*** [NZ_LPUF00000000.1]	(Tavormina et al. 2015)
Methylosarcina	*M. fibrata* AML-C10	0.08	BS, dPPP, FDH, H_4F, H_4MTP, pMMO, pSC, RuMP	5–9 (5)	10–37 (30)	Can grow at NaCl concs. of up to 1% (w/v); grows poorly with methanol	**AML-C10^{T}*** [NZ_ARCU00000000.1]	(Wise et al. 2001)

Methylosoma	*M. difficile* LC 2	0.01	pMMO	5–9 (6–8)	16–30 (25)	NaCl concs. above 0.5% (w/v) inhibit growth; grows well in dilute NMS medium (1/10 conc. of potassium nitrate) and prefers low oxygen tensions; can fix nitrogen	**LC 2^{T}* 16S partial rRNA sequence** [DQ119050]	(Rahalkar et al. 2007)
Methylosphaera	*M. hansonii*		RuMP		0–21 (10)	Requires artificial seawater salts for growth (at concs.70–100%); can grow on methanol and can fix nitrogen	**AM6^{T}* 16S partial rRNA sequence** [NR_026033.1]	(Bowman et al. 1997)
Methylovulum	*M. miyakonense* HT12	0.01	EDD, EMP, dPPP, FDH, H_4F, H_4MTP, Mxa, pMMO, pSC, sMMO, RuMP, TCA, Xox	6–7.5	5–34 (24)	High sensitivity to NaCl, growth inhibited at concs. above 0.2% (w/v)	**HT12^{T}*** [NZ_AQZU00000000.1]	(Iguchi et al. 2011; Hamilton et al. 2015)
Methylothermaceae								
Methylohalobius	*M. crimeensis*	0.03	BS, CBB, EMP, FDH, H_4MTP, H_4F, Mxa, pMMO, pSC, PPP, RuMP, TCA	6.5–7.5	15–42 (30)	Grows optimally at NaCl concs. of 5.8–8.7% (w/v); evidence of PHB production	**10Ki^{T}*** [NZ_ATXB00000000.1]	(Heyer et al. 2005; Sharp et al. 2015)

(*continued*)

Table 1 (continued)

Name	Species	Growth parameters					Culture collection ID [Genome Accession Number (NCBI)]	References
		Growth rate (h^{-1})	Metabolic pathways	pH (opt.)	T °C (opt.)	Others		
Methylomarinovum	*M. caldicuralii*	0.29–0.33	pMMO, RuMP	5–7 (6.4)	30–55 (50)	Optimal growth at NaCl concs. of 3% (w/v)	**IT-9^{T}* 16S rRNA partial sequence** [NR_125449.1]	(Hirayama et al. 2014)
Methylothermus	*M. subterraneus*	0.3	pMMO, RuMP	5.2–7.5 (6.3)	37–65 (60)	Optimal growth at NaCl concs. of 0–0.3% (w/v)	**HTM55 ^{T}* 16S rRNA partial sequence** [NR_113020.1]	(Hirayama et al. 2011)
Alphaproteobacteria								
Methylocystaceae								
Methylocystis	*M. rosea* SV97	NA	FDH, H_4F, H_4MTP, pMMO, SC-EMP	5.5–9.0	5–37 (27)	Does not require NaCl for growth; produces PHB	**SV97^{T}*** [NZ_ARCT00000000.1]	(Wartiainen et al. 2006b)
	Methylocystis ATCC 49242 strain Rockwell	0.16	FDH, H_4F, H_4MTP, Mxa, pMMO, SC-EMP	NA (6.8)	NA (30)	Highly tolerant of ammonium, capable of nitrogen fixation	**ATCC 49242** [AEVM00000000.2]	(Nyerges et al. 2010; Stein et al. 2011)
	M. parvus OBBP	0.12	FDH, H_4F, H_4MTP, Mxa pMMO, SC-EMP	6–8 (6.8)	5–37 (30)	Can produce PHB	**OBBP^{T}*** [NZ_AJTV00000000.1]	(Whittenbury et al. 1970a; del Cerro et al. 2012)
	Methylocystis sp. SC2	NA	FDH, H_4F, H_4MTP, Mxa, pMMO, SC-EMP	NA	NA	Low-affinity pMMO	**SC2** [HE956757.1]	(Dam et al. 2012)

	Methylocystis sp. SB2		FDH, H_4F, H_4MTP, Mxa, pMMO, PPP, SC-EMP, TCA	6–9 (6.8)	10–30 (30)	Facultative methanotroph with EMP pathway	**SB2** [NZ_AYNA00000000]	(Vorobev et al. 2014)
Methylosinus	*M. trichosporium OB3b*	0.12–0.14	FDH, H_4F, H_4MTP, Mxa, pMMO, SC-EMP, sMMO	6–8 (6.8)	5–37 (30)	Well-established catalysis for PHB production, epoxidation; can degrade chlorinated aliphatic hydrocarbons; can produce methanobactins	**OB3b ^{T}*** [NZ_ADVE00000000.2]	(Whittenbury et al. 1970b; Oldenhuis et al. 1989; Stein et al. 2010)
	Methylosinus sp. LW4	0.12–0.15	FDH, H_4F, H_4MTP, pMMO, SC-EMP, sMMO	6–8 (6.8)	5–37 (30)	Strain shows robust growth; can produce methanobactins	**LW4** [ARAB00000000.1]	(Auman et al. 2001; Kenney et al. 2016)
Beijerinckiaceae								
Methylocella	*M. silvestris*	0.014	dPPP, FDH, H_4F, H_4MTP, Mxa, SC-GS, sMMO, TCA	5–7.0 (5.5)	4–30 (25)	Facultative methanotroph; growth is inhibited by NaCl concs. above 0.8% (w/v)	**BL2^{T}*** [NC_011666.1]	(Dunfield et al. 2003; Chen et al. 2010)
	M. tundrae	NA	FDH, Mxa, PPP, SC-GS, sMMO, TCA	4.2–7.5 (5.5)	5–30 (15)	Growth is inhibited by NaCl concs. above 0.8% (w/v); best growth at methanol concs. of 0.5–1% (v/v)	**T4^{T}*** **16S rRNA partial sequence** [NR_025596.1]	(Dedysh et al. 2004)

(*continued*)

Table 1 (continued)

Name	Species	Growth parameters					Culture collection ID [Genome Accession Number (NCBI)]	References
		Growth rate (h^{-1})	Metabolic pathways	pH (opt.)	T °C (opt.)	Others		
Methylocapsa	*M. acidiphila B2*	0.03	BS, FDH, H_4F, H_4MTP, Mxa, pMMO, PPP, SC-GS, TCA	4.2–7.2 (5.5)	10–30 (24)	NaCl inhibits growth at a concs. of 0.5% (w/v); methanol supports growth at concs. below 0.05% (v/v); capable of PHB production	**B2**^{T*} [NZ_ATYA00000000.1]	(Dedysh et al. 2002)
	M. aurea KYG	0.018	BS, FDH, H_4F, H_4MTP, Mxa, pMMO, SC-GS, TCA	5.2–7.2 (6.2)	2–33 (30)	Growth is inhibited with 0.3% (w/v) NaCl; methanol supported growth at concs. below 0.1% (v/v); capable of PHB production	**KYG**^{T*} [JQKO00000000.1]	(Dunfield et al. 2010)
Methyloferula	*M. stellata* AR4	0.005 (CH_4)/ 0.015 (CH_3OH)	BS, CBB, FDH, H_4MTP, Mxa, SC-GS, sMMO, TCA, Xox	3.5–7.2 (5.2)	4–33 (23)	Reduced growth at NaCl concs. above 0.7% (w/v). Optimum methanol concs. between 0.1 and 1% (v/v)	**AR4**^{T*} [NZ_ARWA00000000.1]	(Vorobev et al. 2011; Dedysh et al. 2015)

Candidate phylum NC10								
Candidatus Methylomirabilis								
Methylomirabilis	*M. oxyfera*		CBB, FDH, H_4MTP, Mxa, pMMO	7–8	25–30	Intracellular oxygen production; requires nitrite for growth	**No pure strains** [FP565575.1]	(Ettwig et al. 2010; Rasigraf et al. 2014)
Verrucomicrobia								
Methylacidiphilaceae								
Methylacidiphilum	*M. fumariolicum* SolV	0.07–0.08	BS, CBB, EMP, FDH, pMMO, PPP, TCA, Xox	0.8–5.8 (2)	40–65 (55)	Grows at very low pH; capable of nitrogen fixation	**SolV** [PRJEA85607]	(Pol et al. 2007; Khadem et al. 2011; Anvar et al. 2014)
	M. infernorum V4	0.04	BS, CBB, FDH, pMMO, Xox	1–6 (2–2.5)	60	Capable of nitrogen fixation	**V4** [NC_010794.1]	(Dunfield et al. 2007; Hou et al. 2008)
	M. kamchatkense Kam1	0.01	CBB, EMP, FDH, pMMO, PPP, TCA Xox	2–5 (3.5)	40–60 (55)	Grows in low concs. of methanol (up to 0.0001% w/v); can fixate nitrogen	**Kam1** [NZ_JQNX00000000.1]	(Islam et al. 2008; Erikstad and Birkeland 2015)
Unclassified								
Methylacidimicrobium	*M. cyclopophantes*	0.04	CBB, pMMO, Xox	0.6–6 (1.5–3)	20–49 (44)	ICM system that consists of membrane stacks orthogonal to the cell wall	**3B 16S rRNA partial sequence** [NR_126315.1]	(Van Teeseling et al. 2014)
	M. tartarophylax	0.04	CBB, pMMO, Xox	0.5–5 (1–3)	20–43 (38)	Growth is inhibited by oxygen; no ICM system was observed	**4AC^T* 16S rRNA partial sequence** [NR_126314.1]	

(*continued*)

Table 1 (continued)

Name	Species	Growth parameters					Culture collection ID [Genome Accession Number (NCBI)]	References
		Growth rate (h^{-1})	Metabolic pathways	pH (opt.)	T °C (opt.)	Others		
	M. fagopyrum	0.01	CBB, pMMO, Xox	0.6–6 (1.5–3)	20–39 (35)	No ICM system was observed	**3C^{T*} 16S rRNA partial sequence** [NR_126313.1]	

^{T*}, type strain; BS, *Bifidobacterium* shunt; concs., concentrations; dPPP, dissimilatory pentose phosphate cycle; EDD, Entner-Doudoroff pathway; EMP, Embden-Meyerhof-Parnas pathway; FDH, formate dehydrogenases; H_4MTP, methanopterin-linked C1 transfer; H_4FP, folate-linked C1 transfer; ICM, intracytoplasmic membranes; Mxa, PQQ-linked methanol dehydrogenases; NA, data not available; PHB, polyhydroxybutyrate; NMS, nitrate mineral salts medium; pMMO, particulate methane monooxygenase; pSC, partial serine cycle; pXmo, methane/ammonia monooxygenase-related proteins of unknown function; RuMP, assimilatory ribulose monophosphate pathway; sMMO, soluble methane monooxygenase; TCA, tricarboxylic acid cycle; Xox, PQQ-linked methanol and formaldehyde dehydrogenases

some distant homologs await experimental validation. The sMMO genes seem to be less widespread among the methanotrophs than pMMO genes. However, some methanotrophs of the family *Beijerinckiaceae* only encode sMMO (Dedysh and Dunfield 2014). Moreover, some of these organisms encode additional soluble alkane monooxygenases, with different substrate specificities (propane-specific rather than methane-specific), and these are related to both sMMO and other soluble di-iron center monooxygenases from versatile *Proteobacteria* and *Actinobacteria* (Crombie and Murrell 2014).

The product of methane oxidation is methanol, which is further oxidized to formaldehyde (Anthony 1982). One enzyme that carries out this function, a periplasmic pyrroloquinoline quinone (PQQ)-dependent, calcium-containing methanol dehydrogenase (MDH), was identified over a half century ago (Anthony and Zatman 1964, 1965, 1967a, b) and has now been studied in detail (Anthony 2004). The enzyme consists of two subunits, large and small, MxaF and MxaI, and is functionally linked to a dedicated cytochrome MxaG (Williams et al. 2006). Most known methanotrophs and many non-methanotrophic methylotrophs contain this enzyme and the necessary accessory functions (Chistoserdova and Lidstrom 2013). Rather surprisingly, a novel MDH has been recognized recently, whose single subunit, XoxF, is homologous to MxaF (Chistoserdova 2016). It requires lanthanides, which are rare earth elements, in its active center instead of calcium (Pol et al. 2014; Keltjens et al. 2014). Genetic investigations in model organisms indicated that rare earth elements are not only important for the activity of XoxF enzymes, but that they are also involved in the regulation of the alternative MDH enzymes, by decreasing expression of the *mxa* gene cluster and by increasing expression of *xox* genes (Vu et al. 2016; Chu and Lidstrom 2016; Gu et al. 2016). The formaldehyde oxidation step in the methane oxidation pathway can be catalyzed by several enzymes, including the tetrahydromethanopterin- (H_4MPT-) or tetrahydrofolate-linked and formaldehyde dehydrogenases (possibly also XoxF enzyme). The organisms operating the ribulose monophosphate (RuMP) pathway for formaldehyde assimilation can also oxidize formaldehyde via the oxidative branch of this cycle (Anthony 1982).

The pathways for assimilation of methane carbon have recently undergone a thorough revision through matching recent omics data to established knowledge, in combination with enzyme and metabolite characterization. The gammaproteobacterial methanotrophs typically use the RuMP pathway as their primary route of incorporation of carbon into cellular material. Thermodynamically, the RuMP pathway is the most efficient pathway for methane carbon assimilation. Different variants of the pathway have been described, and it has been shown that the pathway can also contribute to fermentative metabolism (Kalyuzhnaya et al. 2013). A pathway for glyoxylate regeneration by the serine cycle methylotrophs which do not possess a glyoxylate shunt (among them the alphaproteobacterial methanotrophs) remained a mystery for half a century (Anthony 2011) and has been solved only recently as the ethylmalonyl-CoA cycle (EMC; Erb et al. 2007). The discovery of this new pathway also prompted a reevaluation of the amount of carbon assimilated in the form of CO_2 through the multiple carboxylation reactions that are key to this metabolism (Erb et

al. 2007; Chistoserdova et al. 2009; Yang et al. 2013). Interestingly, genomic evidence indicated that the gammaproteobacterial methanotrophs also encode the serine cycle, but not the EMC or glyoxylate shunt (Chistoserdova et al. 2005; Kalyuzhnaya et al. 2015b); the role of this variant serine cycle in these methanotrophs and its significance for methylotrophy metabolism remain to be determined. The carbon assimilation through the Calvin Benson Bassham (CBB) cycle has been now demonstrated in some methanotrophs, such as Verrucomicrobia and Candidate Phylum NC10 (Khadem et al. 2011; Rasigraf et al. 2014).

Thus far, the majority of methanotrophic cultures isolated in pure culture have been described as obligate, i.e., they can use only methane and methanol as sources of carbon and energy. However, the number of facultative methanotrophs (i.e., those able to use multicarbon compounds) has increased over time. Many newly described species fall into groups canonically associated with obligate methanotrophy (Semrau et al. 2008). The first fully authenticated facultative methanotroph, *Methylocella silvestris*, which can grow on either methane or multicarbon compounds, is found among *Alphaproteobacteria* (Dedysh et al. 2005; Im et al. 2010; Theisen et al. 2005). This methanotroph has been shown to be able to consume short-chain alkanes as an alternative or supplement to methane (Crombie and Murrell 2014).

The ability to fix N_2 by Type II methanotrophs and *Mc. capsulatus* (Bath) has been known for some time (Romanovskaia et al. 1980). Genomic evidence resulting from screening for the gene *nif*H, which encodes the Fe protein of nitrogenase, indicates that a number of gammaproteobacterial methanotrophs such as *Methylomonas*, *Methylocaldum*, *Methyloprofundus*, and *Methylobacter* may also fix N_2 (Auman et al. 2001). Type I methanotrophs assimilate NH_4^+ mainly by reductive amination of pyruvate and/or α-ketoglutarate, whereas Type II methanotrophs use the glutamate cycle, i.e., glutamine synthetase (GS) and the glutamine-oxoglutarate amidotransferase (GOGAT) system (see Trotsenko and Murrell 2008).

5 Ecology/Habitats

Methanotrophic bacteria appear to be ubiquitous in the environment and can be isolated from many environments where methane is present. The physiological range of methanotrophy encompasses aerobic and anaerobic zones, acid and alkaline ecosystems, freshwater and moderately saline (up to 24%) environments, and highly oligotrophic and highly contaminated niches. Methane-utilizing bacteria typically flourish at the aerobic-anaerobic interface, but many groups have been detected in a variety of anoxic ecosystems. Various mechanisms have been proposed for growth of methanotrophic bacteria in anoxic environments; among the mechanisms most broadly discussed are intracellular O_2-production (Ettwig et al. 2010), close interactions with cyanobacteria (Milucka et al. 2015), and denitrification (Cao et al. 2019).

Freshwater and sediments harbor a variety of different methanotrophs. Gammaproteobacterial methanotrophs, including *Methylobacter*, *Methylomonas*, *Methylosarcina*, *Methylococcus*, and *Methylosoma*, are particularly predominant in these environments. For example, using three independent techniques, Costello et al.

(2002) have estimated that Type I methanotrophs are ten–hundredfold more abundant than Type II methanotrophs in Lake Washington sediment. In Lake Constance, Type I methanotrophs also predominate (Rahalkar et al. 2007). The study of methanotrophs in marine water columns and sediments is somewhat limited. Species from the *Methylomonas*, *Methylomicrobium*, and *Methyloprofundus* genera have been isolated; however, molecular methods suggest that there remains a wide distribution of yet-to-be-cultivated marine methanotrophs, mainly from *Methylococcaceae*. Upland and forest soils represent the major sink of atmospheric methane, and soils from these environments usually have high affinity for methane. The most frequently detected *pmoA* sequences from upland soils [upland soil cluster α (USCα) and upland soil cluster γ (USCγ)] belong to *Methylocystis*. It is thought that USCα and USCγ belong to uncultivated methanotrophs which can utilize atmospheric concentrations of methane (discussed in Ricke et al. 2005; Knief 2015; Pratscher et al. 2018). Baani and Liesack (2008) have shown that multiple copies of genes encoding pMMO from *Methylocystis* have different affinities for methane and this may explain why *Methylocystis* is ubiquitous in forest and upland soils and a number of other environments. New evidence indicates the existence of *Gammaproteobacteria* methanotrophs with high affinity for methane (Edwards et al. 2017). In some ecosystems, high-affinity methane oxidation has been linked to the activity of conventional methanotrophic communities, consistent with both gamma- and alphaproteobacterial traits (Cai et al. 2016).

Rice cultivation is a major source of atmospheric methane, and its contribution to the global methane budget may continue to grow due to the increasing demand for rice. Studies have shown that rice fields contain highly diverse methanotrophs, including *Methylomonas*, *Methylobacter*, *Methylomicrobium*, *Methylococcus*, *Methylocaldum*, *Methylocystis*, and *Methylosinus*. The distribution and abundance of methanotrophs in rice fields are affected by several factors, including oxygen availability and the growth period of the rice (Eller and Frenzel 2001). No obvious geographical distribution patterns have been found for methanotrophs present in rice fields around the world, although *Methylocaldum* spp. tend to be detected in tropical regions.

Methanotrophs are also found in landfills, e.g., *Methylobacter*, *Methylosarcina*, *Methylomicrobium*, *Methylocystis*, and *Methylosinus*, with species of *Methylobacter* and *Methylocystis* being the most dominant (Cébron et al. 2007; Su et al. 2014).

Methanotrophs have been found in many extreme environments, ranging from 0 °C to 70 °C, salinity from 0% to 20% (NaCl), and pH 1.5–12. Pure cultures of thermophilic, psychrophilic, acidophilic, alkaliphilic, and halophilic methanotrophs have been obtained (reviewed in Trotsenko and Khmelenina 2002; Dunfield 2009). Thermophilic methanotrophs include *Methylococcus capsulatus* Bath which was isolated from a hot spring (Whittenbury et al. 1970a), *Methylocaldum* species (Bodrossy et al. 1997, 1999), and *Methylothermus thermalis,* which was isolated from a Japanese hot spring (Tsubota et al. 2005). Molecular methods suggest that the diversity of thermophilic methanotrophs is even greater, and many species remain to be uncultivated (Eloe-Fadrosh et al. 2016). Three isolated psychrophilic methanotroph strains include *Methylobacter psychrophilus* (Omel'chenko et al. 1996), *Methylosphaera hansonii* which was isolated from an Antarctic lake (Bowman et al. 1997), and *Methylomonas*

scandinavica which was isolated from ground water of Sweden (Kalyuzhnaya et al. 1999). These Type I methanotrophs grow between 5 °C and 15 °C and have a low DNA G + C content (43–54 mol%). Halophilic and alkaliphilic methanotrophs have also been isolated, which mainly belong to the genus *Methylomicrobium* (Trotsenko and Khmelenina 2002). However, culture-independent methods have demonstrated that soda lakes harbor diverse methanotrophs other than *Methylomicrobium*. The isolation and characterization of acidophilic/acid-tolerant methanotrophs started with soils taken from peatlands and acidic forests (Dedysh et al. 2002), which represent one of the major sources of atmospheric methane. Two genera were isolated and validated, *Methylocella* and *Methylocapsa* (Dedysh et al. 2002; Dunfield et al. 2003). Another study reported that *Methylocystis* species may also be acidophilic/acid-tolerant (Dedysh et al. 2007). Three thermophilic and extremely acidophilic *Verrucomicrobia* species growing below pH 2 have been isolated and characterized (Dunfield et al. 2007; Islam et al. 2008; Pol et al. 2007). Two other methanotrophic genera, *Methylacidiphilum* and *Methylacidimicrobium* (Table 1), contain unusual *pmoA* sequences and may have atypical metabolic pathways for carbon metabolism and assimilation (reviewed briefly in Semrau et al. 2008). Initially described as extremophile thermoacidophilic methanotrophs, some members of *Verrucomicrobia* are now found to be mesophilic (Dunfield et al. 2007; Islam et al. 2008; Pol et al. 2007; reviewed in Semrau et al. 2008).

Methanotrophs are also found as symbionts (reviewed in Dubilier et al. 2008). The first methanotroph-based symbiosis to be discovered was in deep-sea mussels at cold seeps in the Gulf of Mexico. Subsequently symbiotic methane-oxidizing bacteria have been observed in other deep-sea mussels, hydrothermal-vent snails, tubeworms, and marine sponges. Although mainly observed in marine environments enriched in methane, methanotroph symbionts have also been found in a species of *Sphagnum* moss in peat bogs in the Netherlands. All of the methanotroph symbionts found in the marine symbioses are phylogenetically related to gammaproteobacterial methanotrophs (cf Type I), whereas the majority of discovered plant symbionts are related to free-living alphaproteobacterial methanotrophs. Thus far, all symbiotic methane oxidizers have resisted all attempts at cultivation in the laboratory. The subject of methanotroph symbioses is a very interesting one for future study and has been described in detail in Petersen and Dubilier (2009).

Cultivation-independent methods (reviewed in McDonald et al. 2008; Eloe-Fadrosh et al. 2016; Chistoserdova and Kalyuzhnaya 2018) have revealed that there are many more methanotrophs present in the environment that await isolation and cultivation in the laboratory. Future challenges will be in isolating methanotrophs that grow on atmospheric concentrations of methane, novel uncultivated species, as well as facultative methanotrophs.

6 Cultivation

Methanotrophs can be readily isolated from most environments using mineral salts medium and high-purity methane (natural gas contains a small proportion of ethane which can poison methanotrophs due to its conversion to ethanol and ethanol by

MMO). The nitrate mineral salts (NMS) medium first described by Whittenbury et al. (1970a) contains magnesium sulfate, potassium nitrate, and calcium chloride, supplemented by a trace element solution. Iron in the form of NaFeEDTA and molybdate are often also added, especially for the enrichment of nitrogen-fixing methanotrophs (minus inorganic nitrogen sources). Ammonia salts can substitute for nitrate as an inorganic nitrogen source but are usually added in lower concentrations since ammonia is a competitive inhibitor of MMO. Copper ions can also be added to ensure good growth since pMMO requires copper. Liquid cultures can be cultivated in flasks or serum vials sealed with airtight seals. Methane can then be injected into the headspace to typically 20% (v/v). The addition of rare earth elements (RRE, lanthanides) might stimulate isolation and growth of methanotrophic bacteria lacking Ca-dependent MDH (Pol et al. 2007). Tungsten supplementation might help to grow microbes with tungsten-dependent formate dehydrogenase or reduce formate excretion by pure cultures (Akberdin et al. 2018b). The addition of balanced micronutrients (Cu, Fe, W, rare earth elements, or Ca) can further improve yields of methanotrophic cells in large-scale cultivation. A number of strategies and optimized protocols for methanotroph cultivation are now available (reviewed in Dedysh SN, Dunfield PF (2014); Vekeman et al. 2017).

Methanotrophs can be grown on agar plates contained within airtight boxes (e.g., Tupperware) or anaerobic jars with a headspace of roughly 20% methane and 80% air. Methanotrophs can often be grown on methanol (high purity) although this is often toxic to some methanotrophs even at low concentrations (0.1%). Some methanotrophs can be "trained" to grow on methanol by building up methanol tolerance. Methanotrophs such as *Methylococcus capsulatus* Bath and *Methylosinus trichosporium* strain OB3b (the two best characterized methanotrophs) can readily be grown on methane in batch and continuous fermenter culture, achieving high cell densities (e.g., 1–5 g dry weight per liter) with specific growth rates approaching 0.2 h^{-1}. There are now reports on other fast-growing traits, such as *Methylomonas* and *Methylomicrobium (currently Methylotuvimicrobium)*, which display growth rates around 0.4 h^{-1} (summarized in Kalyuzhnaya 2016).

Patience is often required in the enrichment and isolation of methanotrophs, and while some methanotrophs yield colonies within 5–7 days, many are slow-growing. For example, it can take 2–4 weeks before enrichments become turbid or colonies appear on agar plates during the isolation of more unusual methanotrophs from exotic environments such as hot springs (e.g., *Methylocaldum*, *Methylocella*). The use of dilute mineral salts medium has been successful in the isolation of moderately acidophilic methanotrophs such as *Methylocapsa* and *Methylocella* from peat bogs (Dedysh 2002). Clearly, the choice of medium and enrichment conditions are important for the isolation of new methanotrophs, and certainly the original NMS medium which has been widely used over the last 40 years can bias selective enrichment of methanotrophs. For example, *Methylococcus capsulatus* (Bath) can easily be re-isolated from the Roman Baths in Bath, UK and forms the dominant methanotroph in enrichment cultures with NMS. However, molecular ecological studies have shown that this is not the dominant methanotroph in this environment, and alternative media and approaches need to be employed to isolate other methanotrophs present in this moderately thermophilic environment (Murrell et al.,

unpublished). Many methanotrophs do not readily store at −80 °C or in cryoprotectants such as glycerol, and so subculturing on agar plates every few weeks is recommended to maintain healthy, viable cultures.

7 Genomics and Genetics

Methylococcus capsulatus (Bath) is probably the best characterized methanotrophs and has been the "workhorse" organism for researchers studying the biology of methane oxidation for over 40 years. The genome of *Mc. capsulatus* (Bath) has been sequenced (Ward et al. 2004) and thus provides a metabolic "blueprint" which enables further study of one-carbon metabolism and the regulation of methane oxidation in this bacterium. Several genomes of different methanotrophic cultures are now available, providing the necessary background information to carry out comparative genomics and to determine experimentally the molecular basis for obligate methanotrophy (reviewed in Wood et al. 2004) in many methane-oxidizing bacteria.

Molecular genetic systems for obligate methanotrophs have been slow in development, largely due to their obligate nature (only growth on methane makes it difficult to select for alternative phenotypes). Fortunately there are now a number of genetic tools available to carry out mutagenesis and expression studies with *Mc. capsulatus* (Bath) and other methanotrophs (e.g., see Csaki et al. 2001, 2003; Sharpe et al. 2007; Theisen et al. 2005). These techniques allow the introduction of broad-host-range plasmids carrying homologous and heterologous genes into methanotrophs, promoter probe fusions, transposon mutagenesis, and mutagenesis by marker-exchange. Homologous expression systems are also available for certain methanotrophs (e.g., see Borodina et al. 2007) in order to study structure and function of MMO. Whole-genome metabolic models coupled with flux-balance modeling for three methanotrophic species have recently been reported. The models summarize the current knowledge of methane utilization, enabling in silico simulation of the catalytic capabilities of methanotrophic cells and streamlining metabolic engineering (reviewed in Akberdin et al. 2018a and in Henard and Guarnieri 2018). The genomics and post-genomics of methanotrophs have been described in Akberdin et al. 2018a, b and in Chistoserdova and Kalyuzhnaya 2018.

8 Research Needs

1. *Core biochemistry and molecular regulation.* While key steps of microbial methane utilization are now well established, there are knowledge gaps which hold back further developments in industrial applications. They include the limited knowledge of the initial steps of methane oxidation driven by pMMO, the lack of molecular data on stress responses, and the impact of environmental perturbations, specifically O_2-limitation. While methanotrophy is highly metabolically redundant, and a number of minerals, such as iron, copper, rare earth

elements, and tungsten, have been shown to impact the carbon flow across primary metabolic pathways, the detailed vision of interactions and responses to variability of nutrients remains to be established. Membrane biogenesis is also a much neglected area of research, and for most methanotrophs, the exact function of these unusual intracytoplasmic membranes and how their synthesis is regulated represents an interesting challenge which will be aided by genome sequence information. The mechanisms of intracellular oxygen production in some anaerobic methanotrophs also await further validation and might provide a great tool for methane-based fermentation processes. The nature of as-yet uncultivated methanotrophs which grow on atmospheric concentrations of methane also needs to be addressed. The extremophile methanotrophs which can grow at very high or very low pH values provide a means to study mechanisms by which these methanotrophs survive and even thrive at extremes. The differences between the metabolism of mesophilic and thermophilic methanotrophs can now also be studied in detail using emerging genome information as metabolic blueprints. Thermophilic methanotrophs may represent a new source of enzymes with potential in biocatalysis.

2. *Taxonomical and functional diversity.* Molecular ecology studies have revealed that there are considerably more methanotrophs present in the environment than are represented in culture collections. Considerable effort now needs to be made in the enrichment and isolation of novel methanotrophs, particularly those from more extreme environments. There are few marine methanotrophs in culture, and their role in the marine environment warrants further study.
3. *Quorum sensing and community interactions.* The mechanisms of cell-to-cell communication in methanotrophic communities are an emerging area which could provide new insights into ecosystem-level methane cycling. A number of methanotroph-associated viruses have been discovered, but their role in the regulation methanotrophic community structures and methane fluxes remains elusive. The symbiotic methanotrophs, which have mostly been characterized in marine environments, have no representatives in culture yet and thus will provide a fascinating area for future study, particularly with respect to the involvement of the methanotroph in supplying nutrients to the host and vice versa. Thus far, there is only one example of a methanotroph-plant symbiont, and again such symbioses will provide a fruitful area for study.

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Facultative Methane Oxidizers

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Abstract

Most aerobic methanotrophic bacteria grow only on C_1 compounds (methane, methanol, formate, formaldehyde, or methylamines). However, facultative methanotrophs are able to use either methane or some non-C_1 compounds as their sole energy sources. The existence of such bacteria was a controversial topic until facultative methanotrophy was conclusively demonstrated in members of the genus *Methylocella*, which are widely distributed in acidic and neutral terrestrial environments. *Methylocella* species are morphologically and genetically unlike obligate methanotrophs in several ways. They lack a particulate, membrane-bound methane monooxygenase that is nearly universal to methanotrophs and instead use only a soluble form of this enzyme for methane oxidation. The latter is repressed if an alternative multicarbon growth substrate is present. *Methylocella* spp. can grow on a range of alternative substrates including acetate, pyruvate, succinate, malate, ethanol, propane, ethane, propanol, propanediol, acetone, methyl acetate, acetol, glycerol, propionate, tetrahydrofuran, and gluconate. More limited facultative methanotrophy has also been demonstrated in several alphaproteobacterial methanotrophs of the genera *Methylocystis* and *Methylocapsa*, as well as in verrucomicrobial methanotrophs of the genus "*Methylacidiphilum*." Unlike *Methylocella* spp., these methanotrophs all possess particulate methane monooxygenase, and growth is limited to only one or two alternative substrates (acetate, ethanol, or H_2, depending on the strain). The metabolic flexibility of facultative methanotrophs offers new biotechnological potential and calls for revising our outlook on methane cycling in the environment.

Abbreviations

GAF domains	(Found in cGMP-phosphodiesterases adenylyl cyclases and FhlA, where FhlA is formate hydrogen-lyase transcriptional activator) are small-molecule-binding domains present in signal transduction proteins in organisms from all phyla
PLFA	Phospholipid fatty acid
pMMO	Particulate methane monooxygenase
RT-PCR	Reverse transcription polymerase chain reaction
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
sMMO	Soluble methane monooxygenase
TCA cycle	Tricarboxylic acid cycle
EMC pathway	Ethylmalonyl-coenzyme A pathway, PrMO Propane monooxygenase, SDIMO Soluble di-iron monooxygenase, ICM Intracytoplasmic pathway membranes

1 Introduction

Most aerobic methanotrophic bacteria are able to grow only on methane, methanol, and in some instances a few other C_1 compounds: formate, formaldehyde, and/or methylamines. (Although methylamines may contain up to three C atoms, they are

considered C_1 compounds because metabolism acts on individual methyl groups.) Facultative methanotrophs, on the other hand, are able to use multicarbon compounds or H_2 as alternative sole energy sources to methane. A debate about facultative methanotrophy began three decades ago with the isolation of *Methylobacterium organophilum* strain XX and the report that this strain could grow on both methane and glucose (Patt et al. 1974). However, later examination of cultures found that they were unable to grow on methane, calling the original report into doubt (Green and Bousfield 1983). Other reports of facultative methanotrophs followed but, as with strain XX, were not verified in subsequent work (reviewed in Dedysh et al. 2004a; Theisen and Murrell 2005; Semrau et al. 2011). For example, a culture named "*Methylobacterium ethanolicum*" was originally proposed to be a facultative methanotroph but was later found to be a tight syntrophic association between a methanotroph and a facultative methylotroph of the genus *Xanthobacter*. Genome sequencing has failed to demonstrate enzymatic systems for methane oxidation in any strain of *Methylobacterium*, the usual suspect in early claims of facultative methanotrophy (Vuilleumier et al. 2009; Marx et al. 2012). Therefore, although early claims of facultative methanotrophs stimulated debate, none were pursued to a point that ruled out culture contamination or poor experimental stringency, and the existence of this metabolic combination was largely discounted.

This changed in 2005 when facultative methanotrophy was conclusively demonstrated in members of the genus *Methylocella* (Dedysh et al. 2005a). This study presented the first unequivocal proof of a methanotrophic bacterium growing on substrates containing C–C bonds. Later, the occurrence of facultative methanotrophy was demonstrated in several other alphaproteobacterial methanotrophs of the genera *Methylocystis* and *Methylocapsa* (Dunfield et al. 2010; Belova et al. 2011, 2013; Im et al. 2011), as well as in verrucomicrobial methanotrophs of the genus "*Methylacidiphilum*" (Mohammadi et al. 2017; Carere et al. 2017). This chapter focuses on these metabolically versatile methanotrophs and explains the genomic background for their metabolic capabilities.

2 *Methylocella*: The Champion of Facultative Methanotrophs

2.1 Cultivation

The first isolates of *Methylocella* were obtained from acidic peat bogs of West Siberia and European North Russia (Dedysh et al. 1998) and described as the novel genus and species *Methylocella palustris* (Dedysh et al. 2000). Later, two more species, *Methylocella silvestris* (Dunfield et al. 2003) and *Methylocella tundrae* (Dedysh et al. 2004b), were isolated from acidic forest and tundra soils, respectively. Characteristics that distinguish these three species are summarized in Table 1.

A key to successful isolation of *Methylocella* spp. was the use of moderately acidic (pH 5.0–5.8) mineral media with a low salt content (see Sect. 2.4).

Table 1 Major characteristics that distinguish three described species of the genus *Methylocella*

Characteristic	*M. palustris*	*M. silvestris*	*M. tundrae*
Cell size, μm	0.6−1.0 × 1.0−2.5	0.6−0.8 × 1.2−2.0	0.6−0.8 × 1.0−1.5
Capsule	+	+	−
Colony color	Semitransparent or opaque white	Semitransparent or opaque white	Cream
Growth in liquid medium	Nonhomogenous	Nonhomogenous	Homogenous
Temperature optimum, °C	20	20–25	15
pH optimum	5.0–5.5	5.5	5.5–6.0
Growth on methanol, % CH_3OH (v/v)	<0.3	<5.0	<2.0
NaCl tolerance, % (w/v)	0.5	0.8	0.8–1.2
Major FAMEs	18:1 *ω*7 *c*, 16:1 *ω*7 *c*	18:1 *ω*7 *c*, 16:1 *ω*7 *c*	18:1 *ω*7 *c*, 16:1 *ω*7 *c*, cy19:0 *ω*8 *c*
G + C content, mol%	61.2	63.1	63.3
Species-specific oligonucleotide probe	Mcell-1026, 5′-GTTCTCGCCACCCGAAGT-3′	Mcells-1024, 5′-TCCGGCCAGCCTAACTGA-3′	Mcellt-143, 5′-TTCCCCGAGTTGTTCCGA-3′
Type strain	K, ATCC 700799	BL2, DSM 15510, NCIMB 13906	T4, DSM 15673, NCIMB 13949

Conventional mineral media for methanotrophs (containing 1.5–3 g l^{-1} of salt) do not support the growth of *Methylocella* spp. A side effect of using low-nutrient media is that relatively low optical densities (OD_{540} 0.1–0.3) are usually achieved in batch cultures. This limitation was overcome by Theisen et al. (2005) by growing *Methylocella silvestris* $BL2^T$ in a fermentor run in a fed-batch mode. In prolonged culture over several weeks, an OD_{540} of 13.2 was achieved on methane, indicating that this facultative methanotroph has potential for biotechnological applications.

2.2 Cell Morphology and Ultrastructure

Methylocella cells are Gram-negative, non-motile, polymorphic rods with rounded ends. They occur singly or in shapeless aggregates. Large, highly refractile intracellular granules of poly-β-hydroxybuturate form at each pole, giving the cells a distinct bipolar appearance under phase-contrast microscopy (Fig. 1a and b). The cell ultrastructure is unique among methanotrophs (Fig. 1b). Regardless of the growth phase or the copper content of the medium, the extensive intracytoplasmic membrane structures typical of obligate proteobacterial methanotrophs are absent from

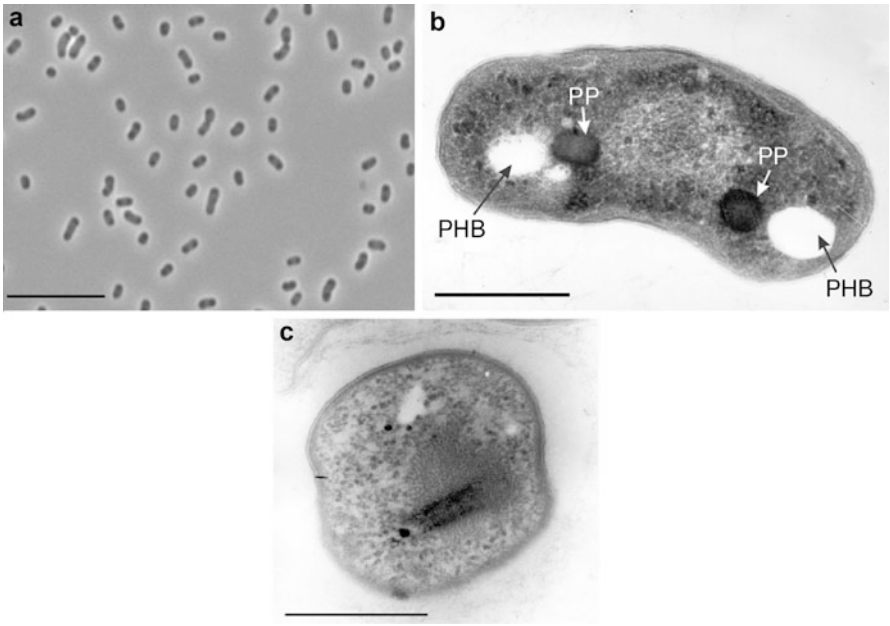

Fig. 1 (**a**) Phase-contrast micrograph of cells of *Methylocella silvestris* $BL2^T$; bar, 10 μm. (**b**, **c**) Electron micrographs of ultrathin sections of vegetative cells of *Methylocella palustris* K^T grown under different conditions; bars, 0.5 μm. Cells grown under normal oxygen tension showed inclusions of poly-β-hydroxybutyrate (PHB) and polyphosphate (PP) granules formed at each cell pole (**b**). Cells grown under oxygen-limiting conditions showed inclusions with periodic macromolecular structure (**c**)

Methylocella cells. Instead, the cells contain a vesicular membrane system composed of small (40–100 nm in diameter) spherical, ovoid, or tube-shaped vesicles formed by cytoplasmic membrane invaginations. These vesicles are bounded by three-layered membranes, and each contains a homogenous matrix of lower electron density than the cytoplasm. Chains of these vesicles are usually located on the periphery of the cells. Another interesting ultrastructural feature observed in cells of *Methylocella palustris* K^T grown under oxygen-limiting conditions (<5% O_2 in the headspace) is that approximately one-third of all cells contain large inclusions with periodic macromolecular structure (Fig. 1c). The nature and function of these inclusions remain unknown.

2.3 Physicochemical Limits

The temperature range for growth of *Methylocella* spp. is 4–30 °C. Different species possess slightly different optima (Table 1). They grow between pH 4.2 and 7.5, with an optimum at pH 5.0–6.0. They are highly sensitive to salt stress and therefore prefer diluted media with a low salt content (0.2–0.5 g l^{-1}). Thus, *Methylocella* spp. can be characterized as psychrotolerant mesophiles and moderate acidophiles with a low salt tolerance. All *Methylocella* spp. utilize ammonium salts, nitrates, yeast extract, and some amino acids as nitrogen sources. They use the glutamate cycle for NH_4^+ assimilation in much the same way as other alphaproteobacterial methanotrophs. When grown in nitrogen-free media, they are able to fix N_2 via an oxygen-sensitive nitrogenase. This autonomy in nitrogen acquisition is probably essential in oligotrophic environments like peat bogs that are highly depleted of available nitrogen compounds.

2.4 Facultative Metabolism: Physiology and Genomics

Originally, *Methylocella* spp. were described as aerobic bacteria capable of growth only on the C_1 compounds methane, methanol, methylamine, and formate. However, it was later observed that they also grew on acetate, pyruvate, succinate, malate, and ethanol (Dedysh et al. 2005a). The species showing the most robust growth on these organic acids and alcohols was *Methylocella silvestris* strain $BL2^T$, which became the model organism for further experiments into facultative methanotrophy (Dedysh et al. 2005a). Culture purity was assured by microscopic observation for the distinct cell morphology of this bacterium, by cloning of 16S rRNA genes, by fluorescence in situ hybridization with strain-specific and genus-specific oligonucleotide probes, and by extinction dilution of cultures grown alternately on methane or acetate as the sole substrate. Growth on either substrate was followed over time using quantitative real-time PCR of the *mmoX* gene, which encodes the α-subunit of soluble methane monooxygenase (sMMO), and verified via cell counts and OD_{600}. Substrate use was followed by gas chromatography and high-performance liquid chromatography (Dedysh et al. 2005a). Counts of *mmoX* increased in parallel with cell counts

during growth on either acetate or methane as the sole substrate, verifying that cells possessing the genetic basis of methane oxidation grew on either substrate. These data demonstrated conclusively that *Methylocella silvestris* BL2^T was not an obligate methanotroph. The growth rate and carbon conversion efficiency were higher on acetate than on methane, and when both substrates were provided in excess, acetate was preferably used, and methane oxidation shut down.

Completion of the full 4.3 Mb genome of *Methylocella silvestris* BL2^T (Chen et al. 2010; Tamas et al. 2014) verified the genetic basis for both methane oxidation and the oxidation of some multicarbon compounds. A complete operon encoding sMMO (but no genes encoding pMMO) was identified, as were genes encoding methanol dehydrogenase, tetrahydrofolate, and the tetrahydromethanopterin-mediated formaldehyde oxidation, and the serine cycle for fixation of C_1 compounds. High activities of hydroxypyruvate reductase and serine-glyoxylate aminotransferase were detected in cell extracts of all *Methylocella* species, verifying the serine pathway (Dedysh et al. 2000, 2004b; Dunfield et al. 2003).

Additionally, smoking guns for catabolism of multicarbon compounds were evident in the genome. The genome encoded two systems to convert acetate to acetyl-CoA: the acetate kinase/phosphotransacetylase pathway and the acetate synthase pathway (Chen et al. 2010; Tamas et al. 2014). Together these explain the strong growth on acetate. Glyoxylate bypass enzymes (isocitrate lyase and malate synthase) to complete the serine cycle and facilitate the assimilation of two-carbon compounds were present. A complete TCA cycle was predicted, which would allow for the metabolism of other organic acids, provided they could be transported inside the cell. A key determinant of obligate methanotrophy is a limited ability to transport potential substrates across the membrane. This may explain the failure of other alphaproteobacterial methanotrophs to utilize organic acids, even though the TCA cycle is complete (Ward et al. 2004; Wood et al. 2004). As expected, many more genes encoding membrane transporters were predicted in the genome of *Methylocella silvestris* compared to the closest obligate methanotroph *Methylocapsa acidiphila*, particularly major facilitator transporters and periplasmic binding proteins (Tamas et al. 2014). This suggested an increased capacity to import potential substrates.

The genome analysis also suggested further metabolic versatility. Besides the operon encoding the di-iron-containing multicomponent soluble methane monooxygenase (sMMO), a gene cluster encoding a second di-iron-containing multicomponent monooxygenase (SDIMO) was predicted. This second SDIMO was shown to be a propane monooxygenase, which acts together with sMMO to facilitate propane oxidation (Crombie and Murrell 2014). Reporter *gfp* fusions as well as knockouts of the hydroxylase α-subunit-encoding genes of each enzyme demonstrated that only the sMMO was needed for methane oxidation, but both the sMMO and PrMO were involved in propane oxidation (Crombie and Murrell 2014). This study extended the range of growth substrates known for this unique methanotroph to include substrates of this monooxygenase, propane and ethane, as well as the downstream intermediates of these oxidations: propanol, propanediol, acetone, methyl acetate, acetol, glycerol, propionate, tetrahydrofuran, and gluconate

(Crombie and Murrell 2014). Whether or not these substrates are also suitable for other species of the genus *Methylocella* has not been verified yet.

2.5 Regulation of Methane Oxidation

Sequencing the complete genome of *Methylocella silvestris* BL2^T demonstrated conclusively that it oxidizes CH_4 via sMMO and lacks a particulate, membrane-bound methane monooxygenase (pMMO) that is found in almost all other methanotrophs (Dedysh et al. 2016). The closely related genus *Methyloferula* is the only other known example of a methanotroph using only sMMO (Dedysh et al. 2016). In *Methylocella silvestris* BL2^T, the *mmoXYBZDC* structural genes encoding sMMO are located upstream of two genes, *mmoR* and *mmoG*, encoding a σ^{54} transcriptional activator and a putative GroEL-like chaperone, respectively (Theisen et al. 2005). The genes are all co-transcribed from a σ^{54}-dependent promoter located upstream of *mmoX*. The organization of structural genes is similar to that observed in other sMMO-possessing methanotrophs. However, unlike in other methanotrophs, the *mmoR* in *Methylocella silvestris* BL2^T does not seem to be transcribed independently of the structural genes *mmoXYBZDC*. The predicted function of MmoR in other methanotrophs is to activate *mmo* gene expression in response to low copper-to-biomass ratios (Csaki et al. 2003). Analysis of the MmoR in *Methylocella silvestris* BL2^T showed that it contained an N-terminal GAF domain not found in the MmoR proteins of *Methylosinus sporium* OB3b or *Methylococcus capsulatus* (Bath). The different domain structure of MmoR in strain BL2^T and the fact that the activator is not transcribed independently from the operon it regulates indicate a novel control mechanism compared to obligate methanotrophs.

In methanotrophs that possess both pMMO and sMMO, sMMO is expressed only under low copper-to-biomass ratios, while pMMO is expressed under high copper-to-biomass ratios (Stanley et al. 1983). This regulation does not exist in *Methylocella* spp. As shown by Theisen et al. (2005), distinct sMMO-specific polypeptides were observed in cells of *Methylocella silvestris* BL2^T grown in both high-copper (1 μM) and low-copper (5 nM) media. The naphthalene assay, routinely used to monitor sMMO activity, gave positive results in both cases. However, *mmo* genes are repressed in response to preferred alternative growth substrates. Three analyses, transcriptional analysis using RT-PCR, SDS-PAGE of cells grown under methane or acetate, and the use of a *gfp* promoter probe construct, all demonstrated that transcription of the *mmo* operon was repressed by acetate (Theisen et al. 2005).

2.6 Phylogeny

The genus *Methylocella* belongs to the *Alphaproteobacteria* family *Beijerinckiaceae*. The *Beijerinckiaceae* is a sister family to the *Methylocystaceae*. However, while the *Methylocystaceae* contains only methanotrophs, the *Beijerinckiaceae* incorporates metabolically diverse bacteria. These include obligate methanotrophs using pMMO

(*Methylocapsa* spp.), obligate methanotrophs using sMMO *(Methyloferula stellata*), facultative methanotrophs using sMMO (*Methylocella* spp.), facultative methanotrophs using pMMO (*Methylocapsa aurea*, see below), non-methanotrophic facultative methylotrophs (*Beijerinckia mobilis*, *Methylorosula polaris*, *Methylovirgula ligni*), facultative phototrophs (*Rhodoblastus* spp.), and non-methylotrophic chemoorganotrophs (*Beijerinckia* spp.), all in a tight phylogenetic cluster (Dedysh et al. 2005b; Dedysh and Dunfield 2016; Dedysh et al. 2016). At one pole of this metabolic diversity is the extremely versatile organotroph *Beijerinckia indica*, which grows on diverse sugars, organic acids, alcohols, and aromatic compounds. At the other pole is *Methylocapsa acidiphila*, a typical specialized obligate methanotroph. These two species share high 16S rRNA gene sequence identity with *Methylocella silvestris* (97.2% and 96.2%, respectively). No other described family of methanotrophs (the *Methylocystaceae*, *Methylococcaceae*, *Methylothermaceae*, or "*Methylacidiphilaceae*") includes such closely related methanotrophs and non-methanotrophs.

2.7 Evolution

In an ecological sense, obligate methanotrophy is an extreme example of lifestyle specialization, presumably in order to increase the growth efficiency on a specific substrate (methane). In a mechanistic sense, obligate methanotrophy is believed to result because key elements of metabolic pathways such as the TCA cycle are absent or nonfunctional (Shishkina and Trotsenko 1982). An example is the absence of functional α-ketoglutarate dehydrogenase in some methanotrophs belonging to the *Gammaproteobacteria* (Wood et al. 2004). However, no such metabolic lesions are known to be universal to methanotrophs. Analysis of the first complete methanotroph genome, that of *Methylococcus capsulatus*, considered this issue (Kelly et al. 2005; Ward et al. 2004). Surprisingly, genes for α-ketoglutarate dehydrogenase and genetic systems necessary for sugar metabolism were found, even though extensive study has shown that this species is an obligate methanotroph unable to grow on sugars or organic acids. It was hypothesized that obligate methanotrophy may instead be related to a limitation of membrane transporters for these substrates rather than the absence of key enzymes (Ward et al. 2004). An organism like *Methylocella*, which grows under acidic conditions, may not need transporters for acetate, since this can diffuse across the membrane when protonated, but is likely to need transporters for efficient use of other compounds.

The metabolic versatility of the family *Beijerinckiaceae* suggests that there has been a great deal of relatively recent adaptation, where lineages have evolved toward, or away from, an obligately methanotrophic lifestyle. Tamas et al. (2014) used comparative genomics to propose that the ancestor of both the *Methylocystaceae* and the *Beijerinckiaceae* was an obligate methanotroph but that many members of the *Beijerinckiaceae* later diversified their range of substrates via lateral gene transfer. Compared to the closest obligate methanotroph *Methylocapsa acidiphila*, *Methylocella silvestris* possessed more genes in the COG categories of

carbohydrate transport and metabolism (G), energy production and conversion (C), and transcription (K). While methanotrophy and methylotrophy genes showed a vertical line of descent in the families *Methylocystaceae* and the *Beijerinckiaceae*, many genes in the COG categories G, C, and K in *Methylocella silvestris* and *Beijerinckia indica* showed phylogenetic and compositional evidence of having been obtained via lateral transfer. This was particularly true of genes encoding membrane transporters and porins. This study supported the hypothesis that a key determinant of obligate methanotrophy is a limited ability to import substrates and that *Methylocella silvestris* has overcome this limitation, possibly via lateral gene transfer of transporter-encoding genes. It is tempting to speculate that the complex internal membrane architecture of the pMMO-containing methanotrophs is a key factor limiting their ability to possess diverse membrane transporters and that the use of only the non-membrane-bound sMMO has allowed *Methylocella* to bypass this problem.

2.8 Detection in Different Environments

Due to the absence of pMMO in *Methylocella* spp., they cannot be detected using a *pmoA*-based PCR assay considered universal and specific for all other known methanotrophs, which is frequently used in microbial ecology studies of methanotrophy (Knief 2015). In addition, *Methylocella* spp. do not contain any of the phospholipid fatty acids (PLFAs) that are considered signatures for type I or type II methanotrophs and thus cannot be specifically targeted by PLFA-based assays. They can in principle be identified via 16S rRNA gene retrieval. However, metabolically diverse members of the family *Beijerinckiaceae* are closely related in their 16S rRNA genes (generally 96–98% sequence identity), so detection of *Methylocella*-like 16S rRNA genes in an environment cannot be taken as clear evidence of facultative methanotrophy, or even of methanotrophy at all. Detection of *Methylocella*-like *mmoX* genes is more convincing evidence of a methanotrophic genotype; however, detected genes may belong to obligate *Methyloferula*-like methanotrophs rather than facultative *Methylocella*-like methanotrophs. Alternatively, DNA-stable isotope probing using $^{13}CH_4$ is also indicative of methanotrophy, but again not necessarily of facultative methanotrophy.

Isolates of *Methylocella* have been grown from *Sphagnum*-dominated wetlands of the boreal zone and tundra (Dedysh et al. 1998, 2004b), acidic temperate forest soil (Dunfield et al. 2003), and a forested swamp (Miller et al. 2004). They have also been enriched from a neutral (pH 6.8–7.3) landfill cover soil in Moscow (Kallistova et al. 2013). *Methylocella*-like gene sequences have been retrieved from a variety of environments; however, because of the caveats noted above, we only cite here a few studies based on *mmoX* detection or on $^{13}CH_4$-SIP combined with detection of 16S rRNA genes. Positive SIP-based detection of *Methylocella*-related methanotrophs has been reported in acidic (pH 4.3–6.8) boreal wetland, grassland, and forest soils (Morris et al. 2002; Radajewski et al. 2002; Putkinen et al. 2014) and in alkaline (pH 9.4) coal mine soil (Han et al. 2009). *Methylocella*-like *mmoX* sequences were

obtained from a variety of moorlands and peatlands (Chen et al. 2008, Rahman et al. 2011). Noteworthy was the detection of *Methylocella*-like *mmoX* in tropical forests, demonstrating that the genus is not limited to temperate or arctic regions (Rahman et al. 2011). The detection in alkaline environments (Han et al. 2009; Rahman et al. 2011) also indicates that the *Beijerinckiaceae* methanotrophs are not limited to acidic environments.

Another tool for detecting *Methylocella* cells in the environment is fluorescence in situ hybridization (FISH) with species-specific, 16S rRNA-targeted oligonucleotide probes. Several specific probes have been developed for this group (Table 1). Using this approach, *Methylocella palustris* was enumerated at greater than 10^6 cells per g of wet peat in a *Sphagnum* bog, making it a numerically predominant methanotroph population (Dedysh et al. 2001).

3 Limited Facultative Methanotrophs

Since the discovery of facultative methanotrophy in *Methylocella*, several other methanotrophs have also been shown to be able to use non-C1 compounds as their sole growth substrate. Unlike *Methylocella*, these methanotrophs all oxidize methane using a pMMO enzyme. The diversity of substrates utilized by *Methylocella silvestris* BL2 far exceeds that of these other methanotrophs, and they have therefore been dubbed "limited facultative methanotrophs" (Dunfield and Dedysh 2014). They use only acetate, ethanol, or H_2 + CO_2, as alternate substrates, and usually grow poorly on these substrates compared to methane. The substrates appear to serve primarily as supplementary energy sources to assist survival during methane starvation. Where *Methylocella* is a Jack of all trades, most other facultative methanotrophs appear to be moonlighting to supplement their income.

3.1 *Methylocystis*

Methylocystis spp. are typical aerobic methanotrophs possessing pMMO and a well-developed ICM system in which pMMO is bound. The first observation of slow growth on acetate was made for *Methylocystis bryophila* strain H2s, which was isolated from northern *Sphagnum*-dominated wetlands and represented a numerically abundant methanotroph population in these ecosystems (Belova et al. 2011, 2013). Strain H2s demonstrated a strong preference for growth on methane with a specific growth rate of 0.06 h^{-1} and a maximal OD_{410} of 0.8 to 1.0. However, clearly detectable, albeit slow, growth with a specific growth rate of 0.006 h^{-1} was also observed on acetate in the absence of methane. The OD_{410} of acetate-grown cultures reached 0.25–0.30 following 3–4 weeks of incubation. The efficiency of carbon conversion to cell material [mean g C_{cell} per g $C_{substrate}$ $\pm$ 1 standard error of the mean (SEM), %] for strain H2s was higher on methane (47.0 $\pm$ 6.0%) than on acetate (18.0 $\pm$ 1.4%). Yet, this methanotroph was able to survive multiple (up to 20) transfers on a medium containing acetate as the sole energy substrate. In cells grown

for several transfers on acetate, ICM were maintained, although in a reduced form, and mRNA transcripts of pMMO were detectable. These cells resumed their growth on methane faster than those kept for the same period of time without any substrate. A reexamination of all type strains of *Methylocystis* species showed that *Methylocystis heyeri* H2^T and *Methylocystis echinoides* IMET10491^T are also capable of slow growth on acetate (Belova et al. 2011). Following these findings, the taxonomic description of the genus *Methylocystis* was emended, in order to reflect the facultative nature of methanotrophy in these bacteria (Belova et al. 2013).

One additional, taxonomically unvalidated member of the genus *Methylocystis*, strain SB2, was also reported to grow on acetate as well as ethanol (Im et al. 2011). Similarly to the other facultatively methanotrophic *Methylocystis* strains, growth on acetate was very slow. However, optimal growth on ethanol was much better and reached 42% of the growth rate on methane. The genome of strain SB2 is 3.7 Mb in size. It contains acetyl-coenzyme A synthetase, all genes encoding enzymes of the TCA cycle, and the ethylmalonyl-coenzyme A (EMC) pathway (Vorobev et al. 2014). Transcriptomic analyses of *Methylocystis* sp. strain SB2 cultures grown on either ethanol or methane revealed that (i) expression of the pathway of methane oxidation and the serine cycle was significantly reduced when grown on ethanol, (ii) expression of the TCA cycle dramatically increased when grown on ethanol, and (iii) expression of the EMC pathway was similar for both substrates. Presumably, this methanotroph converts ethanol to acetyl-coenzyme A, which is then funneled into the TCA cycle for energy generation or incorporated into biomass via the EMC pathway.

3.2 *Methylocapsa aurea*

The substrate use of *Methylocapsa aurea* KYG^T, a pMMO-using methanotroph within the *Beijerinckiaceae*, is similar to that of *Methylocystis bryophila* noted above (Dunfield et al. 2010). This bacterium was isolated from a forest soil in Germany. It grows better on methane (maximum OD_{600} 1.2, $\mu = 0.018\ h^{-1}$) than on acetate (maximum OD_{600} 0.3, $\mu = 0.006\ h^{-1}$). However, the cultures of *Methylocapsa aurea* KYG^T can be maintained continuously on acetate as the sole carbon and energy source without the loss of viability. ICMs are maintained in acetate-grown cells, although they are present in a reduced form. Notably, the capability to grow on acetate could not be detected in two other species of the genus: *Methylocapsa acidiphila* and *Methylocapsa palsarum* (Dedysh et al. 2015).

3.3 *"Methylacidiphilum"*

The proteobacterial methanotrophs *Methylococcus capsulatus* and *Methylosinus trichosporium* express hydrogenases that can reduce NAD^+ to support MMO function (Shah et al. 1995; Hanczár et al. 2002). Although H_2 cannot serve as the sole

energy source during growth of these methanotrophs, H_2 scavenging may provide supplementary energy during growth on methane (Hanczár et al. 2002).

However, methanotrophs of the *Verrucomicrobia* phylum have more recently been shown to be capable of growth via the Knallgas reaction alone in the absence of methane (Mohammadi et al. 2017; Carere et al. 2017). To date, two strains, "*Methylacidiphilum fumariolicum*" strain SolV (Mohammadi et al. 2017) and "*Methylacidiphilum infernorum*" strain RTK17.1 (Carere et al. 2017), have been shown to grow on $H_2 + CO_2$ as the sole energy and carbon sources at low O_2 partial pressures. (Note that "*Methylacidiphilum*" is a proposed name and still not taxonomically validated due to difficulties in depositing the culture in public repositories.) Verrucomicrobial methanotrophs incorporate carbon autotrophically as CO_2 using the Calvin-Benson-Bassham cycle, a key prerequisite for growth via the Knallgas reaction (Hou et al. 2008; Khadem et al. 2011; Sharp et al. 2014). Hydrogenase-encoding gene clusters were identified in each of the genomes of strain SolV (Mohammadi et al. 2017) and strain RTK17.1 (Carere et al. 2017). Strain SolV had a higher diversity of hydrogenase-encoding genes and was shown to express both oxygen-sensitive (*hup*-type) and oxygen-insensitive (*hhy*-type) forms. At below 0.2% O_2, when the *hup*-type hydrogenase was expressed, the growth rate of SolV via the Knallgas reaction reached about 60% of the growth rate achievable on methane (Mohammadi et al. 2017). Although strain RTK17.1 could also grow on $H_2 + CO_2$ alone, its growth under these conditions was seven times slower than its optimal growth rate and nearly ten times lower than the growth rate of SolV on $H_2 + CO_2$. Optimal growth of strain RTK17.1 was observed when H_2 and methane were provided simultaneously (Carere et al. 2017). Verrucomicrobial methanotrophs have been found primarily in volcanic habitats (Op den Camp et al. 2009; Sharp et al. 2014), where the ability to grow on two gases that are commonly produced in geothermal conditions is certainly an adaptive trait. Carrere et al. (2017) suggested that the two studied strains fill slightly different ecological niches: SolV can switch between efficient methanotrophic or hydrogenotrophic growth, while RTK17.1 uses H_2 predominantly as a supplementary mixotrophic substrate to optimize growth and survival under conditions of limited methane and/or O_2 availability.

Indeed, hydrogenotrophic growth is likely to be widespread in methanotrophs. Genomic surveys indicate that all publicly available aerobic methanotroph genomes possess hydrogenases (Greening et al. 2016; Carere et al. 2017). Given that only few methanotrophs are autotrophic via the Calvin-Benson-Bassham cycle, H_2 is unlikely to be used by many methanotrophs as a sole growth substrate. However, H_2 scavenging could be a common strategy to gain supplemental energy (Shah et al. 1995; Hanczár et al. 2002).

3.4 Others

A detailed procedure for differentiating obligate and facultative methanotrophs was proposed by Dedysh and Dunfield (2011). The key steps include strict and clear demonstration of culture purity via several methods, demonstration of methane

oxidation, and elucidation of the genetic machinery for methanotrophy. Some further reports of facultative methanotrophy, while potentially intriguing, fall so short of these standards that we do not note them here. However, the known taxonomic and metabolic diversity of methanotrophs is growing. For example, some methanotrophs have been shown to have a surprising capacity for processes such as fermentation (Kalyuzhnaya et al. 2013) and anaerobic respiration (Kits et al. 2015). Given this widening scope of methanotrophy, it is likely that new versatile methanotrophs will be found in the future.

4 Future Prospects and Research Needs

4.1 Biotechnological Applications

MMOs are promiscuous enzymes that co-oxidize a wide range of hydrocarbons, including several important environmental contaminants such as the halogenated hydrocarbons trichloroethylene and chloroform (Colby et al. 1977; Semrau 2011). Facultative methanotrophs may be particularly useful for bioremediation purposes. The ability of *Methylocystis* strain SB2 to degrade chlorinated hydrocarbons was compared during growth on methane, ethanol, or acetate (Im and Semrau 2011; Yoon et al. 2011). Diverse chlorinated hydrocarbons were co-degraded due to pMMO activity. However, while co-oxidation of the pollutants always inhibited growth on methane via competitive inhibition, growth on ethanol was not affected by most of the chlorinated hydrocarbons tested. Because *pmo* genes were constitutively expressed, alternative substrates allowed the organisms to survive and grow while still co-degrading pollutants (Im and Semrau 2011). The current state of knowledge of methanotrophy as it applies to pollutant degradation and suggestions for future research have been reviewed by Semrau (2011).

Methylocystis bryophila has been identified as a promising candidate for industrial methanol production from methane (Patel et al. 2016). Addition of formate increased methanol production, possibly by altering the electron flow and reductant status of the organism. Explicit comparisons of facultative versus obligate methanotrophs for such industrial conversions and the potential effect of alternative substrates like acetate on electron flow and the production of intermediates have not yet been made.

Methylocella and *Methyloferula* are the only methanotrophs that rely solely on the sMMO enzyme for methane oxidation. sMMO oxidizes a wider range of substrates than pMMO and is often considered the more promising candidate for bioremediation. The sMMO of *Methylocella* is also attractive from a biotechnological viewpoint since expression should not be repressed by copper ions in the environment (Murrell et al. 2000; Theisen and Murrell 2005). In theory, indigenous populations of *Methylocella* might be stimulated with a substrate like acetate for bioremediation of contaminated sites. However, unlike *pmo* genes in the facultative *Methylocystis* strains, *mmo* genes in *Methylocella* are not constitutively expressed but rather repressed by other substrates, so the use of this organism in bioremediation

is not so straightforward. More knowledge of genomic capabilities and gene regulation in this organism would be an aid toward assessing its value in bioremediation.

4.2 Ecological Implications

Our understanding of the ecology of facultative methanotrophy lags behind our understanding of its genetic basis. It is now evident that methanotrophs can be specialists that concentrate on methane or generalists with the ability to metabolize other compounds besides methane. Based on simple ecological theory, a generalist strategy should confer an advantage under conditions of low or fluctuating methane availability, while specialists should dominate under a reliable and large methane source. The limited facultative methanotrophs of the genus *Methylocystis* appear to use alternative substrates primarily to remain energized during methane starvation. Belova et al. (2011) demonstrated a faster recovery after methane starvation when acetate was provided during the starvation phase. Metabolic flexibility in *Methylocystis* spp. also provides one explanation for the exceptionally wide distribution of these methanotrophs in natural environments (Knief 2015).

The ecology of *Methylocella* is more complex, as it is more versatile and methane is not the preferred substrate. Pudasaini et al. (2017) reported growth of *Methylocella*-like bacteria on a soil substrate membrane system, a cultivation strategy aimed at growing K-selected bacteria adapted to low concentrations of substrates. This is a fascinating indication that *Methylocella* may indeed be a K-selected bacterium, probably uncompetitive under excess methane but able to grow and survive in a substrate-limited environment. Crombie and Murrell (2014) suggested that *Methylocella*, given its ability to consume both methane and other alkanes, might be better adapted than other methanotrophs to consuming geological natural gas that typically has higher alkanes in it than biogas. This has not been tested, although intriguingly, *Methylocella*-like bacteria have been detected in weathered shale cliffs (Cockell et al. 2011). Again, the diversity of the *Beijerinckiaceae* means that it is difficult to postulate the physiology of detected strains, but these findings suggest potential avenues of further study. There are few ecological studies actually testing hypotheses about facultative versus obligate methanotrophy in the field, but examining their relative distributions could be fruitful.

In environments where the facultative strategy dominates, methane oxidation activity may be affected by factors other than methane availability, a possibility that has not generally been considered. When acetate was added to batch cultures of *Methylocella* growing on methane, methane oxidation was initially inhibited, but after the acetate was depleted, methane oxidation resumed at a faster maximum rate than in control cultures due to population growth on acetate (Dedysh et al. 2005a). More research is needed to assess both the short-term inhibitory effects of alternate substrates on sMMO and the long-term stimulatory effects these substrates will have on the population sizes of facultative methanotrophs. However, some findings are intriguing. It has been noted that acetate stimulates methane oxidation in tundra soil (West and Schmidt 1999). Inhibition of methane oxidation was observed for mire

soils supplemented with acetate, propionate, or ethanol (Wieczorek et al. 2011). Measurements of methane oxidation rates in paddy soils showed that the methanotrophic activity was inhibited by the addition of acetate compared to the continuous supplementation of methane, although the paddy soil maintained the methane oxidation capacity and recovered following methane supplementation (Leng et al. 2014). Molecular identification of methanotrophs that consumed labelled acetate revealed uncultivated *Methylocystis* species, which are widespread in paddy soils or are associated with rice roots.

In summary, the relative importance of facultative versus obligate methane oxidizers in natural environments remains uncertain and calls for further studies. Habitats dominated by facultative methanotrophs may turn into more potent sources of methane when acetate availability increases. This could be the case when vegetation changes or when exudation patterns of plants change, as could happen under elevated atmospheric CO_2, increased temperature, or increased nitrogen fertilization and deposition. Hence, one research need is to examine conditions, habitat types, or environmental changes that switch facultative methanotrophs from methane to acetate consumption.

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Hormoconis resinae, The Kerosene Fungus 16

Catherine Rafin and Etienne Veignie

Contents

Abstract

The ascomycete *Amorphotheca resinae* Parbery (1969) is widely known by the anamorph name *Hormoconis resinae* (Lindau) Arx & G.A. de Vries or its obligate synonym *Cladosporium resinae* (Lindau) G.A. de Vries. It belongs to Saccharomyceta, Pezizomycotina, Leotiomyceta, Sordariomyceta, Leotiomycetes, Leotiomycetes *incertae sedis*, and Myxotrichaceae. This fungus has been isolated from natural environments (soil, freshwater, and marine) and manufactured environments. In particular, it grows in hydrocarbon-rich substrates such as jet fuel, diesel, petroleum, and wood preserved with creosote or coal tar. In the 1960s, the ascomycete *A. resinae* was reported as one of the most common fuel-deteriorating microorganisms. This species is known colloquially as the kerosene, petroleum, jet fuel, or creosote fungus. It utilizes aliphatic and aromatic hydrocarbons, as well as

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alcohols and acids. The processes involved in *n*-alkane uptake and metabolism by *H. resinae* have been studied in detail, and it has demonstrated a constitutive *n*-alkane-oxidizing system. Its growth can lead to serious biodeterioration of the final product quality, the formation of sludge, and deterioration of pipework and storage tanks, both in the refinery and at the end-user facility. *H. resinae* has a broad distribution and is commonly found in soil or water that could be potential sources of contamination for petroleum tanks, leading to biodeterioration and economic loss. Therefore, a considerable amount of literature has been reported on this species in the twentieth century, corresponding to the increase in the anthropogenic use of petroleum and its refined products. This chapter presents an overview of the research conducted on the so-called kerosene fungus.

1 Introduction

In the numerous conflicts between humankind and microorganisms, a saprotrophic fungus identified as the ascomycete *Amorphotheca resinae* Parbery (1969a), teleomorph, also widely known by the anamorph name *Hormoconis resinae* (Lindau) von Arx & G.A. de Vries or *Cladosporium resinae* (Lindau) G.A. de Vries, has occupied a central position. It was first isolated (and described as *Hormodendron resinae* Lindau) from the resin of *Pinus excelsa* near Hamburg (Germany) in 1906 (Lindau 1906). During the twentieth century, this fungus was widely isolated from natural habitats and considered as a saprotrophic fungus of the soil microbiota (Parbery 1968, 1969c, 1970). It has also frequently been isolated from anthropogenic environments and consequently identified as being responsible for the degradation of manufactured structures in the aeronautical industry (leading to military crashes) and other human activities associated with the use of fuel, diesel, or kerosene as an energy source (Edmonds and Cooney 1967, 1968; Lindley and Heydeman 1983, 1985; Itah et al. 2009; Teh and Lee 1973). In the 1960s, investigators reported that the ascomycete *Amorphotheca resinae* was one of the most common fuel-deteriorating microorganisms. Therefore, this fungus is known colloquially as the kerosene, petroleum, or jet fuel fungus.

Subsequently, numerous research studies have been conducted to clearly identify this fungus and understand its particular metabolism that affects hydrocarbon use. In this chapter, we focused on different aspects of this particular fungus: its taxonomy, distribution, morphology, physiology, as well as considering how to treat contaminated fuels and further research needs.

2 Taxonomy

The ascomycete *A. resinae* Parbery (1969a), teleomorph, is widely known by the anamorph name *Hormoconis resinae* (Lindau) von Arx & G.A. de Vries or its obligate synonym *Cladosporium resinae* (Lindau) G.A. de Vries. According to Kirk (2017), its taxonomic classification is as follows: Fungi, Ascomycota, Eurotiomycetes, not assigned, *Amorphothecaceae*, and *Hormoconis* (http://www.speciesfungorum.org/Names/SynSpecies.asp?RecordID=326157).

Table 1 Nomenclature and synonyms of *Amorphotheca resinae*

Teleomorph: *Amorphotheca resinae* Parbery, Australian J. Bot. 17: 340. 1969a
Anamorph
Hormodendrum resinae Lindau, in Rabenh. Krypt.-Fl., 2, 1 (Pilze) 8: 699. 1907
Cladosporium avellaneum G.A. de Vries, contribution to the knowledge of the genus *Cladosporium*, Uitg. Druk. Hollandia, p. 56, 1952
= *Cladosporium avellaneum* f. *albidum*
= *Cladosporium avellaneum* f. *avellaneum*
= *Cladosporium avellaneum* f. *sterile*
= *Cladosporium avellaneum* f. *viride*
Cladosporium resinae (Lindau) G.A. de Vries, Antonie van Leeuwenhoek 21: 167. 1955
= *Cladosporium resinae* f. *albidum*
= *Cladosporium resinae* f. *avellaneum*
= *Cladosporium resinae* f. *resinae*
= *Cladosporium resinae* f. *sterile*
Hormoconis resinae (Lindau) von Arx & G.A. de Vries in von Arx, Verh. K. Ned. Akad. Wet., Afd. Natuurk. 61: 62. 1973
= Hormoconis resinae f. resinae

The numerous synonyms with the references available in the literature are detailed in Table 1.

Using micromorphological and cultural characteristics, phylogenetic analysis based on large subunit rRNA gene and ribosomal internal transcribed spacer (rRNA-ITS) sequences, and provisions of the International Code of Botanical Nomenclature, Seifert et al. (2007) attempted to resolve the taxonomic and nomenclatural confusion due to the use of the epithet *resinae* for three species of *Cladosporium*-like Hyphomycetes. Indeed, the type specimen *Hormodendrum resinae*, which is the basis for the use of the epithet *resinae* for the creosote fungus (either as *Hormoconis resinae* or *Cladosporium resinae*), represents the mononematous synanamorph of the synnematous, resinicolous fungus *Sorocybe resinae*, called the resin fungus (Table 2).

Seifert et al. (2007) clearly demonstrated that the teleomorph name *A. resinae* referred to a different species other than the anamorph *Sorocybe resinae* (resin fungus), and the names were split. *A. resinae* belongs to the family Myxotrichaceae, whereas *S. resinae* is related to *Capronia* (Chaetothyriales, Herpotrichiellaceae). The actual classification available for *A. resinae* is Fungi, Ascomycota, Saccharomyceta, Pezizomycotina, Leotiomyceta, Sordariomyceta, Leotiomycetes, Leotiomycetes *incertae sedis*, Myxotrichaceae, and *Amorphotheca resinae*. These authors emphasize that the use of the name *Cladosporium avellaneum* G.A. de Vries has never been in doubt, and it would be possible to retain this species as the type of *Hormoconis*.

3 Occurrence and Case History

The information on almost all the strains of *H. resinae* (Lindau) von Arx et de Vries anamorph (teleomorph *A. re*sinae Parbery) is deposited in collections with information on their isolation source (Table 3), independent of the misidentification

Table 2 Nomenclature and synonyms for the creosote and resin fungi showing the use of the same basionym for the two fungi

Creosote fungus
Teleomorph: *Amorphotheca resinae* Parbery, Australian J. Bot. 17: 340. 1969a
Anamorph *Hormodendrum resinae* Lindau, in Rabenh. Krypt.-Fl., 2, 1 (Pilze) 8: 699. 1906 ≡ *Cladosporium resinae* (Lindau) G.A. de Vries, Antonie van Leeuwenhoek 21: 167. 1955 ≡ *Hormoconis resinae* (Lindau) von Arx & G.A. de Vries, in von Arx, Verh. K. Ned. Akad. Wet., Afd. Natuurk. 61: 62. 1973 = *Cladosporium avellaneum* G.A. de Vries, contribution to the knowledge of the genus *Cladosporium*, Uitg. Druk. Hollandia, p. 56, 1952
Resin fungus
Mononematous synanamorph: *Hormodendrum resinae* Lindau, in Rabenh. Krypt.-Fl., 2, 1 (Pilze) 8: 699. 1906 ≡ *Cladosporium resinae* (Lindau) G.A. de Vries, Antonie van Leeuwenhoek 21: 167. 1955 ≡ *Hormoconis resinae* (Lindau) von Arx & G.A. de Vries, in von Arx, Verh. K. Ned. Akad. Wet., Afd. Natuurk. 61: 62. 1973 Synnematous anamorph: *Sorocybe resinae* (Fr.) Fr., Summa Veg. Scan. 2: 468. 1849 ≡ *Racodium resinae* Fr. ≡ *Sporocybe resinae* (Fr.) Fr. ≡ *Dendryphion resinae* (Fr.) ≡ *Stysanopsis resinae* (Fr.) Ferr.

The "false" names and synonyms for the anamorph of the resin fungus are indicated in blue text. The second nomenclatural solution proposed by Seifert et al. (2007) would have switched the blue text to black for the creosote fungus and would simultaneously switch the equivalent black text to blue for the mononematous synanamorph of the resin fungus (Seifert et al. 2007)

highlighted above. Moreover, we avoided confusion in this paper by using the name *Hormoconis resinae* as anamorph of *Amorphotheca resinae*.

C. resinae (Lindau) de Vries was first isolated (as *Hormodendron resinae* Lindau) from the resin of *Pinus excelsa* near Hamburg in Europe (Lindau 1906). Since then, it has been isolated worldwide, in Europe (Hendey 1964; Nicot and Zakartchenko 1966), Africa (Arx 1973), North or South America (Christensen et al. 1942; Marsden 1954; Bento 2001; Bento and Gaylarde 2001), Antarctica (Kerry 1990), Asia (Goto et al. 1975), and Oceania, particularly in Australia (Parbery 1969b) and New Zealand (Sheridan et al. 1972).

This fungus colonizes natural as well as manufactured substrates (listed in Table 3). Parbery (1968) isolated it from soil in Australia, and, subsequently, it was isolated from other natural environments: the atmosphere (Parbery 1971) as well as fresh, estuarine, and marine waters, especially in oil-polluted water (Ahearn and Meyers 1972). Since it has been found to be associated with the resins of *Picea pungens*, pines and cedars (Christensen et al. 1942), Parbery (1969c, 1970) emphasized that certain organic natural substances could serve as potential growth substrates for *H. resinae* in soils, i.e., vegetable oils, leaf waxes, keratin, and chitin. It is considered as a widely distributed and natural saprotrophic fungus of the soil microflora (Domsch et al. 2007).

Table 3 List of strains of *Hormoconis resinae* (Lindau) von Arx et de Vries anamorph (teleomorph *Amorphotheca resinae* Parbery) deposited in collections with information on their isolation source

Accession number[a]	Deposition form	Source	Geographical location	GenBank[b]	References
ATCC® 200942™ CBS 406.68		Soil	Australia	AF393726	Parbery (1969b) Martin-Sanchez et al. (2016)
ATCC® 90773™		Soil	The Netherlands		
ATCC® 22065™ IFO 31696		Soil	Australia		
MUCL 15069	*Cladosporium avellaneum* f. *viride* de Vries *Cladosporium resinae* (Lindau) de Vries *Cladosporium resinae* f. *albidum* de Vries *Cladosporium resinae* f. *avellaneum* de Vries *Sorocybe resinae* (Fries) Fries	Soil	Canada		Bhatt (1970)
ATCC® 34012™	*Cladosporium resinae* f. *albidum* de Vries	Plant detritus	Japan		Goto et al. (1975)
ATCC® 38834™		Jet fuel tank			May and Neihof (1979)
ATCC® 52833™	*Cladosporium resinae* (Lindau) de Vries	Diesel fuel tank	UK		Lindley and Heydeman (1983, 1985, 1986a, b)
ATCC® 64053™	*Cladosporium resinae* (Lindau) de Vries	Diesel fuel tank	New Zealand		
CBS 176.62	*Cladosporium resinae* f. *avellaneum*	Aircraft fuel tank	Philippines		

(*continued*)

Table 3 (continued)

Accession number[a]	Deposition form	Source	Geographical location	GenBank[b]	References
ATCC® 20495™ CBS 174.61 IMI 344050	*Cladosporium resinae* (Lindau) de Vries	Aircraft fuel tank		X67708 X68143	Fagerström et al. (1990) McCleary and Anderson (1980) Vainio et al. (1993) Joutsjoki and Torkkeli (1992)
ATCC® 34066™	*Cladosporium resinae* (Lindau) de Vries	Aircraft jet fuel			Rubidge (1974, 1975)
CBS 177.62 IMI 089559i [MB#315230]	*Cladosporium resinae* f. *avellaneum*	Aircraft kerosene filter	The Netherlands		
IMI 358574		Fuel	Brazil		Lopes and Gaylarde (1996)
ATCC® 22712™	*Cladosporium resinae* (Lindau) de Vries	Jet fuel			Cooney (1969) Cooney and Kula (1970) Clark and Hufford (1979) Edmonds (1965) Edmonds and Cooney (1967, 1968) Smucker and Cooney (1981) Walker and Cooney (1973)
ATCC® 66289™	*Cladosporium resinae* (Lindau) de Vries	Jet fuel	New Zealand		
ATCC® 22711™	*Cladosporium resinae* f. *avellaneum* de Vries	Jet fuel	Canada	ITS EU030278 LSU EU030280	Carson and Cooney (1988a, b) Cooney (1969) Cooney and Felix (1970) Cooney and Proby (1971) Cooney et al. (1968) Edmonds (1965)

					Edmonds and Cooney (1967, 1968) Goswami and Cooney (1999) Seifert et al. (2007) Siporin and Cooney (1976) Turner et al. (1980) Walker and Cooney (1973)
ATCC® 66684™		CCA[c]-treated southern pine pole in storage	USA		Wang and Zabel (1990)
ATCC® 66685™		CCA-treated *Douglas*-fir pole	USA		Wang and Zabel (1990)
ATCC® 11873™ CBS 183.54 IMI 089838	*Cladosporium resinae* *Cladosporium resinae* f. *avellaneum*	Creosote-treated wooden pole	USA		Marsden (1954)
ATCC® 11841™ CBS 184.54 IMI 089837	*Cladosporium resinae* de Vries f. *resinae*	Creosote-treated wooden pole	USA	AY251067EU040231	Marsden (1954) Crous et al. (2007)
ATCC® 66686™		Penta-treated southern pine pole	USA		Wang and Zabel (1990)
CBS 173.61 IMI 090126	*Cladosporium resinae* f. *albidum* *Cladosporium resinae*	Bitumen-treated cardboard in gold mine	South Africa		Arx (1973)
ATCC® 11272™ CBS 185.54 MUCL 10101	*Cladosporium resinae* f. *albidum*	Cosmetic face cream	The Netherlands		de Vries (1952, 1955)
ATCC® 11274™ CBS 187.54	*Cladosporium avellaneum* f. *viride*	Ointment	The Netherlands		de Vries (1955)

(*continued*)

Table 3 (continued)

Accession number[a]	Deposition form	Source	Geographical location	GenBank[b]	References
MUCL 10104 IMI 49621					
ATCC® 11273™ CBS 186.54 IMI 49620	*Cladosporium avellaneum* de Vries f. *avellaneum*	Ointment	The Netherlands		de Vries (1952, 1955)
ATCC® 26227™	*Cladosporium resinae* f. *avellaneum* de Vries	Sole of foot of man	New Zealand		
CBS 159.58 MUCL 10099	*Cladosporium resinae*	Perfume manufactory	USA		Arx (1973)
CBS 161.59	*Cladosporium resinae* f. *avellaneum*	Kaopectate			

[a]ATCC, American Type Culture Collection, VA, USA; CBS, Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; IFO, Institute for Fermentation, Osaka, Japan; IMI, International Mycological Institute, CABI-Bioscience, Egham, Bakeham Lane, UK; MUCL, Mycotheque de l' Université Catholique de Louvain, Louvain-la-Neuve, Belgium

[b]ITS, internal transcribed spacer regions and 5.8S rRNA gene; LSU, partial 28S rRNA gene

[c]CCA, chromated copper arsenate

H. resinae has been found in a wide variety of habitats, most typically fuel storage tanks. In particular, *H. resinae* commonly contaminates fuel-oil storage tanks (Teh and Lee 1973; Lindley and Heydeman 1986a, b; Seifert et al. 2007) and distribution systems such as petrol station storage tanks before filtration, petrol station pumps, and vehicle injection pumps, especially in Brazil where the problems of hydrocarbon fuel storage are particularly serious for diesel fuel (Bento and Gaylarde 2001). In fact, in Brazil, during the 1990s, the "urban (or metropolitan)" diesel introduced onto the market was characterized by a lower permitted sulfur content (0.5% maximum) that enhanced microbial activity (Gaylarde et al. 1999). Moreover, some additives used to improve the stability of this fuel also act as nutrients for microorganisms. Both conditions explain why, in Brazil, diesel was the fuel that was most susceptible to diverse types of microbial contamination problems.

H. resinae has also been frequently isolated in both commercial and military aircraft jet fuel systems (Itah et al. 2009) and diesel fuel used in ships (Hettige and Sheridan 1984). The contamination of fuel oil can occur from different potential sources, such as soil, air, water, and contaminated pipelines or tanks with no obvious prevalent route of contamination. The development of the fungus in fuel tanks is favored by the following parameters: the presence of water (from condensation on tank walls, air moisture, insufficient drainage, or water added during ballast operations in ships), the oxygen content, and the presence of nutrients, in particular, phosphorus, nitrogen, and iron (Gaylarde et al. 1999).

This fungus has also been found in manufactured products such as palings, railway sleepers, and tarred wood (Christensen et al. 1942; Lopez et al. 1990). It has particularly been reported on creosoted *Eucalyptus* poles used for electricity lines in Buenos Aires in Argentina and Santa Fe, New Mexico, and in the USA, after isolation from small portions of damaged poles placed on agar media (Lopez et al. 1990; Ribichich and Lopez 1996). It was suggested that the capacity of *H. resinae* to degrade and thus detoxify creosote allowed other fungi to break down the wood (Ribichich and Lopez 1996). Therefore, this species is also known colloquially as the creosote fungus.

The consequences of mycelial growth in storage tanks and fuel systems include the formation of microbial mats that can block pipes, valves, and filters and adhesion of the mycelia to the metal surface, resulting in microbial-induced corrosion of storage and distribution systems. This is particularly due to the production of organic acids by the fungus (Hendey 1964; Gaylarde et al. 1999), which induces severe biodeterioration, especially in aluminum or steel tanks. In culture, *H. resinae* grows at the interface of diesel fuel/Bushnell-Haas mineral medium forming mycelial mats; in situ these mats would adhere to tank walls (Hettige and Sheridan 1989). Some more anecdotal isolation sources are cosmetic face creams, ointments, perfumes, and Kaopectate (de Vries 1952, 1955).

H. resinae can use a variety of hydrocarbons in the absence of organic nitrogen and can grow on substrates that are recalcitrant to attack by numerous other organisms, which is consistent with the view that it can proliferate in ecological niches where other organisms do not (Christensen et al. 1942; Parbery 1969c; Cofone et al. 1973). Therefore, Parbery (1969c) suggested that *H. resinae* is not a

strong competitor for the nutrients generally available to a wide range of other soil-inhabiting fungi. Furthermore, this author supposed that to compete with other soil organisms, *H. resinae* would need to utilize substrates not generally available to them. Its metabolism strongly suggests that this is quite a specialized fungus.

4 Morphology and Culture Conditions

The fungus *H. resinae* produces lightly pigmented, warty conidiophores, and branched, acropetally developing chains of lightly pigmented ameroconidia lacking conspicuous scars (Seifert et al. 2007; Fig. 1). These images were originally published by Seifert et al. (2007) from the strain of *A. resinae* isolated from jet fuel by P. Edmonds (culture, DAOM 170427 = American Type Culture Collection [ATCC] 22,711, Canada, British Columbia, source unknown, isol. "Mrs. Volkoff," Jul. 1969).

The information on the carbon source requirements of one isolate of *H. resinae* reveals that it grows in vitro on a variety of mono- and disaccharides, and its growth is best supported by xylose, maltose, cellobiose, and mannose, in that order, followed by other monosaccharides including glucose (Sheridan et al. 1972). Concerning enzyme production, Fujii et al. (2010) isolated one *Amorphotheca* strain from dead beech trees possessing polysaccharolytic activities, especially amylolytic, pectinolytic, and mannanolytic, but not cellulolytic activity, implying that this fungus could play an important role in dead-tree decomposition after de-lignification by basidiomycetes. Two extracellular glucoamylases, glucoamylase P and S, were purified to homogeneity from the culture medium of *H. resinae* (ATCC 20495; formerly *C. resinae*) (Fagerström et al. 1990). Their apparent molecular masses (71 and 78 kDa for glucoamylase P and S, respectively) and catalytic properties were in agreement with those previously reported in the literature (McCleary and Anderson 1980). This isolate also produces alpha-D-glucosidase and alpha-amylase.

5 Ecophysiology

H. resinae is a dematiaceous (phaeoid) fungus and belongs to a heterogeneous collection of darkly pigmented fungi. This pigmentation is due to the presence of melanin, a pigment of high-molecular weight composed of various types of phenolic or indolic monomers usually associated with protein and often carbohydrates. San-Blas et al. (1996) described in detail the cell wall composition of a strain of *H. resinae* isolated from Venezuelan oil reservoirs. This pigment is highly advantageous for fungi in withstanding various environmental extremes and confers them with a competitive advantage in some environments, particularly in extreme conditions such as temperature, pH, and pressure (Hendey 1964). For instance, the optimal growth temperature of *H. resinae* was reported to be approximately 30 °C with a range of 5–40 °C and a pH range of 2–10 with the optimum toward the acidic end of the range. Hendey (1964) also indicated that its spores are resistant to a wide range

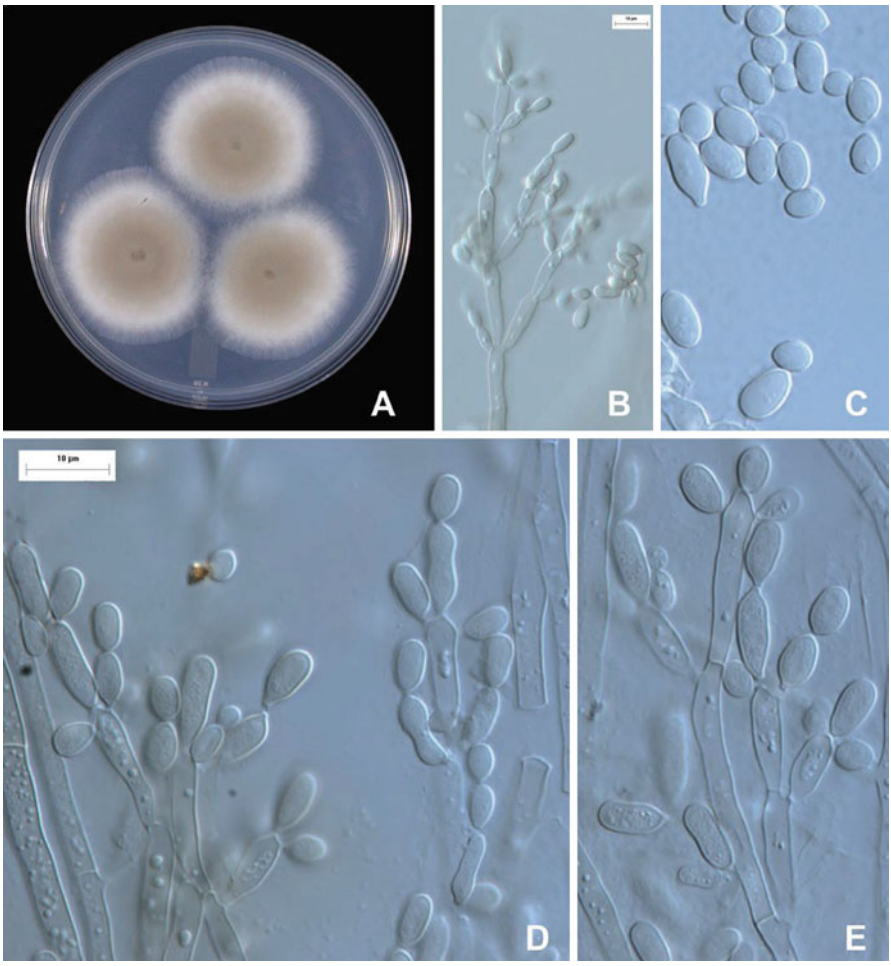

Fig. 1 *Hormoconis resinae* anamorph of *Amorphotheca resinae*, colony characteristics, and anamorph micromorphology. (**a**) A 10-day-old colony on potato dextrose agar. (**b**, **d**, and **e**) Micromorphology of conidiophores showing acropetal conidial chains, ramoconidia, and conidia. (**c**) Conidia. For **c** and **e**, see scale bar in **d** (originally published in Seifert et al. 2007, published with kind permission of ©Springer Science+Business Media New York, 2017. All rights reserved)

of temperatures. This eurythermal adaptation could confer *H. resinae* with tolerance to a wide range of thermal stresses, permitting its survival in very different ecological niches from high to extremely low temperatures, with the latter perhaps explaining why it is commonly found in fuel used in high-altitude flights (Allsopp and Seal 1986). Likewise, *H. resinae* was isolated from the Chernobyl area after the nuclear reactor accident in 1986 (Dighton et al. 2008) in heavily contaminated sites, together with *Cladosporium sphaerospermum*, *Cladosporium herbarum*, *Alternaria alternata*, and *Aureobasidium pullulans*, which are other melanized fungi (Zhdanova et al. 2004). The predominance of melanized fungi suggests that pigmentation

provides tolerance to radiation (Tugay et al. 2006, 2007). Indeed, a remarkable aspect of melanin is its ability to absorb all types of electromagnetic radiation, which endows it with the capacity for both radioprotection and energy transduction (Dighton et al. 2008). This capacity may enable fungi to be used in the bioremediation of radioactively contaminated sites and cleanup of industrial effluents.

6 *n*-Alkane Uptake and Metabolism

H. resinae is one of the most studied fungi for its ability to grow on *n*-alkanes. As described above, this fungal species has largely been involved in aircraft fuel tank contamination and biodeterioration (Lindley 1995). Therefore, in the 1970s and 1980s, numerous research studies were conducted to understand the mechanism involved in fungal *n*-alkane assimilation. Before their incorporation into the fungal cell, water insoluble *n*-alkanes must be emulsified. Compared with yeasts, which produce surface-active emulsifying agents for *n*-alkane assimilation (Goma et al. 1973), *H. resinae* produces only small quantities of active surfactants such as fatty acids or phospholipids (Cooney and Proby 1971). In fact, the uptake of *n*-alkanes by *H. resinae* first involves passive adsorption of hydrocarbons through the cell surface (Lindley and Heydeman 1986a). The fungus grew better on *n*-alkanes with a chain length of 10–18 carbons (Cofone et al. 1973; Teh and Lee 1973; Lindley and Heydeman 1985) in contrast to *n*-alkanes with 6 or 8 carbons, which supported little or no growth of the fungus and were rather toxic (Walker and Cooney 1973). Furthermore, the growth of *H. resinae* on *n*-alkanes with 12 carbons and above shows a lag phase, the duration of which increases with the length of the alkane chain (Lindley and Heydeman 1985). Finally, in cytological studies conducted on hydrocarbon-grown cells, large membrane-bound vesicles and lysed spheroplasts yield *n*-alkanes (Carson and Cooney 1988a), suggesting that unaltered hydrocarbons accumulated in the fungal cell (Cooney et al. 1980; Smucker and Cooney 1981). After the passive adsorption of *n*-alkanes onto the cell wall, their entrance into the cytoplasm requires energy consumption (Lindley and Heydeman 1983, 1986b), and they could be assimilated via an apparently active transport (Lindley 1995). However, presently, the precise mechanism by which *n*-alkanes are transported into fungal cells remains unknown.

In the presence of glucose and hexadecane, *H. resinae* surprisingly preferentially used the hydrocarbon rather than glucose (Siporin and Cooney 1976). This result indicated the presence of a constitutive *n*-alkane-oxidizing system in *H. resinae* (Walker and Cooney 1973). In fact, the initial attack on *n*-alkanes was generally from the terminal methyl group by a constitutive *n*-alkane monooxygenase. This enzyme is NADH-dependent and belongs to the P450 monooxygenase class (Walker and Cooney 1973; Goswami and Cooney 1999). The subsequent catabolic steps used enzymes specific to long-chain alcohols (fatty alcohol oxidase) and aldehydes (fatty aldehyde dehydrogenase) to yield a fatty acid. Goswami and Cooney (1999) localized fatty aldehyde dehydrogenase in the fungal cytoplasm and fatty alcohol oxidase bound to the mitochondrial membrane. The produced fatty acids were classically activated into an acyl-CoA ester via an acyl-CoA synthetase (Lindley 1995). Cytological studies demonstrated the likely role of microbodies when *H. resinae* was

cultivated on *n*-alkanes as carbon and energy sources (Smucker and Cooney 1981; Carson and Cooney 1988a). In this case, they observed a proliferation of microbodies in the fungal cells that could play a role in lipid degradation via β-oxidation and glyoxylate bypass. However, the proliferation of the microbodies was fungal-strain dependent (Turner et al. 1980), and their presence was not a prerequisite for *n*-alkane oxidation by *H. resinae* (Carson and Cooney 1988b). The processes involved in *n*-alkane uptake and metabolism by *H. resinae* are summarized in Fig. 2.

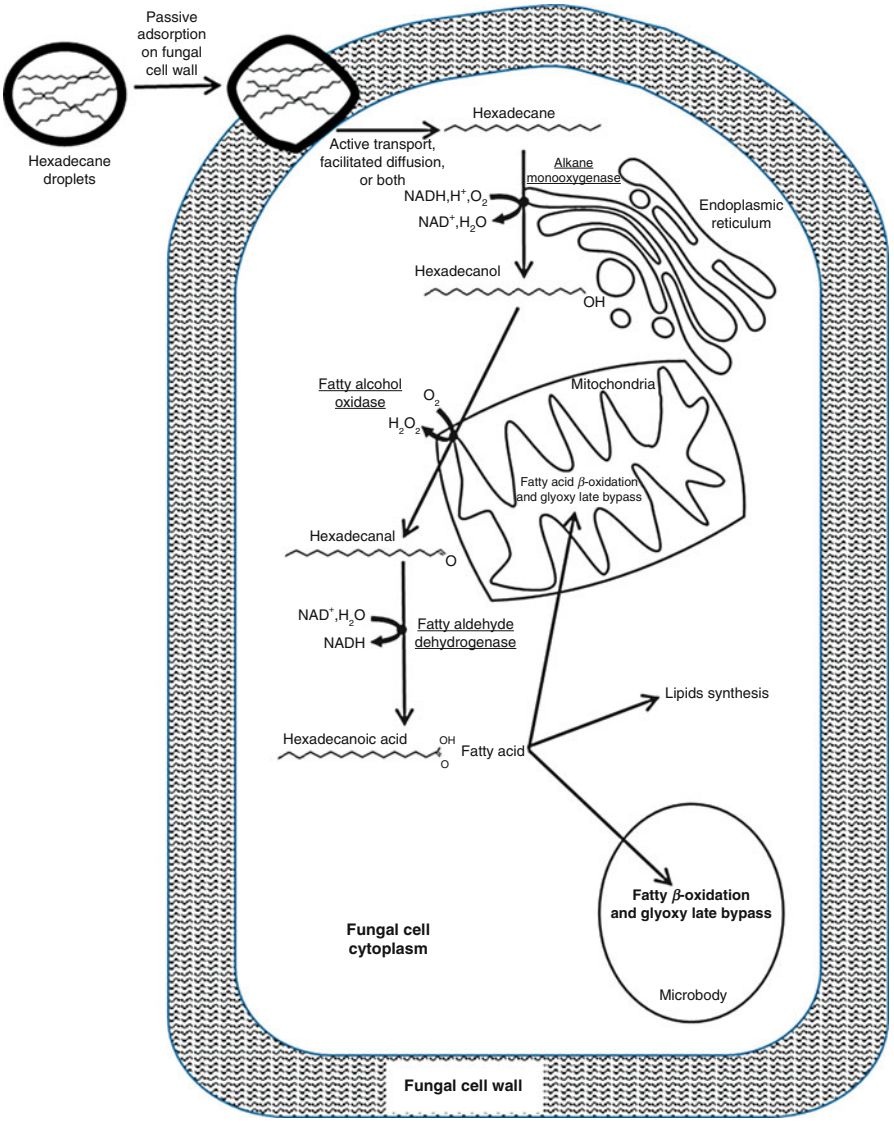

Fig. 2 Oxidative pathway and subcellular location of hexadecanes by *Hormoconis resinae*. (Adapted from Lindley 1995; Goswami and Cooney 1999)

7 Detection in Fuel

Following the discovery of the role of *H. resinae* in the contamination of fuel, intensive research has been conducted for its identification and quantification. Methods based on cultivation and morphological criteria were used at first. However, the economic interest of the petroleum industry and jet-aircraft incidents soon led to the development of new effective methods for *H. resinae* detection in fuel. The dip-slide method, which is the so-called total aerobic counts, was one of the first fast and effective techniques available on the market (Bailey and May 1979). In this technique, small agar-medium-coated slides allow the enumeration of aerobic microorganisms (Gaylarde 1990). Nevertheless, this technique was not specific for *H. resinae*. This drawback was also found for the commercial Boron® Microtube Monitor Test kit. Moreover, both methods required a certain level of expertise and needed several days to obtain a result (Lopes and Gaylarde 1996; Gaylarde et al. 1999). To establish a specific test for *H. resinae*, Lopes and Gaylarde (1996) produced a specific rabbit antiserum against this fungus. This immune serum allowed the development of a very sensitive immunofluorescence test, which could theoretically detect 0.5 fungal propagules/mL. Today, a commercial detection kit, FUELSTAT® DIESEL Test kit, based on a similar immunoassay technique is available and permits the detection and quantification of *H. resinae* in a few minutes. More recently, using a quantitative polymerase chain reaction (Q-PCR) protocol, Martin-Sanchez et al. (2016) developed a very specific method for *H. resinae* detection, which is 100-times more efficient than the immunofluorescence test. They concluded that the incidence of *H. resinae* was probably overestimated in the past because of the misidentification of some fungal strains using the morphological methods. Currently, molecular techniques in combination with culture-dependent approaches allow more reliable identification.

8 Treatments and Prevention of Biodeterioration

Passman (2013) reviewed in detail the control of microbial contamination in fuels and fuel systems since 1980. As the methods proposed were not specific to *H. resinae*, we simply summarized the main ones used for monitoring and treating the development of the microorganisms involved in fuel and fuel system biodeterioration. Passman (2013) rightly emphasized that the best control treatment is prevention, i.e., acting on the primary factors contributing to microbial contamination and its subsequent proliferation in fuel systems, which are climate, engineering (system design), fuel chemistry, product inventory control (throughput rates), housekeeping, and maintenance. The presence of water is required in fuels for fungal growth. Therefore, even if the total removal of water may be impracticable, there is a consensus that frequent water removal reduces biodeterioration risk (Passman 2013). Underground storage tanks (UST), where drainage is quite difficult, could be replaced by aerial tanks as in Brazil (Gaylarde et al. 1999) or inclined horizontally at 30° to facilitate water drainage. Other techniques for eradicating contaminant

microbes are tank cleaning and treatment with biocides. Bento and Gaylarde (1996) found that an isothiazolone mixture and a quaternary ammonium compound were the most effective among four biocides tested on bacteria and fungi isolated from diesel fuels. The same isothiazolone mixture was active against biofilms of *H. resinae* on a metal surface at 50 ppm but was much less effective when sulfate-reducing bacteria (SRB) were also present (Guiamet and Gaylarde 1996). Fuel additives also affect *H. resinae*. Indeed, to prevent microbial degradation of jet fuel, the biocidal anti-icing inhibitor diethylene glycol monomethyl ether (DiEGME) is routinely added to the fuel. Nevertheless, Rabaev et al. (2009) demonstrated that the maximal degradation of DiEGME was obtained with the specific jet fuel utilizing microbial strains, *H. resinae* and *Pseudomonas aeruginosa*, in a carbon-free mineral medium. On the contrary, other research studies suggest that certain new additives do not affect fungal growth (Bento and Gaylarde 1998).

9 Research Needs

Fundamentally, even if *n*-alkanes penetrate into the fungal cell by an apparently active transport process (Lindley and Heydeman 1983, 1986a, b; Lindley 1995), the precise underlying molecular mechanisms involved in their transport through the fungal cell wall and membrane remain unknown. Mutational studies of specific proteins involved in the incorporation of *n*-alkanes or the structure/function studies of particular enzymes could be appropriate tools to elucidate this particularly elusive mechanism.

In vivo cytological studies could also be performed using specific fluorescent markers to precisely identify the intracellular organelles involved in *n*-alkane transport and storage in fungal cells. Smucker and Cooney (1981) and Carson and Cooney (1988a, b) observed the proliferation of microbodies in the fungal cells that could play a role in lipid degradation via β-oxidation and glyoxylate bypass, but the precise nature of these organelles currently remains unknown.

Mishra et al. (2010) reported the extracellular synthesis of stable gold nanoparticles by this fungus through the extracellular bioreduction of $Au^{(3+)}$ to $Au^{(0)}$ by its biomass. The ubiquity of this fungus in the soil suggests that this property could have a biotechnological application in the remediation of soils contaminated with heavy metals. Such a cheap source of material would enable the cost-effective preparation of various gold-based nanostructures using the fungus as possible eco-friendly nano-factories.

H. resinae also has novel applications in the critical field of biorefinery processing. Indeed, for cellulosic ethanol production, pretreatment of lignocellulose is the key step to obtaining fermentable sugar in enzymatic hydrolysis. During this pretreatment, the over-degradation of partial cellulose, hemicellulose, and lignin generates various compounds such as furan derivatives, especially furfural and the 5-hydroxymethylfurfural (HMF), organic acids, and phenolic compounds, which severely inhibit the subsequent enzymatic hydrolysis and ethanol fermentation. Therefore, the removal of these inhibitory compounds or “detoxification” is a

prerequisite step and remains one of the most expensive ones. The kerosene fungus strain, *A. resinae* ZN1, was isolated from the microbial community growing on the pretreated corn stover material (Zhang et al. 2010). This strain has demonstrated an extraordinary capacity for fast and complete degradation of furan derivatives into corresponding alcohol and acid forms (Zhang et al. 2010; Wang et al. 2015), with numerous advantages, such as no energy input, no wastewater generation, no sterilization requirement, and a wide lignocellulose feedstock spectrum. These advantages make it possible for industrial applications with fast and efficient biodetoxification. Moreover, the elucidation of its molecular degradation metabolism will facilitate the detoxification of the pretreated lignocellulose biomass and provide the metabolic pathway information for more powerful biodetoxification strain development. To identify the degradation mechanism of *A. resinae* ZN1, Wang et al. (2015) investigated the transcription levels of 137 putative genes involved in the degradation of furfural and HMF in *A. resinae* ZN1 using a quantitative real-time PCR method under the stress of furfural, HMF, and their secondary metabolites, furfuryl alcohol and HMF alcohol, respectively. The genes responsible for the furfural and HMF degradation to the corresponding alcohols and acids in *A. resinae* ZN1 were clearly identified based on the analysis of the genome annotation, the gene transcription data, and the inhibitor conversion results. The *A. resinae* ZN1 genome is approximately 54.47 Mb (https://www.ncbi.nlm.nih.gov/genome/?term=txid5101[Organism:exp). These genetic resources provided important information for understanding the mechanism of furfural and HMF degradation and the subsequent modification of highly tolerant strains used for biorefinery processing. The genome sequencing of *A. resinae* DAOM 194228 is also in progress, funded by Genome Canada.

Recent progress in omics-based techniques could improve our understanding of the following aspects of *H. resinae*. (i) The real occurrence of *H. resinae* in various natural and industrial environments could be determined using metagenomics. (ii) Its metabolism could be further elucidated using methods such as transcriptomics and proteomics to characterize properties such as the enzymes involved in plant cell wall decomposition either in natural habitats or in fuel-polluted products. (iii) The precise *n*-alkane catabolic pathway of this organism could be deciphered using a combination of approaches, such as metabolomics.

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Global Consequences of Ubiquitous Hydrocarbon Utilizers

17

Roger C. Prince

Contents

Abstract

Hydrocarbon-degrading organisms are found throughout the biosphere – from the poles to the tropics, from the depths of the sea to the upper atmosphere, and from the deepest mines to mountaintops. Some are opportunistic – able to degrade hydrocarbons but also other substrates – while others appear to have specialized on hydrocarbons to the exclusion of other foods. They impinge on human activities in many ways – they remove seeped and spilled hydrocarbons from

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water and soil and also from wastewater and commercially contaminated air. But they also consume hydrocarbons in reservoirs, fuel, and lubricant tanks with important deleterious effects, and they are implicated in the "souring" of hydrocarbon reservoirs and in the corrosion of oil pipelines. Our challenge is to encourage the former and discourage the latter – both offering opportunities to improve on current practice as we understand the responsible organisms more thoroughly.

1 Introduction

From the simplistic Darwinian perspective of natural selection, it is no surprise that hydrocarbon utilizers are ubiquitous. Hydrocarbons provide a rich source of carbon and energy – indeed the best – for those organisms able to consume them, and there is an essentially continuous and substantial influx of such molecules into the environment from natural seeps, biosynthesis, partial combustion, and anthropogenic sources. There is thus an enormous selection pressure maintaining such organisms, and they are everywhere. Oil-degrading microorganisms have been isolated from the Arctic (Crisafi et al. 2016), the Antarctic (Vázquez et al. 2013), and essentially everywhere in between. They have been found in coal and oil shale mines (Penner et al. 2010; Permanyer et al. 2010), in deep oil reservoirs (Kato et al. 2001), and in alpine soils (Margesin et al. 2003). Hydrocarbon degraders have also been isolated from the depths of the ocean (Bazylinski et al. 1989; Wang et al. 2008) and from arid deserts (Abed et al. 2015). There is also convincing evidence of viable bacteria in the upper atmosphere (Smith 2013), and while it seems that the original *Bacillus stratosphericus* has been lost (Liu et al. 2015), an isolate, albeit from a harbor, has the ability to grow on fluoranthene and other hydrocarbons (Hentati et al. 2016).

But if hydrocarbon-degrading microbes are ubiquitous, how distinct are different populations? Such a question is relatively easy to address when dealing with large eukaryotes, although even here there are surprises, such as the recent recognition of two distinct species of African elephant (*Loxodonta*; Roca et al. 2001). Microbial ecosystems are far harder to assess; there is the fundamental difficulty of defining exactly what constitutes a genus (see ▶ Chap. 1, "Prokaryotic Hydrocarbon Degraders," Prince RC, Amande TJ, and McGenity TJ, this volume); the enormous microbial populations in most environments, of the order of a million cells per ml in the ocean (Amaral-Zettler et al. 2010), and of ten billion per gram of soil (Raynaud and Nunan 2014); our ignorance of the life-span of relatively inactive microorganisms (Roszak and Colwell 1987; Blagodatskaya and Kuzyakov 2013); and the kinetics of mixing in the ocean and atmosphere (Waterhouse et al. 2014; Holtslag et al. 2013). Nevertheless it seems safe to say that oil-degrading microbes are ubiquitous, and at least some in every environment are so well adapted to their conditions that they will respond and "bloom" if there is a sudden influx of hydrocarbons into that environment.

This chapter will consider the implications of the activities of this global microbiota and how their activities impact our lives (Fig. 1). At the outset we must recognize that hydrocarbons are unusual substrates, especially when our attention

Fig. 1 Oil-degrading microbes have both beneficial and deleterious effects on our lives

is focused on crude oils and fuels – they are insoluble, and degradation must take place at an oil-water interface. If the goal of human intervention is to maximize the rate of biodegradation of spilled oil, the most beneficial approach will be to maximize this interface, perhaps by using dispersants if the spill is at sea (Prince 2015) or by tilling soil (Raymond et al. 1976). If it is to minimize the biodegradation of stored fuel in tanks, then the simplest approach is to minimize, and preferably eliminate, the presence of any underlying water layer in such tanks. Oils and fuels are also unusual in providing substantial amounts of carbon and energy but none of the other biologically essential elements, notably biologically available nitrogen and phosphorus, and it has been known for more than a century that oil degradation depletes the natural levels of these elements (Gainey 1917). Atlas and Bartha (1972) and Raymond et al. (1976) were probably the first to show that adding these elements had a noticeable stimulatory effect on oil biodegradation when there was a lot of oil in the local system. As we will see, this provided the basis for successful bioremediation of oiled shorelines and contaminated soil.

2 Hydrocarbon Microbiology That Is Beneficial to Humans

The principal boon of hydrocarbon microbiology is the fact that microbes essentially completely consume hydrocarbons, whether from natural seeps, accidental spills, or industrial processes. Initial metabolism by organisms that degrade hydrocarbons can

release up to 70% of the carbon as CO_2, with the majority of the rest becoming microbial biomass (Bouchez et al. 1996; Solano-Serena et al. 1999). And some co-metabolism, such as that by methanotrophs with their promiscuous methane monooxygenases, may be responsible for removing trace levels of contaminants even when they are so dilute that no organism can actively grow on them (Hazen 2010).

2.1 Cleansing Oceans

The role of hydrocarbon-degrading microbes in maintaining our environment relatively oil-free should not be underestimated – the "best estimate" that natural oil seeps contribute 600,000 tonnes of oil a year to the world's oceans (National Research Council 2003) indicates that about a cm of oil would accumulate on the ocean's entire surface every 500 years in the absence of biodegradation – obviously it does not. In fact the vast majority of the hydrocarbons in seep oil are degraded quite promptly, although some large cyclic hydrocarbons and the pigmented asphaltenes and resins may persist for a long time. Perhaps the best-studied seeps are the Coal Oil Point seeps in the Santa Barbara Channel, CA (Hornafius et al. 1999; Quigley et al. 1999; Farwell et al. 2009), that release some 80 tonnes of hydrocarbon gases (mainly methane and ethane) and 20 tonnes of heavy oil a day. Only 1% of the methane seems to be emitted to the atmosphere; the rest is consumed by methanotrophs in the water column (Mau et al. 2007). The oil is a typical immature oil from Southern California, with an API gravity near 10 – it barely floats but does rise to the surface where it evaporates and undergoes biodegradation. There is a substantial fallout plume of biodegraded oil over 90 km^2 (Farwell et al. 2009), and tarry residues linger on local shorelines, but adverse environmental effects seem restricted to limited seabird mortality (Henkel et al. 2014).

Of course the uncertainties around the size of seepage input to the world's oceans are large, between 200,000 and 2,000,000 tonnes per year (National Research Council 2003), but to put the amount into perspective, the enormous spill from the tragic *Deepwater Horizon* blowout has a "best estimate" of 466,000 metric tonnes, albeit from a single source (Barbier 2015). Fortunately such enormous spills are rare – the only other comparable accident, the 1979 blowout of the *Ixtoc I* well in the Bay of Campeche, was about the same size (Jernelöv and Lindén 1981), as was the criminal release of oil into the Persian Gulf by Iraqi forces during their eviction from Kuwait in the 1991 Iraq War (Readman et al. 1996).

Most spills are much smaller (National Research Council 2003), and many "disappear" from the environment without human intervention. Oil spill response plans typically expect skimming to remove the bulk of any spilled oil (Fingas 2012), and this can be achieved if the volumes are relatively small, and equipment is near to hand, such as in harbors. But spills at sea spread rapidly, and the logistics of getting booms and skimmers to a spill site often frustrate efforts to corral and remove the oil. Despite the involvement of more than 600 skimmers, less than 10% of the oil from the tragic *Deepwater Horizon* accident was recovered (McNutt et al. 2012). Responders then turn to dispersants to encourage the dispersion of floating

oil into the water. Dispersants are complex and quite precise mixtures of surfactants (Prince 2015) formulated to incorporate into an oil slick to minimize the turbulence required to break the slick into tiny droplets. Severe weather will often disperse crude oils without the need for dispersant, as happened with the 1993 *Braer* spill off Orkney (Harris 1995), but dispersants aid this quite natural phenomenon in calmer seas. Once oil is dispersed into tiny droplets, it tends to dilute to the sub-ppm level in hours, and microbial degradation proceeds such that oil's apparent half-life is of the order of a week or two (Prince et al. 2017b). At such dilutions, the naturally low levels of biologically available nitrogen, phosphorus, iron, etc. are sufficient to provide the oil-degrading microbes with these requirements.

There is exaggerated concern that dispersants are significantly toxic chemicals that will add additional insult to an environment already assaulted by an oil spill. In fact dispersants are typically composed of components that are "Generally Regarded as Safe" (Burdock and Carabin 2004) and have acute toxicity equivalent to standard household dishwashing liquids (Word et al. 2014). They are dispensed at a nominal rate of 5 gallons of dispersant per acre, 47 L/hectare (see Prince 2015). Dispersed crude oil IS significantly toxic, whether dispersed with or without the aid of dispersants, but it dilutes to levels below acute concern within hours. It is important to note that choosing not to use dispersants will likely have serious alternative adverse effects – if dispersant strategies are not implemented soon after the release of oil to the environment, oil will be subject to photochemical oxidation and polymerization, both of which appear to retard biodegradation (Garrett et al. 1998; Aeppli et al. 2012), and oil will likely strand on shorelines.

2.2 Cleansing Shorelines

Oil that arrives on a sandy shoreline may be amenable to mechanical collection (Fingas 2012), and oil that gets into porous sandy beaches may undergo almost as rapid degradation as in open water (Huettel et al. 2018). But oil that penetrates into a more granular shoreline, such as a gravel beach, may resist cleanup and may exhibit very slow biodegradation. An environmentally responsible approach to speeding natural biodegradation is to add fertilizer nutrients to at least partially ameliorate the nutrient limitation of that biodegradation. This was the successful strategy used in the *Exxon Valdez* response, where dispersants were not used and most of the oil reached the shore (Bragg et al. 1994; Prince et al. 2017a). Of course fertilizer application strategy has to be considered carefully, for oil-degrading microbes are not the only nutrient-limited organisms in such an environment – algal growth would also be stimulated if fertilizer nutrients reached them in any significant amount. The *Exxon Valdez* response used two different fertilizers, an oleophilic liquid – essentially a microemulsion of urea in oleic acid – and an encapsulated inorganic fertilizer, to direct fertilizer to the oil-degrading microbes (Bragg et al. 1994; Prince et al. 2017a), and no increase in algal abundance was detected, nor were any other potential adverse impacts.

2.3 Cleansing Soils

Oil is also spilled on soils – in fact the largest spill in the United States was most likely the Lakeview Gusher in the San Joaquin Valley, which flowed at 18,000 barrels a day for 18 months from March 15, 1910 (SJV Geology 2015). Even larger amounts were deliberately released onto Kuwait's deserts by retreating Iraqi troops in 1991; much was burnt (Husain 1995), but staggering amounts remain (see Balba et al. 1998). Again microbiology eventually removes (or will remove) the vast majority of the hydrocarbons in that oil, leaving the dark asphaltenes and resins that in many respects are essentially indistinguishable from humic and fulvic acids in soils (Stevenson 1994). Stimulating this biodegradation, either in situ or in excavated biopiles, is a major part of the remediation industry (see National Research Council 1993; Firth et al. 2016; O'Brien et al. 2017). Even desert soils are potentially amenable to bioremediation (Balba et al. 1998; Godoy-Faúndez et al. 2008).

2.4 Cleansing Groundwater

Although most hydrocarbons have very minimal solubility, the small aromatics, especially benzene, toluene, ethylbenzene, and the xylenes (BTEX), are soluble enough that they leach out of spilled oils and fuels and contaminate groundwater. Biotreatment of groundwater is widely practiced (see Firth et al. 2016), and one of the earliest bioremediation patents addressed this target (Raymond 1974). McHugh et al. (2013) report that over 12,000 sites with groundwater contamination were treated biologically between 2001 and 2011 in California alone. Such contamination is usually quite shallow, and biodegradation usually begins aerobically. As oxygen is consumed, microbes turn to a succession of other electron acceptors, typically nitrate, then manganese (IV), iron (III), sulfate, and finally carbon dioxide (McMahon and Chapelle 2008), and in many cases this involves a succession of degrading organisms.

2.5 Cleansing Air

Microbes play an important role in atmospheric chemistry, especially in clouds (Delort et al. 2010), but it is not yet clear whether significant hydrocarbon oxidation occurs there. Biogenic isoprene is an important atmospheric component that gives rise to noticeable haze (Lee and Wang 2006), and it would not be surprising if microbes exploited this resource.

Engineered microbiological treatments for contaminated air are beginning to be widely used (Delhoménie and Heitz 2005; Quijano et al. 2017; Luengas et al. 2017). Microbes are encouraged to grow on porous biological material, such as peat or compost, or an inorganic, perhaps ceramic, support (Togna and Singh 1994), and contaminated air is blown through the system. Providing that the microbial supports

are kept adequately moist, air filters can be very effective and make a sensible addition to fume hoods that handle significant volumes of hydrocarbons.

2.6 Cleansing Wastewater

The invention of the activated sludge process in the early years of the twentieth century was surely one of the milestone accomplishments of modern urban civilization (Ardern and Lockett 1914). This highly aerobic process has no problem with low concentrations of most hydrocarbons, although it does not do a particularly good job on low levels of multi-ringed aromatic hydrocarbons (Ozaki et al. 2015). Oil refinery wastewater plants are usually "protected" by dissolved air flotation devices to skim dispersed oil (Radzuan et al. 2016) but are then particularly effective at degrading dissolved hydrocarbons and even naphthenic acids (Misiti et al. 2013). Despite its age, activated sludge technology remains under active improvement (van Loosdrecht and Brdjanovic 2014).

2.7 Bioprocessing

Hope springs eternal, and there are perennial expectations that bioprocessing will enter crude oil refineries for biodesulfurization, biodenitrogenation, and biodemetallation or even to cleave large molecules to more valuable small ones (e.g., Singh et al. 2012). Microbes with promising activities appear to be ubiquitous, but despite much research, commercialization always seems to be on the horizon. A major hurdle is that refiners are notoriously skeptical of having any water in their processes – the first step in any refinery is desalting and dewatering the incoming crude oil. Even if refiners were to be interested, the volumes being processed are such that diluting them with water in a bioreactor is unappealing.

Significant efforts to grow hydrocarbon-biodegrading microbes to provide a source of protein began in the 1960s when oil was cheap (Mateles et al. 1967; Laskin 1977; Bornstein et al. 1982), and interest continues, especially with methane (Pieja et al. 2017; Cantera et al. 2018).

2.8 Microbially Enhanced Oil Recovery

Another perennial hope is that microbial processes might be harnessed to mobilize oil in reservoirs, perhaps by producing surfactants in situ, generating gases to increase reservoir pressure, or to plug "thief zones" where waterflood bypasses reservoir oil pockets. Some approaches add microbes, while others rely on indigenous microbes. The field is replete with promising anecdotal success (Rudyk and Søgaard 2011; Chai et al. 2015; Zhao et al. 2015), but it does not appear to be a commercially important technology yet.

An alternative suggestion for getting energy from reservoirs with residual oil is to encourage methanogenesis in situ by the indigenous anaerobic microbes (Suflita et al. 2004; Jones et al. 2008; Gieg et al. 2008).

3 Hydrocarbon Microbiology That Is Detrimental to Humans

While hydrocarbon biodegradation is a truly beneficial phenomenon in some of the cases described above, it is not always benign. If it occurs in oil reservoirs, it may consume a valuable resource before it can be harnessed, and if it occurs in pipelines, it can cause corrosion. Once a fuel or refined product has been manufactured, it is important to preserve it for use rather than see it consumed by microbial degradation. It is not the loss of fuel that is the principal concern here, but rather the by-products of microbiological growth that cause problems – microbial clogging of fuel lines, filters and injectors, and corrosion of tank materials.

3.1 Consuming or Spoiling Oil Reservoirs

Crude oils truly are "fossil fuels" – the average age of commercially valuable reservoirs is about 100 million years (Tissot and Welte 1984). Thus even if biodegradation is quite slow, there has potentially been plenty of time for biodegradation to occur if it ever started. In fact several of the largest oil reservoirs, such as in the Orinoco Belt of Venezuela and the Canadian oil sands, contain biodegraded crude oils that are viscous and difficult to produce by conventional means. Microbes prefer smaller hydrocarbons, and removing them from a mature crude oil leaves a viscous semisolid. It is not yet clear whether the biodegradation in those resources was aerobic, relying on the slow flow of meteoric water with some oxygen, anaerobic, or both (Larter et al. 2003, 2006; Bennett et al. 2013), but it does seem clear that pasteurization above about 80–90 °C inhibits biodegradation (Wilhelms et al. 2001). Yet many reservoirs at up to 80 °C have a microbiome of both Bacteria and Archaea, even before waterflooding (L'haridon et al. 1995; Gao et al. 2016; Nie et al. 2016). Unfortunately for this chapter, most studies are genomically focused, and there has been little work on the appetites of these microbes toward hydrocarbons. Nevertheless, biodegradation in reservoirs consumes the choicest molecules in a crude oil, decreasing its value and increasing production costs, and accurate prediction of its occurrence in newly discovered reservoirs would be a valuable tool for oil exploration.

An additional deleterious effect in oil reservoirs is "souring." A "sour oil" reservoir may contain either a lot of organic sulfur, dissolved H_2S and mercaptans or both, while "sour gas" is typically a natural gas (principally methane) containing more than 4 ppm H_2S. Hydrogen sulfide is of course acutely toxic, and it is also corrosive (Smith and Joosten 2006). Providing the sour nature of the oil or gas is known during development, appropriately resistant steels and safety measures will be used, but since these are an additional expense, they are not routinely used for

"sweet" reservoirs – those containing much less than 1 ppm H_2S. Unfortunately "sweet" reservoirs sometimes become "sour," posing major problems for production engineers. Both thermochemical and biological processes generate H_2S in reservoirs (Gaspar et al. 2016), but it has been suspected for almost a century that sulfate-reducing microbes are responsible for the majority of souring occurring after production has begun (Ginter 1930). These microbes are taxonomically diverse (Muyzer and Stams 2008), including both Bacteria and Archaea, and they grow in environments where no higher potential oxidant such as nitrate or oxygen is available. Their organic substrate in reservoirs and pipelines remains somewhat controversial, but it is generally thought to be short-chain volatile fatty acids such as acetate and propionate (Eden et al. 1993). Whether these are relics of the initial diagenesis of kerogen into petroleum (Carothers and Kharaka 1978) or are by-products of microbial activity (Magot et al. 2000) is unclear. There is also growing evidence that sulfate-reducing microbes can consume petroleum, particularly alkanes, cycloalkanes, and simple aromatics (Rueter et al. 1994; Caldwell et al. 1998; Townsend et al. 2004), and it remains a strong possibility that hydrocarbon-degrading microbiology is a significant part of the problem.

Souring of sweet reservoirs typically only begins after secondary production has been initiated, and water is being injected to drive residual oil toward production facilities. Seawater is often used as an inexpensive but sulfate-rich injection fluid that likely brings microbes to the reservoir even if it was sterile when discovered (Terry and Rogers 2015). A variety of mitigation options for reservoir and pipeline souring have been proposed, including removing sulfate and phosphate from the injection water (Henthorne and Wodehouse 2012; McElhiney 2008), UV sterilization (Wardell et al. 1986), a variety of biocides (McGinley and van der Kraan 2012), and the injection of nitrate (Hitzman et al. 1995). The latter is widely used and is thought to have several modes of action; nitrate is a much more oxidizing species than sulfate, so it may locally raise the ambient redox potential to prevent sulfate reduction (Jenneman et al. 1986). Certainly its reduction provides far more free energy than the reduction of sulfate, encouraging sulfate-reducing bacteria that can reduce nitrate to prefer this substrate and allowing nitrate-reducing bacteria to outcompete sulfate-reducing bacteria (Callbeck et al. 2013). Some nitrate-reducing bacteria use H_2S as a substrate, so their metabolism actively removes preformed sulfide (Gevertz et al. 2000), and Suri et al. (2017) suggest that this is in fact the major effect. Furthermore, the nitrite intermediate in nitrate reduction is a well-known inhibitor of some of the enzymes of sulfate reduction (Rajeev et al. 2015).

Two classes of oil reservoirs have been treated extensively with nitrate to minimize souring and corrosion. One is relatively high-temperature reservoirs in the North Sea that are injected with seawater, and sometimes with produced water, to maintain pressure within the reservoir to aid production. Perhaps the most studied is the Halfdan reservoir in the Danish North Sea (Gittel et al. 2009). This hot (80 °C) chalk reservoir is operated from platforms on the Dan field, and the deaerated injection water has been treated with 1 mM nitrate from the first injections of seawater in 2001. Injection water facilities are also treated weekly with the bactericide bis[tetrakis(hydroxymethyl)phosphonium] sulfate (THPS). The nearby Dan

reservoir is produced with very similar technology from a very similar reservoir without nitrate addition, providing a potential "control" site (e.g., Gittel et al. 2009, 2012). Presumably the operators were concerned that the injection of vast amounts of seawater (83 billion liters by the end of 2011) would cool the reservoir and allow sulfate-reducing activity. The nitrate addition seems to be successful: produced water from the untreated Dan field has 5 ppm sulfide, while produced water from Halfdan has 0.1 ppm sulfide (Gittel et al. 2009). By the end of 2011, they had used 2.6 tonnes of $Ca(NO_3)_2$ per day for a total of almost 7000 tonnes!

The other group of reservoirs where there are published data on the use of nitrate to inhibit souring is low-temperature reservoirs, especially in Canada. Here the conditions for microbial growth likely exist throughout the reservoir if an electron acceptor was to become available, and sulfate is often present in injected fluids, particularly when they include treated municipal wastewater. One example is the Colville field in Saskatchewan, discovered in 1951, that has been producing with water injection since 1958. The bottom hole temperature is 28 °C, and the oil is a heavy asphaltic crude (API gravity of 13°). Produced water is the dominant injection fluid (Jenneman et al. 1999), and sulfide levels, while erratic, ranged from a few to 200 ppm. Thus biocides and corrosion inhibitors were required to protect the production equipment. The addition of ammonium nitrate (400 ppm) and monobasic sodium phosphate (12 ppm), designed to encourage the growth of sulfide-oxidizing nitrate-reducing microbes, decreased sulfide levels, in some cases to below detection limits, within 2 weeks (Jenneman et al. 1999). Another example is the Medicine Hat Glauconitic C field in Alberta, another shallow (1000 m), low-temperature (30 °C) field producing heavy oil by water injection. The produced water is sulfate-free, but the injection water contains 0.8 mM sulfate, and increasing sulfide concentrations in the gas phase began to be measured in 2006. 2 mM nitrate has thus been added to the injection water since 2007. The nitrate lowered the total aqueous sulfide output of the production wells by 70% in the first 5 weeks, followed by recovery. Better results were obtained by alternating weeks of nominally 14 mM and 2 mM nitrate. In fact the nominally 14 mM nitrate was injected as a very concentrated solution that likely peaked at 760 mM for an hour and was followed by up to 0.6 mM nitrite in the produced water of some wells (Voordouw et al. 2009). Nitrate (5 mM) has also been used to inhibit microbial souring in a water/oil separation tank (15 mM sulfate, 34 °C) on a Brazilian offshore oilfield platform (Jurelevicius et al. 2008).

3.2 Spoiling Oil in Storage and Pipelines

Corrosion in oil pipelines and well equipment can be a very serious problem, and much of it is attributed to microbially induced corrosion, especially by sulfide-producing bacteria as described above (e.g., Liang et al. 2014; Skovhus et al. 2017). A particular aspect of microbial corrosion is that it tends to lead to pitting and pinhole failures, attributed to biofilm phenomena (Skovhus et al. 2017). As discussed above for oil reservoirs, it is not clear how much of this corrosion is due to hydrocarbon degraders per se and how much to their co-conspirators that are

metabolizing small fatty acids and other compounds that may be either relics of ancient diagenesis (Carothers and Kharaka 1978) or more recent intermediates of hydrocarbon degradation (Magot et al. 2000). Biofuels offer a particularly favorable substrate for corrosive organisms (Liang et al. 2018).

Sulfate-reducing organisms are not the only ones causing corrosion in oil storage facilities – El-Sayed et al. (1996) showed that hydrocarbon-degrading bacteria degraded polyurethane tank coatings, and Uchiyama et al. (2010) have described a methanogen that corrodes crude oil storage tanks, although it may be growing exclusively on abiotic hydrogen from the iron. Whatever its origin, microbial corrosion leads to enormous costs – likely many billions of dollars annually worldwide (see Bartling 2016).

3.3 Spoiling Fuel in Storage

Biodegradation during fuel storage and during use in vessels, planes, and vehicles can be a major problem if enough biomass accumulates to clog lines and filters or to cause corrosion. The "poster child" of this phenomenon is the Ascomycete fungus *Hormoconis resinae*, and its anamorph *Amorphotheca resinae*, often known as the petroleum or creosote fungus (see ▶ Chap. 16, "*Hormoconis resinae*, The Kerosene Fungus," Rafin C and Veignie E, this volume), although it is by no means the only problematic organism (Hill and Hill 2008). Clogging of fuel lines and filters has caused substantial economic harm, especially with the addition of biofuels such as fatty acid methyl esters to diesel and ethanol to gasoline (Lee et al. 2010; McFarlane 2011). Microbial growth also enhances corrosion and crack propagation of iron (Sowards et al. 2014) and erosion of polyethylene (Restrepo-Flórez et al. 2015). Minimizing water in the fuel is the major preventative, but this requires constant vigilance as tanks warm and cool, allowing condensation of atmospheric water. Biocide treatments are thus often used as well (McFarlane 2011).

3.4 Spoiling Industrial Lubricants

Biodegradation also plays an unwelcome role in the biodeterioration of cutting fluids (Hill 1977) and is additionally worrisome if the contamination involves pathogens such as mycobacteria (Chang and Adriaens 2007). Again a scrupulous attention to minimizing water and the use of biocides are required (Lee 1995).

4 Research Needs

Research in these areas is proceeding apace, especially in identifying the major microbial contributors to the phenomena discussed here. Less studied areas include the fate of the hydrocarbons themselves, which could be followed with careful isotope analysis, since petroleum compounds are typically so old that they no longer

contain residual natural 14C. This would provide insights into how important fossil hydrocarbons are to each environment, and what fraction of the biomass in that environment comes from fossil fuels.

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Occurrence and Roles of the Obligate Hydrocarbonoclastic Bacteria in the Ocean When There Is No Obvious Hydrocarbon Contamination

18

Tony Gutierrez

Contents

Abstract

The obligate hydrocarbonoclastic bacteria (OHCB) are an intriguing group of microorganisms for their unique ability to utilize hydrocarbons almost exclusively as a sole source of carbon and energy. Based on their narrow nutritional requirement for hydrocarbons as their major food source, these organisms are nonetheless found distributed throughout the global ocean and not confined to regions where there is an obvious source of petrochemical contamination from either anthropogenic (e.g., oil spills) or natural (e.g., oil seep) sources. The OHCB have been found in seawater and sediment samples collected from remote oligotrophic regions, such as Arctic and Antarctic waters, where there is no obvious

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hydrocarbon pollution. Some recently discovered OHCB have not yet been found in oil-contaminated sites. Collectively, this suggests that these organisms would likely be acquiring hydrocarbon or hydrocarbon-like substrates from sources other than the obvious oil spills and oil seeps. This chapter therefore provides a look at the various possible sources from which the OHCB could acquire hydrocarbons that may play an important part to sustaining their existence in remote and "pristine" marine environments.

1 Introduction

Obligate hydrocarbonoclastic bacteria (OHCB) exhibit the distinct ability for utilizing hydrocarbons almost exclusively as a sole source of carbon and energy. Interestingly, their global distribution appears to be solely confined to the marine environment. The importance of OHCB in the removal of hydrocarbon pollutants is evidenced in the wealth of reports documenting their strong enrichment at contaminated marine environments (Head et al. 2006; Yakimov et al. 2007 and references therein), and the recent Deepwater Horizon oil spill is a testament to this. Members that specialize in the degradation of linear or branched saturated hydrocarbons include *Alcanivorax*, *Oleiphilus*, *Oleispira*, *Oleibacter*, *Thalassolitus*, and *Marinobacter*, whereas two genera, *Cycloclasticus* and *Neptunomonas*, comprise species that specialize in the degradation of polycyclic aromatic hydrocarbons (PAHs). In recent years, three other obligate hydrocarbonoclastic taxa (*Algiphilus aromaticivorans*, *Polycyclovorans algicola*, and *Porticoccus hydrocarbonoclasticus*) were discovered from the phycosphere of marine eukaryotic phytoplankton (Gutierrez et al. 2012a, b, 2013a). Interestingly, these organisms are not detected at oil-contaminated sites by cultivation methods or sequencing surveys, and their role in the degradation of hydrocarbons in the ocean remains poorly understood. Furthermore, these and the more common OHCB (*Alcanivorax*, *Oleiphilus*, *Oleispira*, etc.) have been found in shallow, deep, coastal, and offshore waters throughout the world's oceans and seas, albeit at low abundances (<1% of the total bacterial community) and where there is no obvious source of hydrocarbon contamination (Head et al. 2006; Yakimov et al. 2007). While a discussion on the survival of these specialist bacteria in oligotrophic environments would require a look at how they overcome any number of physiological constraints, such as extreme cold tolerance and micronutrient limitation, this is beyond the focus of this chapter. As a rule, the OHCB can use one or two low-molecular-weight organic acids (e.g., acetate, pyruvate), but not able to metabolize sugars and amino acids. Their survival in the ocean could be assumed to be, at least in part, dependent on their ability to access an available source of hydrocarbon or hydrocarbon-like (e.g., fatty acids, fatty alcohols, alkenones) substrates. Hence, this chapter provides an overview of the ways in which these organisms sustain an existence in "pristine" marine environments where there is no obvious source of hydrocarbon contamination.

2 Sustained Existence of the OHCB from Atmospheric Deposition of Hydrocarbons and Their "Grasshopping" on the Sea Surface

The sea surface microlayer represents the boundary layer (typically 40–100 μm) between the atmosphere and underlying water column of the ocean. A number of organic substances accumulate at this layer, including amphiphilic, hydrophilic, and hydrophobic species that exhibit a strong interfacial affinity. The burning of organic matter, from fossil fuels to the burning of fuels like wood, cigarettes, forest fires, and the incomplete combustion of petrol by motor vehicles, is a significant source of environmental hydrocarbons, especially PAHs, which can enter marine environments through atmospheric fallout (Ding et al. 2007; Genualdi et al. 2009). Several studies have focused on the accumulation of organic pollutants, such as hydrocarbons, on the sea surface microlayer, showing it to be a sink for these chemicals in even remote areas (Wurl and Obbard 2004; Stortini et al. 2008) where there is no obvious source of hydrocarbon contamination. A study by Fuoco et al. (2005) showed that the sea surface microlayer can accumulate hydrocarbons, such as PAHs, with an enrichment factor ranging from 3 to 40 for individual organic pollutants compared to subsurface seawater. In remote regions, such as Antarctica, sources of hydrocarbon input to surface waters include melting waters from pack-ice and continental ice and snow which contain hydrocarbons that had originally been captured into the ice from the atmosphere (Fuoco et al. 2005). Wet deposition (rainfall) has also been described as a mechanism contributing to the input of hydrocarbons to the sea surface (Lim et al. 2007). In open ocean areas that are subject to high rainfall, wet deposition may be a significant contributor to providing the OHCB with a source of hydrocarbons.

Hydrocarbons can travel on air currents many hundreds of miles across the ocean from their point of origin. Even after travelling and then settling on the sea surface microlayer, they can re-enter the atmosphere to be carried away again and be deposited many miles elsewhere. This long-range atmospheric transport and deposition of hydrocarbons on the sea surface is a major route for their introduction to the ocean, even in remote regions. As the hydrocarbons tend to move in stages, or hop, from one location to another, this has been referred to as the grasshopper effect (Wania and Mackay 1996) – a process that has been extensively studied for persistent organic pollutants (POPs) (Iwata et al. 1993; Jones and de Voogt 1999; Lohmann et al. 2007; Jurado and Dachs 2008). The process involves the repeated volatilization and deposition of these chemicals, delivering them at multiple locations across two or more seasons. It could contribute significantly to seeding pristine marine environments with these chemicals, therein provisioning resident communities of the OHCB with hydrocarbons which are their preferred carbon and energy source.

Hydrocarbons preferentially accumulate at the sea surface microlayer compared to the underlying water column (Stortini et al. 2008). Upon deposition to the sea surface microlayer, if not subsequently re-entered into the atmosphere by

volatilization, the settled hydrocarbons have a tendency to enter the underlying water column by association with particulate organic matter, such as transparent exopolymer (TEP) or marine snow (Cripps 1990; Stortini et al. 2008). Considering that eukaryotic phytoplankton cells have an affinity to adsorb, like a sponge, hydrocarbons from the surrounding seawater environment (see Sect. 4), periods of low primary productivity would minimize this. Hence, it has been reported that during seasons of low primary productivity, the transportation of hydrocarbons to remote regions, such as the Arctic, is maximized because these chemicals can grasshop more frequently rather than be captured like a glue by "sticky" phytoplankton cells. Lower temperatures have also been shown to positively influence the association of hydrocarbons, in particular PAHs, to particulate matter (Meyers and Quinn 1973), where these chemicals enter the water column more readily in cold remote regions. The settling of particulate-bound hydrocarbons to the seafloor should be considered part of the biological pump, especially in areas of high primary productivity. The whole process, commencing with atmospheric deposition of hydrocarbons to the sea surface microlayer to their association with particulate matter (e.g., phytoplankton cells) and subsequent transportation to the seafloor, provides a route for hydrocarbons to reach OHCB within the whole water column expanse in regions where there is no obvious source of hydrocarbon contamination.

3 Sustained Existence of the OHCB from "Nonfossil Fuel" Hydrocarbons

3.1 Extraterrestrial Sources of Hydrocarbons

Aliphatic and aromatic hydrocarbons have been part of the Earth's composition since its formation approximately 4.5 billion years ago (Dalrymple 2001; Wood et al. 2006) – a time well before the formation of fossil fuels (e.g., crude oil/petroleum, coal, oil shales, tar sands, natural gas). In fact, hydrocarbons are ubiquitous in the universe as they are created by interstellar radiation and are present in cosmic dust (Allamandola et al. 1989; Kwok and Zhang 2011; Tielens 2008). It is hypothesized that the Earth's prebiotic oceans were covered with oil slicks (Hadean – Archaean eon), with photochemical alkylation of polycyclic aromatic hydrocarbons (PAHs) by UV radiation resulting in the formation of primitive pigments (Mahajan et al. 2003). During the famous meteorite cascade of approximately 3.8 billion years ago, carbonaceous chondrites and comets collided with the Earth's crust, bringing with them huge quantities of aromatic and aliphatic hydrocarbons, among other organic species (Gargaud et al. 2010; Martins et al. 2008; Gomes et al. 2005; Kvenvolden et al. 1970). Considering that an estimated 40,000 tons of cosmic dust falls to Earth each year, this brings with it considerable quantities of hydrocarbons that find their way into the seas and oceans (Galliano et al. 2008; Zook 2001). In the absence, therefore, of any anthropogenic and/or natural seepage of oil hydrocarbons, this route of cosmic hydrocarbon deposition may provide the OHCB with a constant source of these substrates and explain their ubiquitous occurrence in pristine oligotrophic

marine environments, such as in Antarctic waters. Whether this cosmic source of hydrocarbons would suffice to support communities of OHCB in pristine regions is a facet of the global hydrocarbon cycle in the ocean that remains unresolved.

3.2 Geological Sources of Hydrocarbons

The spatial distribution of hydrocarbons in the ocean can be influenced by their release from natural geological sources, such as from hydrothermal vents, and other tectonic-active sources, such as volcanoes. Since the first discovery of hydrothermal PAHs at the Guaymas Basin, Gulf of California, in 1978 (Simoneit et al. 1979), several studies have since reported the occurrence of PAHs in hydrothermal vent plumes (Li et al. 2012 and references therein). For example, studies on the sediments of the Andaman Backarc basin in the Indian Ocean showed that hydrothermal surface sediments of this site were found predominated by alkyl naphthalenes and alkyl phenanthrenes (Chernova et al. 1999; Venkatesan et al. 2003), whereas PAHs found in the hydrothermal solid structures, such as sulfide deposits (Simoneit et al. 2004) and chimneys (Simoneit and Fetzer 1996), were higher in high-molecular-weight PAHs (e.g., coronene). Volcanic eruptions are one of the most important natural sources of pollutants (Delmelle et al. 2002; Stracquadanio et al. 2003). In a recent study by Kozak et al. (2017), the authors showed that surface waters of the Arctic are markedly polluted with PAHs, likely from volcanic eruptions at higher latitudes, such as Iceland. The constant flow of PAHs into the ocean from hydrothermal vents and volcanic eruptions underwater or on land should be considered a major natural source of these chemicals to the OHCB.

3.3 Biogenic Sources of Hydrocarbons

Since the advent of photosynthetic reaction centers that date back to their formation approximately 3.4 billion years ago – e.g., the appearance of photosynthetic matts discovered in Australia (Gargaud et al. 2010; Noffke et al. 2013) – these light-harvesting centers could potentially act as a source of carbon and energy to OHCB. One of various types of photosynthetic reaction centers is chlorophyll-*a*, which is an aromatic porphyrin ring containing a magnesium atom at its center and a phytol (diterpene hydrocarbon) side chain. These biogenic chemicals are in fact precursors in the formation of fossil fuels, like crude oil, and have been used as biological markers for determining the source organisms that formed the oil (Petrov 1987). The first organisms to evolve this light-harvesting antenna were cyanobacteria, and over the course of evolution many other types of phytoplankton (diatoms, dinoflagellates, coccolithophores) evolved chlorophyll-*a* and other types of photosynthetic pigments. The presence of chlorophyll in the sea is significantly correlated with hydrocarbon content, and the cellular membranes of microorganisms and higher organisms, as well as the membranes surrounding the internal organelles of eukaryotic cells, all contain a diversity of *n*-alkane chains, such as those comprising

phospholipids and hopanoids which have aliphatic tails or side chains (Schoell et al. 1992; Sohlenkamp and Geiger 2016). Chlorophyll a, which is the central pigment in photosynthesis, can make up anywhere between 0.3% and 5% dry weight of a microalgal cell (Meeks 1974; Kirk 1983). During the death phase of phytoplankton blooms in the euphotic zone, microalgal cells lyse and their cellular contents enter the microbial loop in the water column which re-mineralizes their organic matter. The extracellular release of aliphatic/aromatic hydrocarbon-rich photosynthetic pigments and cell membrane phospholipids would, therefore, potentially provide a source of carbon and energy to OHCB, possibly re-vitalizing these organisms after a period of extensive hydrocarbon depletion between phytoplankton blooms.

The isoprenoids phytane and pristane, which are found in crude oil and coal extracts (Brooks et al. 1969), also have an origin with chlorophyll and can serve as a source of carbon and energy for the OHCB. Both of these compounds are ubiquitous in ancient sediments and are formed via the transformation, largely through prokaryotic biodegradation, of phytol – the esterifying alcohol of cyanobacterial and green-plant chlorophylls (e.g., Chl a) (Didyk et al. 1978). Tocopherols of plants and phytoplankton are also sources of pristane (Goossens et al. 1984). In sediments, particularly those in extreme environments, the compound archaeol, which is a core lipid in Archaea, has been reported as an important source of phytane (Brassell et al. 1981; Rowland 1990). Other biogenic sources of pristane and phytane in the marine environment have been reported. For example, pristane was found in high concentrations (1–3% of body lipid) in several species of calanus (*Calanus finmarchicus*, *C. glacialis*, and *C. hyperboreus*), and at lower concentrations in a wide range of other planktonic animals, with phytol suggested as its precursor in herbivorous zooplankton (Blumer et al. 1963; Blumer et al. 1964). Prokaryotes play a key role in the formation of pristane and phytane in the marine environment (Rontani and Bonin 2011) whereby these compounds could serve to support communities of the OHCB.

Another important nonfossil fuel hydrocarbon in the marine environment is isoprene, which is the second most abundant natural hydrocarbon (after methane) that is emitted into the atmosphere. It is a volatile low-molecular-weight hydrocarbon ($CH_2{=}C(CH_3)CH{=}CH_2$) that is common in terrestrial plants, marine algae, and even human breath (Hryniuk and Ross 2009; Shaw et al. 2010). In an estuarine environment, isoprene production was found to be lower at night and at low tide, and its role has been suggested to maintain an organism's cell membrane structure in times of stress (Exton et al. 2012). Acuña Alvarez et al. (2009) observed that microbial degradation of isoprene is significant, and McGenity et al. (2012) posited that its production by algae could sustain populations of hydrocarbon-degrading bacteria during periods when there are no obvious anthropogenic sources of crude oil or petrochemical contamination. Microalgae are known to synthesize isoprene (Fall and Copley 2000; Kuzma et al. 1995) and include species belonging to the genera *Thalassiosira*, *Skeletonema*, *Chaetoceros*, and *Emiliania* (Shaw et al. 2010; Exton et al. 2012). It has also been shown that some bacteria, such as OHCB, are able to utilize the isoprenoid hydrocarbon side chains of photosynthetic pigments (Rontani et al. 1999; Zsolnay 1973). In fact, petroleum hydrocarbons contain alkanes with a range of isoprenoid side chains (up to C_{45} isoprenoids) that are thought to be

remnants of biogenic molecules (e.g., plastoquinone isoprenoid side chains, phytol/chlorophyll, vitamin A, or vitamin E side chains) (Petrov 1987).

The carcasses of whales and other marine mammals and fish that are rich in oils (e.g., C_{12}–C_{26} lipids) can also provide a biogenic source of alkanes (Petrov 1987) to populations of the OHCB in the absence of petrogenic carbon inputs. Sunken whale carcasses offer a unique opportunity to select for specialist microorganisms, such as the OHCB, in geographically remote locations where nutrients are in poor availability (Rouse et al. 2004). Several strains of *Neptunomonas japonica*, for example, were isolated from sediment adjacent to a sperm whale carcass off the coast of Kagoshima, Japan (Miyazaki et al. 2008). These bacteria could play an important role in the decomposition of oil-rich animals in the sea, as well as provide an opportunity for discovering novel lipid-degrading enzymes for commercial use, such as for low-temperature detergents.

Chemical characterization of two open-ocean sites found that CDOM contains a suite of chlorinated aromatic compounds, such as polychlorinated biphenyl carboxylic acids, and based on the global inventory and isomer distribution of this class of compounds, these chemicals were proposed to be produced by in situ biological activity, likely of microbial origin (Repeta et al. 2004). This biogenic source of chlorinated hydrocarbons could also serve as a source of carbon and energy to the OHCB, such as *Alcanivorax* (Li and Shao 2014).

4 Sustained Existence of the OHCB by Nonhydrocarbon Carbon Sources

Seawater constitutes acomplex mixture of dissolved and particulate organic matter – DOM and POM, respectively. With respect to total dissolved organic carbon (DOC) content, the world's oceans contain an amount that is comparable in mass to the carbon in atmospheric CO_2 (Hansell and Carlson 1998). While the bulk of DOC pool in the ocean remains largely uncharacterized, there are three major chemical compound classes that have been identified: carbohydrates (mono- and polysaccharides or EPS), proteins, and lipids. Both DOC and POC serve as a source of carbon and nutrients to heterotrophic microorganisms, including to possibly also the OHCB whereby these organisms could contribute to the degradation of this material and its overall turnover in the ocean through the stages of a labile to semi-labile and finally recalcitrant form. Extracellular vesicles of bacteria are portions of their cell membranes that have budded off. These are lipid rich, found commonly within biofilms (Mashburn and Whiteley 2005), and specifically within EPS (Biller et al. 2014), and may serve as a viable source of carbon and energy to the OHCB in surficial sediment and solid structures where biofilms are commonly found.

Aside from the many salts and dissolved inorganic and organic carbon (DIC, DOC) that constitute seawater, the ocean also contains enormous quantities of transparent exopolymer (TEP), particulate organic matter (POM), and an abundance of planktonic eukaryotic organisms (e.g., microalgae), all of which could potentially act as a surface for the OHCB to inhabit and to acquire sustenance in the form of

carbon and energy. Much of the DOC in the ocean water column exists as EPS biopolymers (ca. 10–25% of total oceanic dissolved organic matter) that undergo reversible transition between colloidal and dissolved phases (Verdugo 1994; Chin et al. 1998). Much of thisEPS is produced by bacteria and is of glycoprotein composition (Verdugo et al. 2004; Long and Azam 1996). The amino acid and peptide components of these glycoprotein biopolymers have been reported to be amphiphilic (Gutierrez et al. 2009a, b; Verdugo et al. 2004), allowing them to interact with hydrocarbons and influencing their biodegradation (Gutierrez et al. 2013b). In remote areas where there is no obvious source ofpetrochemical contamination, EPS may play an important role in sequestering background concentrations of hydrocarbons and provisioning these compounds to the OHCB in a more concentrated form. During phage-mediated lysis of bacterial and eukaryotic phytoplankton cells, cellular components, including lipids and metabolites of central metabolism, are released into the water column, any of which could serve as substrates to support communities of the OHCB. Members of the OHCB, such as *Alcanivorax* and *Marinobacter*, have been reported living associated with eukaryotic phytoplankton (Amin et al. 2009; Green et al. 2004, 2006) and would be expected to contribute to the recycling of highly labile cell components in the photic zone as part of the microbial loop. Of the various genera that comprise the OHCB (Sect. 1), members of the *Marinobacter* have a high degree of variation in hydrocarbon use and would thus be expected to contribute more to the recycling of algal components as part of microbial loop.

It is well established that marine snow and other forms of macroscopic particulate matter harbor communities of bacteria that are distinct from those that are free-living in their surrounding seawater environment (DeLong et al. 1993). Recent work investigating marine oil snow (MOS) formation has shown EPS, such as bacterial glycoproteins, to play a key role in MOS formation and in sequestering oil hydrocarbons (Arnosti et al. 2016; Gutierrez et al. 2013b; Passow et al. 2012), where the OHCB and other oil-degrading bacteria have been found enriched (Bælum et al. 2012; Arnosti et al. 2016; Duran Suja et al. 2017).

Plant matter contains lignin, humic and fulvic substances, waxes, terpenes, and squalene (Becker 1997), which are rich in aromatics and hydrocarbon side chains. Many types of PAH-containing compounds that are natural occurring in soil are plant-derived (Singer et al. 2003). These compounds can enter the sea through river effluents and from surface runoff. This terrestrial-derived organic material largely contributes to the pool of chromophoric dissolved organic matter (CDOM) in the ocean (Blough and Del Vecchio 2002; Del Vecchio and Blough 2004), which could serve as a semi-labile carbon source for the OHCB.

5 The Curious Case of Hydrocarbon Degraders Living with Phytoplankton: Friends with Benefits?

The ecology of hydrocarbon-degrading bacteria and their interactions with, and close dependence on, other microorganisms and higher organisms is an area that is in a relatively nascent stage of understanding. The cell surface of eukaryotic

phytoplankton (i.e., the "phycosphere") is recognized as a unique niche where bacteria inhabit and acquire nutrients, carbon and energy, including hydrocarbon and hydrocarbon-like substrates. Communities of bacteria associated with phytoplankton are often different from the free-living communities (Crump et al. 1999), suggesting an association that is inextricably linked by some form of symbiosis. In general, the success of an algal-bacterial relationship has much to do with access, by the bacteria, to an available source of carbon and energy in the form of algal exudates (Bell and Mitchell 1972; Myklestad 1995) and reciprocated benefits to the phytoplankton through bacterial-mediated trace metal/nutrient bioavailability (McGenity et al. 2012).

A number of studies have reported the isolation of oil-degrading bacteria, including the OHCB, from laboratory cultures of phytoplankton that have been maintained in continuous cultivation for decades, or identified through sequencing surveys in freshly collected field samples of phytoplankton where there was no obvious source ofpetrochemical input (see below). The occurrence of the OHCB with phytoplankton may be more frequent and important in the global ocean than what may be perceived from the limited studies that have reported their association. Studies that explore the presence and dynamics of the OHCB in natural seawater samples do not often fractionate the various microbial populations (e.g., the bacteria from the microalgae) that constitute the water sample. As such, any identified isolate or Operational Taxonomic Unit (OTU) of the OHCB, from any given water sample, should not be assumed to have existed in either a free-living or associated state. Quite often, these organisms are thought to have existed in the free-living state within the water from which they were isolated or identified. Since the early 1900s, it had been recognized that the attachment of microbes to surfaces offers them a greater degree of environmental stability compared to the free-living (non-attached) state (ZoBell and Allen 1935). It is now widely accepted that many bacteria and other microorganisms occur in association, commonly in a biofilm state (Decho and Gutierrez 2017). Hence, many of the OHCB identified in the plethora of reports in the literature that describe their isolation, molecular identification, and/or dynamics may likely have been associated with phytoplankton. Underlying this association brings to light important questions surrounding the evolution of this symbiosis and role of these organisms in the global hydrocarbon cycle. Several studies provide evidence for the omnipresence of OHCB with phytoplankton, constituting members across the three major lineages (dinoflagellates, diatoms, coccolithophores), in the marine environment (Green et al. 2004, 2006; Gutierrez et al. 2012a, b, 2013a, 2014).

Phytoplankton can be a biogenic source of hydrocarbons (Chisti 2007; Schirmer et al. 2010), such as synthesizing PAHs (Andelman and Suess 1970; Borneff et al. 1968; Gunnison and Alexander 1975) and translocating these chemicals into the algal cell wall (Gol'man et al. 1973; Gunnison and Alexander 1975; Pastuska 1961; Zelibor et al. 1988). *Botryococcus braunii*, for example, produces a range of hydrocarbons (*n*-alkadienes and trienes) (Metzger and Largeau 2005). Compared to the surrounding seawater environment, the phycosphere can thus become concentrated with PAHs. As these chemicals are a preferred carbon and energy source for several groups of OHCB, this PAH-enriched biotope (i.e., the phycosphere of phytoplankton) represents an attractive niche where these organisms are found.

Many phytoplankton also produce long-chain hydrocarbon-like compounds, such as alkenones (Marlowe et al. 1984), and as discussed in Sect. 3.3 almost all produce the volatile hydrocarbon isoprene (Shaw et al. 2010; Exton et al. 2012) – substrates that would serve as an available source of carbon and energy for growth to the OHCB. Liu et al. (2013) showed that species of cyanobacteria can produce hydrocarbons, and in a recent ground-breaking study, Lea-Smith et al. (2015) described the "short-term hydrocarbon cycle" in the ocean where the *n*-alkanes pentadecane (C_{15}) and heptadecane (C_{17}) are produced by *Prochlorococcus* and *Synechococcus* – two of the most abundant cyanobacteria in the ocean (Scanlan et al. 2009). Importantly, this study suggested that this cyanobacteria-mediated production of these hydrocarbons – estimated at 308–771 million tons per year – could support populations of heterotrophic hydrocarbon-degrading bacteria in the ocean. This is a staggering amount from biogenic production by these cyanobacteria as it equates to ~1% of the bioavailable DOC in the ocean (Lea-Smith et al. 2015). The authors showed that these *n*-alkanes could support a population of *Alcanivorax borkumensis* – a marine OHCB that is not abundant in the marine photic zone – at cell abundances as low as 1.5×10^1–4.1×10^3 cells per ml.

Hence, OHCB, like *A. borkumensis*, that are found in pristine marine environments would be well poised to respond to and degrade hydrocarbons in the event of an oil spill. Supporting this, a study by Mishamandani et al. (2016) showed that the bacterial community associated with a laboratory culture of the marine diatom *Skeletonema costatum* was tuned to respond to and degrade aromatic hydrocarbons when challenged with crude oil. In a subsequent study investigating this with a field sample, the oil biodegradation potential of the phytoplankton-associated bacterial community was found to exceed that of the free-living community and notably showed a preference to degrade substituted and nonsubstituted PAHs (Thompson et al. 2017). These studies provide evidence of compartmentalization of hydrocarbon-degrading capacity in the marine water column, where the hydrocarbon-degrading bacterial population found associated with phytoplankton is better tuned to degrading hydrocarbons in the event of a spill than that by the community of planktonic free-living bacteria.

In addition to synthesizing hydrocarbons, phytoplankton have also been reported to passively adsorb hydrocarbons from the surrounding seawater environment which could also support the association of a community of OHCB. A study by Binark et al. (2000) showed that cell surfaces of various marine eukaryotic phytoplankton were able to adsorb up to 14 different types of PAHs at relatively high concentrations. Field studies performed in the Baltic Sea have shown a high correlation between particulate organic carbon and the removal of PAHs from the water column, particularly during periods of the year corresponding to peaks in phytoplankton blooms (Witt 1995, 2002). Similarly, a study by Kowalewska (1999) showed phytoplankton cells to significantly contribute to the transport of PAHs from the upper layers of the Southern Baltic ecosystem to the sea floor by sedimentation. These studies suggest that phytoplankton cells act as a "sponge" for adsorbing PAHs in the water column. Although the hydrophobic nature of PAHs ($\log K_{ow} = 3$ to 8) greatly limits their solubility in seawater, this property would, however, favor their

adsorption to phytoplankton cells (Evans et al. 1990; Lee et al. 1978), as was quantitatively demonstrated with different PAHs (Kowalewska 1999). Considering the narrow nutritional spectrum of the OHCB, the phycosphere could be perceived as an asylum for these organisms by way of providing them with an available, possibly also constant, source of hydrocarbons. This is advantageous for these organisms, especially in remote and oligotrophic waters where the provision of these substrates is at a premium. Therefore, whether through biogenic synthesis or adsorption of PAH molecules from the surrounding seawater environment, the phycosphere of phytoplankton cells could be considered a "hot spot" for the OHCB.

6 Research Needs

The OHCB appear to be confined to marine environments, where they are commonly found to become strongly enriched at oil contaminated sites. These organisms have been found in shallow, deep, coastal, and offshore sites around the world, and intriguingly in remote "pristine" areas, such as the Arctic and Antarctica, where there is no obvious hydrocarbon contamination. As discussed, there are several sources from which hydrocarbons, albeit in minute quantities, can find their way into the ocean and support the occurrence of the OHCB in remote areas. The association of some taxa of OHCB with phytoplankton raises important questions with respect to their evolutionary history and ecology. The biogenic production of hydrocarbon and hydrocarbon-like compounds by phytoplankton is one facet of the hydrocarbon cycle in the ocean that could play a significant role in supporting communities of the OHCB, including those that are free-living in the surrounding seawater environment. On a global scale, extraterrestrial inputs of hydrocarbons may also contribute to supporting these types of bacteria, but whether this is significant compared to other hydrocarbon sources remains poorly resolved. Further to this, there is a need to determine the absolute/relative contribution of different sources of hydrocarbon inputs into the ocean. Increasing our knowledge on the ecology of the OHCB in the ocean, including the niche sites where these organisms may be found, could help improve our understanding of their ecological roles and how these organisms acquire sustenance in the form of their preferred carbon source – hydrocarbons – in regions where there is no obvious hydrocarbon input.

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Hydrocarbon-Degrading Microbes as Sources of New Biocatalysts

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Abstract

Petroleum hydrocarbons, including those discharged to the marine environment, are metabolized through different catabolic pathways by a number of microorganisms. Each hydrocarbon-degrading microorganism produces interesting enzymes for degrading alkanes and/or aromatic compounds that allow them to be used as sources of carbon and energy, and thus, these microbes occupy hydrocarbon-rich ecological niches. Their diversity and hydrocarbon-degrading metabolic abilities have been extensively examined in multiple environmental and phylogenetic contexts. Genes encoding enzymes involved in degradation, such as alkane hydroxylases and other monooxygenases, P450 cytochromes, rubredoxin reductases, and ferredoxin reductases, have been examined by genome analysis, and a number of them have been successfully cloned, expressed, purified, and their activities confirmed. However, in these microorganisms, the accumulated information regarding other types of enzymes, particularly those most used at industrial level, is limited. Here, we compile information about the accumulated enzymatic knowledge of obligate marine hydrocarbonoclastic bacteria (OMHCB), key players in bioremediation of hydrocarbons in contaminated marine ecosystems. We focused on bacteria of the genera *Cycloclasticus*, *Alcanivorax*, *Oleispira*, *Thalassolituus*, and *Oleiphilus*. Enzymatic data of these representative OMHCB members are restricted to enzymes of the class hydroxylases, cytochrome P450, dioxygenases, synthases, dehalogenases, ligases, and mostly for hydrolases with a typical α/β hydrolase fold. Despite the limited information reported, the available data suggest that these organisms may be important sources of industrial biocatalysts, the analysis of which may deserve deeper investigation. Comparative information is provided regarding the occurrence of key biotechnologically relevant ester-hydrolases in the genomes of OMHCB and suggesting which of the OMHCB may potentially have higher promise as a source of biocatalysts. We also discuss how the properties of these enzymes could be biologicallly important for these bacteria, as some of them can convert a broad range of compounds.

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1 Introduction

Hydrocarbons are generally toxic and persistent molecules and their release can seriously harm the environment. Hydrocarbons are degraded by indigenous microorganisms that can use these compounds as carbon source thanks to their specialized metabolism (Cappello et al. 2007; Ron and Rosenberg 2014). Blooms of hydrocarbon-degrading bacteria (HDB) occur after crude-oil spills or hydrocarbon release (Yakimov et al. 2005). Such blooms may occur frequently as oil spills represent around 10% of global marine oil pollution (GESAMP 2007). In pristine sites, these microorganisms typically comprise less than 1% of the total bacterial population, but they proliferate rapidly when oil contamination occurs, sometimes accounting for up to 90% of the microbial community (Kasai et al. 2002; Cappello et al. 2007; Manilla-Pérez et al. 2010; Bargiela et al. 2015). In all cases, the first step in hydrocarbon biodegradation is the molecule activation presumably between two carbon atoms. The resulting compounds are then transformed through specific catabolic pathways that converge in common intermediates (Díaz et al. 2013; Boll et al. 2014; Bargiela et al. 2017).

1.1 Principal Hydrocarbon-Degrading Bacteria

The different groups of aerobic hydrocarbon-degrading bacteria are specialized in the degradation of a particular range of compounds. Each microorganism possesses different metabolic abilities, allowing the degradation of different types of substrates. Among the HDB is the group of obligate marine hydrocarbonoclastic bacteria (OMHCB), which can only use hydrocarbons as sole carbon and energy source, in addition to some organic acids, such as acetate and pyruvate (Head et al. 2006; McGenity et al. 2012; Joye et al. 2016). To our knowledge, the marine environment is the only place known to date where obligate hydrocarbon degraders have been found. Most representative OMHCB belong to the class *Gammaproteobacteria*, one of the most physiologically and phylogenetically diverse class of Bacteria, consisting of 15 orders (Gutierrez 2017). Within OMHCB we focus on the genera *Alcanivorax*, *Cycloclasticus*, *Oleispira*, *Thalassolitus*, and *Oleiphilus* (Harayama et al. 2004; Yakimov et al. 2007; Manilla-Pérez et al. 2010; Liu and Liu 2013), which are almost undetectable in pristine marine environments, but are among the most abundant bacteria in polluted marine sites.

Alcanivorax is one of the most studied genera, which currently comprises 11 recognized and one proposed species Ca. *A. indicus.* The vast majority of them (*A. borkumensis*, *A. dieselolei*, *A. gelatiniphagus, A. hongdengensis*, *A. jadensis*, *A. venustensis*, and Ca. "*A. indicus*") grow in presence of *n*-alkanes and branched alkanes, with the inability to use any carbohydrate or amino acid as carbon source. The genome of *A. borkumensis* SK2 was the first sequenced OMHCB genome (Yakimov et al. 1998, 2007; Golyshin et al. 2003). Bacteria of the genera *Thalassolituus* (Yakimov et al. 2004), *Oleiphilus* (Golyshin et al. 2002), and *Oleispira* (Yakimov et al. 2003) also have a high specificity for aliphatic alkanes (Yakimov et al. 2004). Members of the genus *Cycloclasticus* (Dyksterhouse et al.

1995) grow on minimal medium supplemented with poly-aromatic hydrocarbons (PAHs) like naphthalene, phenanthrene, or anthracene as sole carbon source (Harayama et al. 2004; Messina et al. 2016). Within the *Gammaproteobacteria*, other recent obligate polycyclic aromatic hydrocarbon (PAH)-degrading specialists have been described, which almost exclusively use PAHs as sole source of carbon and energy and live in association with marine phytoplankton, including bacteria from the genera *Polycyclovorans*, *Algiphilus*, and *Porticoccus* (Gutierrez et al. 2012, 2013, 2015a, b).

Beyond the OMHCB, other bacterial groups are able to metabolize hydrocarbons that have been recently reviewed by Prince et al. (2018). In this chapter, we focus on the accumulated enzymatic knowledge of OMHCB, key players in the bioremediation of hydrocarbons in contaminated marine ecosystems, particularly during crude-oil spills (Kasai et al. 2002; Cappello et al. 2007; Yakimov et al. 2007; Manilla-Pérez et al. 2010; Bargiela et al. 2015).

1.2 Genomes and Genes Encoding Degrading Enzymes from OMHCB

Imagine the microbial communities responding to hydrocarbon-uptake and how variable this can be (Kostka et al. 2011; Gutierrez et al. 2013). The most obvious response will be production of enzymes supporting the degradation of such hydrocarbons to intermediates feeding into central metabolism (Lu et al. 2011; Mason et al. 2012; Kimes et al. 2013; Mason et al. 2014). These microorganisms may contain versatile enzymes that not only allow microorganisms to metabolize multiple molecules in nature, but also produce enzymes that are potentially valuable to multiple biotechnological processes. The enzyme arsenal of OMHCB has been examined mostly in the context of alkane and aromatics degradation, revealing their diversity and versatility to facilitate pollutant degradation (Kasai et al. 2003; Schneiker et al. 2006; Teimoori et al. 2011, 2012; Golyshin et al. 2013; Kube et al. 2013; Naing et al. 2013; Messina et al. 2016).

Genomes of representative cultivable OMHCB capable of degrading a number of crude-oil components have been reported (Table 1) and their genomic basis established. They include genomes from alkane degraders such as *Alcanivorax* (Golyshin et al. 2003; Schneiker et al. 2006; Lai et al. 2012a; Lai and Shao 2012a, b; Luan et al. 2014; Miura et al. 2014; Barbato et al. 2015; Zhang et al. 2016), *Oleispira* (Kube et al. 2013), *Oleiphilus* (Toshchakov et al. 2017), *Thalassolituus* (Golyshin et al. 2013; Dong et al. 2014) and from PAH-specializing degraders such as *Cycloclasticus* (Messina et al. 2016; Toshchakov et al. 2017). Currently (February 2018), the genomes of seven species of the genera *Alcanivorax* are available (Lai et al. 2012a; Lai and Shao 2012a, b; Parks et al. 2017; Fu et al. 2018), with that of *A. borkumensis* SK2 being the first to be sequenced (Schneiker et al. 2006) (Table 1). Genomes of *Cycloclasticus,* including those of *Cycloclasticus* sp. 78-ME (Messina et al. 2016), *Cycloclasticus* sp. strain PY97M (Cui et al. 2013), and *Cycloclasticus* sp. strain P1 (Lai et al. 2012b), are available. Within the genera *Oleispira,*

Table 1 Current (February 2018) protein-coding genes, theoretical and with activity experimentally confirmed, present in the genomes of representative OMHCB

	No. of proteins in the genome	Accession number of genome sequence	No. of proteins with confirmed enzymatic activity[a]
A.borkumensis^T	2,750	NC_008260	19[b]
A. diselolei	4,362	CP003466	1[c]
A. jadensis	3,266	ARXU01000000	n.d.
A. hondengengensis	3,416	AMRJ00000000	n.d.
A. nanhaiticus	3,778	ARXV00000000	n.d.
A. pacificus	3,669	CP004387	n.d.
A. xenomutans	4,275	CP012331	n.d.
Cycloclasticus sp. 78-ME	2,585	FO203512	7[d]
Cycloclasticus sp. strain PY97M	2,264	ASHL00000000	n.d.
Cycloclasticus sp. strain P1	2,249	CP003230	n.d.
O. antarctica RB-8	3,919	FO203512	15[e]
T. oleivorans MIL-1	3,603	HF680312	n.d.
O. messinensis ME102	5,502	PRJNA362330	n.d.

[a]Enzymes others than those involved in biodegradation steps such as alkane hydroxylases and other monooxygenases, P450 cytochromes, rubredoxin reductases, ferredoxin reductases, and dioxygenases. Abbreviation: *n.d.*, not described

[b]Enzymes with confirmed activity included one fatty acid ω-hydroxylase from the P450 (CYP)153 family, one succinate coenzyme A ligase, one TesB-like thioesterase, and 16 ester-hydrolases

[c]The enzyme with confirmed activity included a haloalkane dehalogenase

[d]Enzymes with confirmed activity included seven ester-hydrolases, four of them belonging to the MCP family. Structure is available for one enzyme with dual ester-hydrolase: MCP hydrolase activity (PDB 4I3F)

[e]Enzymes with confirmed activity included one pyrophosphatase, one phoshonoacetaldehyde hydrolase, two fumarylacetoacetate isomerase/hydrolase, two 2-keto-3-deoxy-6-phosphogluconate aldolase, one amidohydrolase, one isochorismatase hydrolase, one transaldolase, one glycerophosphodiesterase, one dihydroorotate oxidase, two ester-hydrolases, and two chaperones. The crystal structures for 12 of these enzymes (3I4Q, 3IRU, 3L53, 3LAB, 3LNP, 3LQY, 3M16, 3QVM, 3QVQ, 3V77, 3VCR, 3I6Y) and one protein with unknown function (3LMB) are available

Thalassolituus, and *Oleiphilus*, only the genomes of *Oleispira antarctica* RB-8 (Kube et al. 2013), *Thalassolituus oleivorans* MIL-1, and *Oleiphilus messinensis* ME102 (Toshchakov et al. 2017) have been sequenced and are available (Table 1).

The production and characterization of some of the key catabolic enzymes participating in degradation steps in some of the above OMHCB members have been examined. Thus, examples exist for the successful cloning, expression, production, and characterization of alkane hydroxylases (Hara et al. 2004; van Beilen et al. 2004; Miri et al. 2010; Naing et al. 2013) and other monooxygenases (Wang and Shao 2012), P450 cytochromes (Jung et al. 2016), rubredoxin reductases (Teimoori et al. 2011), and ferredoxin reductases (Teimoori et al. 2012) from *Alcanivorax* strains. The

biochemical information of such enzymes for *Cycloclasticus* strains is limited to few PAH dioxygenases (Kasai et al. 2003; Shindo et al. 2011) and cytochrome P450 (Misawa et al. 2011). For other OMHCB, no examples have been described reporting the successful cloning, purification, and characterization of such enzymes.

2 Biotechnologically Relevant Enzymes from OMHCB

The accumulated level of information regarding the characteristics and potential of other types of enzymes present in the genomes of OMHCB is limited (Table 1). This limited knowledge is particularly noticeable for enzymes that are most commonly used at an industrial level, aldo-keto reductases, transaminases, and serine ester-hydrolases from the structural superfamily of α/β-hydrolases (Ferrer et al. 2015; Martínez-Martínez et al. 2017). Below, we compile the reported cases in which enzymes others that alkane hydroxylases, monooxygenases, P450 cytochromes, rubredoxin reductases, and ferredoxin reductases have been reported. Some of these enzymes have been used for the production of valuable molecules.

2.1 Biotechnologically Relevant Enzymes from *Alcanivorax* Strains

Until February 2018, and to the best of our knowledge, a total of 10 enzymes with potential biotechnological uses have been reported from bacteria of the *Alcanivorax* genus, the most explored species being *A. borkumensis* SK2 (Table 1). A recent example is the production of ω-hydroxy palmitic acid from palmitic acid via *A. borkumensis* SK2 fatty acid ω-hydroxylase (CYP153A13) from the P450 (CYP)153 family (Jung et al. 2016). A high product yield of 4.6 g/L of ω-hydroxy palmitic acid was achieved in fed-batch resting cell reactors when *Escherichia coli* cells expressing CYP153A13 were confronted with 5.1 g/L palmitic acid for 30 h, at 30 °C. Production and characterization of other enzymes from *A. borkumensis* SK2 have been reported. They include two ester-hydrolases for oil degradation (Kadri et al. 2018), a succinate coenzyme A ligase with a broad substrate range and useful for the formation of succinate analogues in vitro (Nolte et al. 2014), and a TesB-like thioesterase for the production of extracellular polyhydroxyalkanoates (Sabirova et al. 2006). Recently, three hydrolases from *A. borkumensis* SK2 were found to be efficient for either hydrolyzing poly-(DL-lactic acid) polyester (ABO2449) (Hajighasemi et al. 2016) or esters containing *p*-nitrophenyl and α-naphthyl blocks, with acyl chains ranging from acetate to laurate, as well as tri-acyl-glycerols (preferably tributyrin) and other short-chain esters (Tchigvintsev et al. 2015). Notably, polyester-degrading activity of ABO2449 was found in emulsified and solid poly-(DL-lactic acid), with the capacity to degrade solid material being of high industrial interest. ABO2449 had highest esterase activity at 30–37 °C and retained 32% of its maximal activity at 4 °C, suggesting that it is a cold-adapted esterase, which is consistent with the ability of *A. borkumensis* to grow at 4 °C. Cold-active esterase has been also

reported in *A. dieselolei* (Zhang et al. 2014a); the enzyme did show high stability in the presence of solvents. A haloalkane dehalogenase from *A. dieselolei* B-5 with potential use for biocatalysis and bioremediation applications has been also reported (Li and Shao 2014). An activity assay with 46 halogenated substrates indicated this enzyme possessed broad substrate range, with preference for brominated substrates and chlorinated alkenes. The dehalogenase was most active in the range from 20 °C to 50 °C, with an optimal at 50 °C. Also, a 5-enolpyruvyl-shikimate-3-phosphate synthase for the biosynthesis of aromatic amino acids has been reported from the deep-sea bacterium *Alcanivorax* sp. L27 through screening the genomic library (Zhang et al. 2014b). Its optimal temperature was 50 °C, and it retained 20% of its activity at 0 °C. To the best of our knowledge, no enzyme with confirmed activity has been described for any of the genomes of the other five species of the genera *Alcanivorax* with genome sequences available (Table 1).

Taken together, the available biochemical data revealed that broad substrate range and optimal temperatures of up to 20–50 °C and capacity to retain some activity at 4 °C are common features of enzymes from bacteria of the genus *Alcanivorax*.

2.2 Biotechnologically Relevant Enzymes from *Cycloclasticus* Strains

Until February 2018, and to the best of our knowledge, a total of six enzymes with potential biotechnological uses have been reported from bacteria of the genus *Cycloclasticus* (Table 1). Bioconversions in which hydroxyl groups were regio- and stereo-specifically introduced into various substituted naphthalenes, such as 1-methoxy- and 1-ethoxy-naphthalenes, methylnaphthalenes, dimethylnaphthalenes, and naphthalenecarboxylic acid methyl esters, and β-eudesmol, have been reported. Thus, the conversion was performed by recombinant *E. coli* cells expressing an aromatic dihydroxylating dioxygenase from *Cycloclasticus* strain A5 (Shindo et al. 2011); 10 novel prenyl naphthalen-ols were produced by combinatorial bioconversion when cells expressing the dioxygenase were allowed to react with a number of aromatic substrates at 25 °C. Similarly, *E. coli* cells expressing cytochrome P450 BM3 variant from *Cycloclasticus* strain A5 were capable of introducing at 25 °C a hydroxyl group regio- and stereo-specifically into a sesquiterpene β-eudesmol (Misawa et al. 2011). Also, four hydrolases from the α/β hydrolase family of *Cycloclasticus* sp. ME7 have also been reported with the unusual capacity to efficiently hydrolase C–O and C–C bonds in a broad spectrum of substrates (Alcaide et al. 2013). These enzymes were most active at 40–55 °C, although they retained high activity at temperatures as low as 4–20 °C.

Taken together, as for enzymes from *Alcanivorax*, the available biochemical data suggest that some of the enzymes from *Cycloclasticus* are also characterized by an unusually broad substrate range and are capable of performing well at temperatures of up to 25–55 °C, but also capable of retaining activity at temperatures as low as 4 °C. The only crystal structure reported is from the dual ester-hydrolase:meta-cleavage product (MCP) hydrolase from *Cycloclasticus* sp. ME7 (PDB 4I3F; Alcaide et al. 2013).

2.3 Biotechnologically Relevant Enzymes from *Oleispira* Strains and Other OMHCB

Bacteria of the *Oleispira* genus are the OMHCB for which the most industrially relevant enzymes have been reported currently (February, 2018) (Table 1). The analysis of 15 proteins (including, chaperones, hydrolases, and oxidases) from *O. antarctica* RB-8 revealed that most of its enzymes are functioning suboptimally at temperatures close to that being optimal for bacterial growth. However, their activities at 4 °C are nevertheless sufficient to facilitate the active growth of this bacterium in polar (and deep sea) waters (Ferrer et al. 2003; Goral et al. 2012; Lemak et al. 2012; Kube et al. 2013). Enzymes with confirmed activity included a pyrophosphatase (OLEAN_C30460), a glycerophosphodiesterase (OLEAN_C34790), a dihydroorotate oxidase (OLEAN_C16020), and three ester-hydrolases (OLEI01171, OLEAN_C09750, and OLEAN_C31070) from *O. antarctica* RB-8 (Lemak et al. 2012; Kube et al. 2013). The structures of four of these enzymes have been reported (PDB 3I4Q, 3QVM, 3I6Y, and 3QVQ; Lemak et al. 2012; Kube et al. 2013). These enzymes have in common showed salt-stimulation and optimum temperatures of 15–25 °C, although some of them were found to be most active at 35–50 °C (OLEAN_C34790) and some retained high activity level at 4 °C (OLEAN_C09750); however, their substrate profile was not evaluated in a broad context, so that their biotechnological potential remains to be established. Additionally, although experimental confirmations are needed, the activity of one phoshonoacetaldehyde hydrolase, two fumarylacetoacetate isomerase/hydrolase, two 2-keto-3-deoxy-6-phosphogluconate aldolase, one amidohydrolase, one isochorismatase hydrolase, and one transaldolase were putatively suggested by their X-ray structural determinations (3IRU; 3LAB; 3 L53; 3LNP; 3LQY; 3 M16; 3 V77; 3VCR). The structure of a protein with unknown function from this bacterium is also available (3LMB; Kube et al. 2013). Finally, two proteins from the strain RB-8, which do not have enzymatic activity but chaperone activities, were published (Ferrer et al. 2003) and patented, and their expression in mesophilic bacteria such as *E. coli* allows growth at temperatures as low as 4–10 °C.

Taken together, *Oleispira antarctica* RB-8 is the bacterium from the OMHCB for which more extensive biochemical and structural data are available, and the accumulated data suggest its enzymes as being most active at 15–25 °C, but some of them are capable of performing well at 50 °C, and others retain more than 80% activity at temperatures as low as 4 °C. Until February 2018, no examples were found that report enzymes from bacteria of the genera *Thalassolituus* and *Oleiphilus* (Table 1).

3 Ester-hydrolases from OMHCB

Ester-hydrolases are a class of enzymes widely distributed in the environment and with important physiological functions; this is why at least one can be found in each bacterial genome (Ferrer et al. 2015). They include hydrolases that are among the most important industrial biocatalysts, and extensive biochemical knowledge has

been accumulated from many microorganisms (Ferrer et al. 2015). As mentioned above, currently (February 2018), five ester-hydrolases from the α/β hydrolase family from *A. borkumensis* SK2, four from *Cycloclasticus* sp. ME7, and three from *O. antarctica* RB-8 have been isolated and characterized. By using both genomic and metagenomics approaches, the latter to avoid cultivation and thus access the widest possible genomic variability (Distaso et al. 2017), a wide set of 145 ester-hydrolases from environmentally and phylogenetically diverse origin have been isolated and their substrate specificity reported (Martínez-Martínez et al. 2018). A subset of 25 of them were isolated from OMHCB, particularly from bacteria of the genera *Alcanivorax* (11 in total), *Cycloclasticus* (12), *Thalassolituus* (1), and *Oleiphilus* (1). The source of the 25 enzymes was the genomes of three cultivable OMHCB, *A. borkumensis* SK2, *Cycloclasticus* sp. 78-ME, *O. messinensis* ME102^{T}, and clone libraries created from chronically polluted seawater samples from Milazzo harbor (Sicily, Italy), Messina harbor (Sicily, Italy), Bizerte lagoon (Tunisia), Ancona harbor (Ancona, Italy), and crude oil enrichment from coastal seawater (Kolguev, Russia). The description of this new set of enzymes from OMHCB allows us to further investigate their features and relevance, which will be discussed below.

3.1 Diversity and Divergence at the Sequence Level

The sequences of the 25 ester-hydrolases from OMHCB recently described by Martínez-Martínez et al. (2018) were compared with the sequences available in the NCBI nonredundant public database. Protein sequences were 51.3–99.7% similar to noncharacterized homologous proteins in the database. The pairwise amino acid sequence identity ranged from 0.5% to 99.6%. The pairwise amino acid sequence identity for all 11 ester-hydrolases from *A. borkumensis* SK2 ranged from 2.8% to 23.3%. The pairwise amino acid sequence identity for all 12 ester-hydrolases from *Cycloclasticus* sp. 78-ME ranged from 7.0% to 65.8%, except two enzymes which share 99.6% identity at the amino acid sequence level. The only ester-hydrolase from *Thalassolituus* shows a pairwise comparison of up to 23% to all other ester-hydrolases, and the one from *O. messinensis* ME102T up to 23.4%. BLAST searches were performed for all query sequences by running NCBI BLASTP against the current version of the Lipase Engineering Database using an E-value threshold of 10^{-10}. All, but four, were unambiguously assigned to some of the 14 existing families (F) of the Arpigny and Jaeger classification (Arpigny and Jaeger 1999; Ferrer et al. 2015), which are defined based on amino acid sequence similarity and the presence of specific sequence motifs. These included sequences with a typical α/β hydrolase fold and conserved G-X-S-X-G (FI: 3, FIV: 3, FV: 5, FVI: 1, and FVII: 1) or G-X-S-(L) (FII: 1) motifs and sequences with a serine beta-lactamase-like modular (non α/β hydrolase fold) architecture and a conserved S-X-X-K motif (FVIII: 4). An additional set of seven sequences were assigned to the MCP hydrolase family. Noticeably, all seven sequences encoding hydrolases from the MCP family have been isolated from *Cycloclasticus*; this observation is consistent with the fact that this enzyme participates in the degradation of aromatics such as catechol and biphenyl,

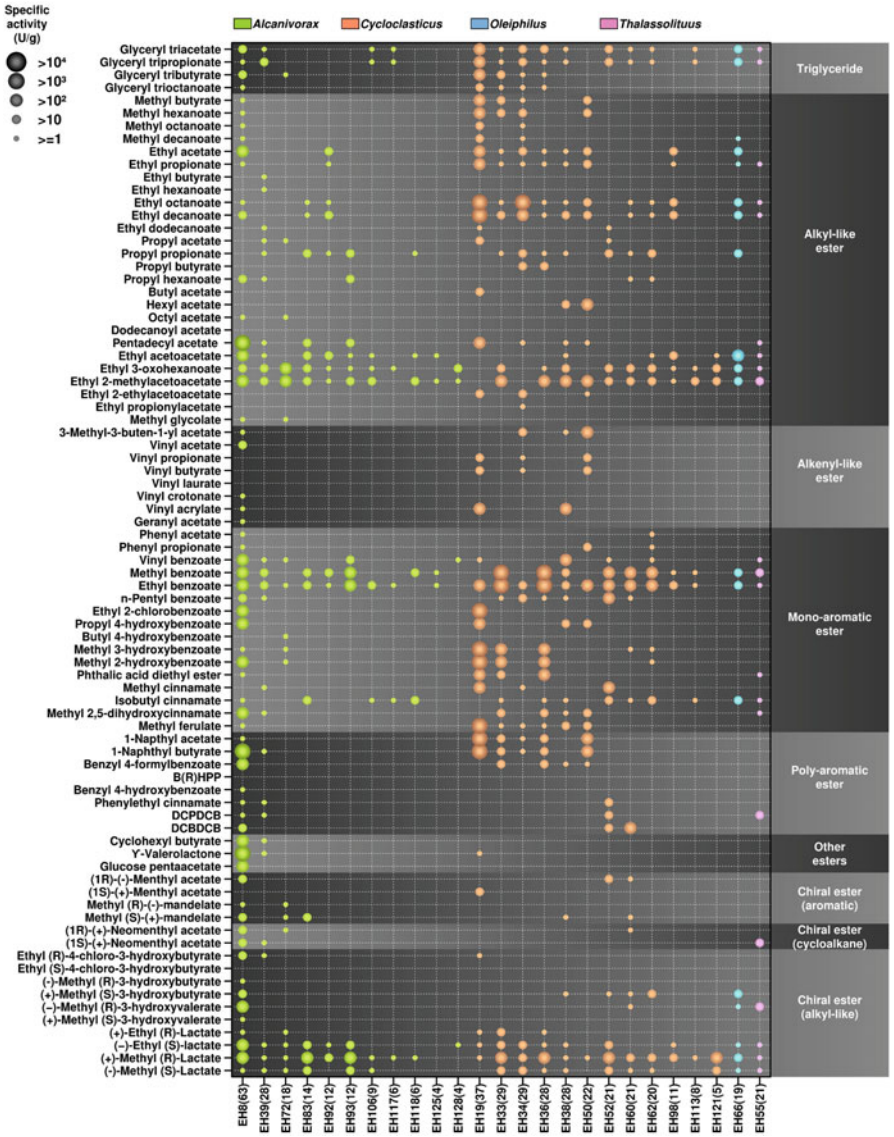

Fig. 1 Substrate ranges of ester-hydrolases from OMHCB Ester-hydrolases. The ID and characteristic of each of the ester-hydrolases is reported elsewhere (Martínez-Martínez et al. 2018). The ID code representing each ester-hydrolase is given at the bottom. Each hydrolase is named based on the code "EH," which means ester-hydrolase, followed by an arbitrary number for the most to least promiscuous enzyme, following the criteria reported by Martínez-Martínez et al. (2018). The number in bracket indicates the number of esters hydrolyzed by each enzyme, which are listed in the left side. The figure was created with the R language console using information about the specific activity in units/g protein (measurable by the size of the circle; see legend on the left side) at 30 °C and pH 8.0 of the analyzed enzymes against a set of 96 substrates tested by Martínez-Martínez et al. (2018). Only the esters for which activity was detected for any of the ester-hydrolases

for which this bacterium is specialized compared to *Alcanivorax*, *Thalassolituus*, and *Oleiphilus*, which are alkane degraders.

Taken together, the primary sequence analysis suggests that the diversity of polypeptides encoding ester-hydrolases from OMHCB is not dominated by a particular type of protein or highly similar protein clusters but consists of diverse nonredundant sequences assigned to multiple folds and subfamilies, which are distantly related to known homologs in many cases. It also revealed the extensive divergence at the sequence level between and within ester-hydrolases from OMHCB.

3.2 Biotechnologically Relevant Characteristics

Experimental data on substrate conversion (i.e., units g^{-1}) followed for 24 h at pH 8.0 and 30 °C were reported for each of the 25 ester-hydrolases from OMHCB for 96 distinct esters. These include esters with variation in size of acyl and alcohol groups and with growing residues (aromatic, aliphatic, branched, and unbranched) at both sides, leading to more challenging substrates because a larger group adjacent to the ester bond increases the difficulty of conversion. Halogenated, chiral, and sugar esters, lactones and an alkyl di-ester, were also included. The substrate profiles of all 25 ester-hydrolases, when tested with a set of 96 chemically and structurally distinct esters, are summarized in Fig. 1. The enzymes had a substrate spectrum that ranged from 62 to 4 substrates. According to recently established criteria (Martínez-Martínez et al. 2018), 2 out of 25 would fall into the category of ester-hydrolases with prominent substrate promiscuity (capable of hydrolyzing 30 or more esters) and 16 into the category of ester-hydrolases with moderate substrate promiscuity (capable of hydrolyzing from 10 to 29 esters), and only seven hydrolyzed nine or fewer esters. The percentage of ester-hydrolases showing moderate-to-prominent promiscuity accounted for around 73% when considering all 145 enzymes previously reported by Martínez-Martínez et al. (2018) and which assigned to at least 10 phyla and 40 genera. This percentage is similar to that found for ester-hydrolases of OMHCB (18 out of 25; or 72%), which suggests that, at least in terms of substrate scope, the hydrolytic potential of ester-hydrolases from these OMHCB is as good as that of other bacterial groups. This is important from a practical point of view, as, along with requirements of a technical nature such as selectivity, scalability, and robustness, a narrow substrate spectrum is one of the most frequent problems for industrial enzyme applications (Martínez-Martínez et al. 2018). A consensus exists that "the more substrates an enzyme converts the better," opening application ranges with consequent reduction of the production cost of multiple enzymes.

Fig. 1 (continued) from OMHCB (81 esters out of 96 tested) are listed in the figure. Abbreviations as follows: DCBDCB, 2,4-dichlorobenzyl 2,4-dichlorobenzoate; DCPDCB, 2,4-dichlorophenyl 2,4-dichlorobenzoate; B(R)HPP, benzyl (R)-(+)-2-hydroxy-3-phenylpropionate

As shown in Fig. 1, each of the 25 enzymes is characterized by distinct substrate spectra in agreement with the differences at the sequence level. The two ester-hydrolases from *Oleiphilus* and *Thalassolituus* were characterized by a narrow substrate scope, but the low number of enzymes characterized so far from these bacteria does not allow conclusions to be drawn about whether this is a common feature among ester-hydrolases from these bacteria. However, as the number of ester-hydrolases from *Alcanivorax* (11 in total) and *Cycloclasticus* (12 in total) was in the same range, the differences in substrate scope could be analyzed. As shown in Fig. 1, we could observe that the capability to convert large aromatic and sterically hindered esters such as benzyl (*R*)-(+)-2-hydroxy-3-phenylpropionate, benzoic acid-4-formyl-phenylmethyl ester, 2,4-dichlorophenyl 2,4-dichlorobenzoate, 2,4-dichlorophenyl 2,4-dichlorobenzoate, and diethyl-2,6-dimethyl 4-phenyl-1,4-dihydro pyridine-3,5-dicarboxylate was most frequent among ester-hydrolases from *Cycloclasticus* (8 out 12 enzymes), compared to enzymes from *Alcanivorax* (1 out 11 enzymes). This was also noticed when examining the hydrolytic capacity towards mono-aromatic esters, such as benzoate esters that were also hydrolyzed by most *Cycloclasticus* esterases, compared with *Alcanivorax* ester-hydrolases. This agrees with the fact that members of the genus *Alcanivorax* grow commonly in the presence of *n*-alkanes and branched alkanes, with the inability to use, in the majority of the cases, PAHs, whereas members of the genus *Cycloclasticus* commonly grow on PAHs. It is thus plausible that the ability of *Cycloclasticus* ester-hydrolases to convert hindered esters is a reflection of the adaptation of this bacterium to manage complex aromatic molecules. Ester-hydrolases capable of converting polyaromatic esters have been identified also by metagenomic-based mining of a naphthalene-enriched community (Martínez-Martínez et al. 2014), suggesting that genomes and metagenomes from PAH-polluted environments may be a source of enzymes with a higher probability to convert hindered molecules.

The biotechnological potential of ester-hydrolases can be evaluated not only by their substrate scope, but also by their ability to convert important molecules in synthetic organic chemistry such as *p*-hydroxybenzoic acid (paraben) esters and related esters (2- or 3-hydroxybenzoates). Two *Alcanivorax* ester-hydrolases and six from *Cycloclasticus* had the capacity to hydrolyze these chemicals, which demonstrates that these organisms' enzymes have the potential to perform conversions of industrially relevant molecules.

Interestingly, ester-hydrolases from all four OMHCB were able to release free acid from methyl esters of hydroxycinnamic acids, such as ferulic and cinnamic esters. This finding was unexpected since cinnamoyl/feruroyl ester hydrolases are abundant in microbes involved in fiber breakdown processes (for review see Wong 2006), but not in marine microorganisms. In addition, one of the *Alcanivorax* ester-hydrolases hydrolyzed glucose esters. The physiological role of this type of activity is normally associated with the cleavage of ester bonds to remove the acetyl moieties from complex polymers (Wong 2006). The deacetylation process increases biodegradability and renders complex polysaccharides more accessible to the attack of other polysaccharide hydrolytic enzymes (Biely et al. 1985; Grohman et al. 1989; Blum et al. 1999; Christov and Prior 1993). Taking this

into account, it appears that ester-hydrolases from OMHCB, i.e., bacteria widespread in oceans after petroleum spills, might have the potential capacity to contribute to the degradation of complex carbohydrates or polymers (i.e., coming from algae) present in the oceans.

Finding versatile biocatalysts that are chirally selective is of interest for the pharmaceutical and chemical industry (Coscolín et al. 2018). The experimental data on substrate conversion against a number of chiral esters (including (*R*) and (*S*) enantiomers) have been also reported for the 25 ester-hydrolases from OMHCB (Martínez-Martínez et al. 2018; Coscolín et al. 2018). The reported data revealed the distinct capacity of ester-hydrolases from OMHCB to hydrolyze 16 chiral esters that included: (*R*) and/or (*S*) enantiomers of menthyl acetate, methyl mandelate, neomenthyl acetate, ethyl 4-chloro-3-hydroxybutyrate, methyl 3-hydroxybutyrate, methyl 3-hydroxyvalerate, and methyl and ethyl lactate. Preferred enzymes are those that show stringent selectivity, although it is commonly considered that enzymes with a selectivity factor of 25 or above begin to have commercial value (Coscolín et al. 2018). As shown in Fig. 1, a number of ester-hydrolases were capable of hydrolyzing only one of the enantiomers for some of the esters, namely, 9 out 11 from *Alcanivorax*, 8 out of 12 from *Cycloclasticus*, 1 from *Oleiphilus*, and 1 from *Thalassolituus*.

3.3 Promiscuous Character of Ester-hydrolases from OMHCB: Physiological Implication

The relevance of substrate promiscuity is indisputable as the operating basis for biological processes and cell function (Martínez-Martínez et al. 2018). For example, the evolutionary progress of enzymes from lower to higher substrate specificity allows the recruitment of alternate pathways for carbon cycling and innovations across metabolic subsystems and the tree of life by maximizing the growth rate and growth efficiency. In general, promiscuous enzymes enable an energetically more favorable lifestyle for a cell than specialized enzymes, and therefore, the cell does not require many different enzymes to take up substrates, favoring genome minimization and streamlining. In addition, the extension of the substrate scope without compromising primary or ancestral enzyme activities is a major driver of microbial adaption to extreme habitats.

Out of the 11 ester-hydrolases from *Alcanivorax* reported by Martínez-Martínez et al. (2018), 10 were retrieved from the genome of *A. borkumensis* SK2 (Schneiker et al. 2006). One additional ester-hydrolase (named EH92) was isolated from a clone library created from chronically polluted seawater samples from Ancona harbor (Ancona, Italy); taxonomic binning of the DNA fragment containing the gene encoding this ester-hydrolase associated it to *Alcanivorax*, although its assignation to strain SK2 could not be unambiguously confirmed. Additionally, Tchigvintsev et al. (2015) and Hajighasemi et al. (2016) reported three ester-hydrolases, one capable of hydrolyzing poly-(DL-lactic acid) polyester, from the genome of *A. borkumensis* SK2. Finally, the presence of a lipase and an esterase in protein extracts from strain SK2 was also confirmed by Kadri et al. (2018), although their identities and

sequences were not confirmed. The genome of strain SK2 consists of a circular chromosome with 2,755 predicted coding sequences. Thus, genes encoding ester-hydrolases with confirmed activity represent about 0.55% of the total genes (15 out of 2,755 genes). Out of the ester-hydrolases from the genome of SK2, one (named EH8 following the nomenclature by Martínez-Martínez et al. 2018) showed a prominent promiscuity level being capable of converting 63 chemically and structurally diverse esters out of 96 tested, whereas the other nine were only capable of converting fewer than 28 esters (Fig. 1). Promiscuous ester-hydrolases thus represent a minor percentage in the genome of *A. borkumensis* SK2, and possibly other OMHCB. It is thus plausible that *Alcanivorax* developed a limited number of highly promiscuous ester-hydrolases, which may have a major biological role that is yet to be established. This enzyme was capable of degrading acetyl moieties from molecules that are known to be included in complex polymers, and thus, its role in microbial survival by expanding the set of potential substrates that can be used as carbon source cannot be ruled out. An additional function of esterases in bacteria such as *Alcanivorax* is supporting degradation of linear alkanes. Here, a specific aerobic degradation mechanism designated as subterminal oxidation pathway exists as an alternative path to the well-explored terminal degradation. This pathway requires a monooxygenase (MO), an alcohol dehydrogenase (DH), a Baeyer Villiger monooxygenase (BMVO), and finally an esterase. The genetic background of this degradation pathway has presently not been described; but loci encoding putative BMVO, DH, and esterase can be identified in the genome of *A. borkumensis* suggesting an operon organization of the respective genes. It is plausible that some of the esterases described to date from *Alcanivorax strains* may be related to or identical with the enzymes which cleave alkanes producing acetates.

Out of the 12 ester-hydrolases from *Cycloclasticus*, seven were retrieved from the genome of *Cycloclasticus* sp. 78-ME (Alcaide et al. 2013; Messina et al. 2016). The genome of strain 78-ME consists of a circular chromosome and a plasmid with 2,585 predicted coding sequences. Thus, genes encoding ester-hydrolases with confirmed activity represents about 0.3% of the total genes (seven out of 2,585). The remaining five ester-hydrolases were isolated from clone libraries created from chronically polluted seawater samples from Milazzo harbor (Sicily, Italy), Messina harbor (Sicily, Italy), and Bizerte lagoon (Tunisia); taxonomic binning of the DNA fragment containing the gene encoding these ester-hydrolases associated them to *Cycloclasticus*. The seven ester-hydrolases from the genome of strain 78-ME showed a capacity to hydrolyze from five to 37 esters. Four of them belong to the MCP family, two showed a typical α/β hydrolase fold with a conserved G-X-S-X-G or G-X-S-(L) motif, and one was not classified into existing families. Hydrolases from the MCP family hydrolases are known to help in the degradation of aromatic pollutants such as catechol and biphenyl (Alcaide et al. 2013). However, the ability of such enzymes to convert molecules other than fission products of bicyclic (i.e., 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate) and monocyclic (i.e., 2-hydroxy-6-oxohepta-2,4-dienoate) aromatics is uncommon. The available biochemical data suggest that *Cycloclasticus* has developed an arsenal of hydrolases with broad substrate range that not only participate in the degradation of pollutants, but also expand the pool of substrates capable of being used by this bacterium. Hence, these

types of enzymes may contribute to the global carbon cycling processes and for channeling complex substrates into the common catabolic pathways, including recalcitrant organic pollutants.

4 Concluding Remarks and Research Needs

Crude oil pollution and the chemical diversity of its components, in combination with environmental constraints such as depth, oxygen concentration, temperature, nutrient input, and other physical and chemical factors, may distinctly influence microbial populations and the biodegradation processes they mediate in response to accidental crude-oil spills in seawater and seawater sediments. A particular group of marine bacteria, so-called OMHCB, are known to be among those that increase most in response to oil spills. Their analysis by cultivation approaches in combination with genomic and metagenomics analysis has confirmed that they are widespread in marine environments. Their versatile ability to degrade a number of pollutants through a series of catabolic enzymes has also been extensively investigated. Accumulated information exists about their taxonomic diversity, genomes, and genes. However, this understanding has not been as well developed for investigating enzymes with potential biotechnological use. This is particularly noticeably given OMHCB are widespread microorganisms in marine environments, particularly after oil spills, cultivable members of all known genera are available, and genomes are available. Recent investigations of a number of enzymes from key OMHCB members have revealed they contain enzymes, not only supporting the degradation of pollutants to be used as carbon sources, but also other enzymes capable of expanding the potential carbon sources to be used by those bacteria and also capable of converting a wide portfolio of molecules. Accumulated knowledge revealed that the properties of these enzymes, particularly their substrate scope, may be a reflection of the microbial metabolism and its adaptation to the respective environment. The ability of OMHCB enzymes to convert multiple substrates could relevant to their survival in nature as few of them can convert a large number of compounds, normally present simultaneously in natural environment where oil spills may occur. Whatever the biological meaning of the broad substrate scope of some of the OMHCB enzymes, the available data suggest that OMHCB bacteria contain in their genomes multiple ester-hydrolases (at least 15 confirmed in the genome of *A. borkumensis* SK2, and seven in the genome of *Cycloclasticus* sp. 78-ME) capable of converting multiple molecules, while at the same time being stereo-selective, two features highly desired in industrial settings. Recent studies support this suggestion, since a functional genome analysis of the recently described marine bacterium *Pseudomonas aestusnigri* revealed not only the presence of a highly specialized metabolism, but also of 22 different putative esterase genes which represent 0.62% of all predicted coding sequences (Gomila et al. 2017). Actually, there is a need to generate more information that helps to decipher not only the industrial potential of available enzymes from OMHCB, but also to recover new ones by genome mining of already available genomes (Table 1) or metagenomic mining in contaminated

marine sites and to investigate their distribution in the environment and their biochemical and structural properties and biotechnological potential.

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