Edouard Jurkevitch
Robert J. Mitchell *Editors*

The Ecology of Predation at the Microscale

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This book is dedicated to the students, post-doctoral fellows, technicians and administrative staff that, along the years, have immensely contributed to the knowledge and understanding shared in it, and to enable our research.

Edouard Jurkevitch and Robert J. Mitchell

Preface

Fifteen years have elapsed since the publication of "*Predatory Prokaryotes – Biology, Ecology and Evolution*" the first and, as of today, only book describing the prokaryotic predators of prokaryotes. It also addressed some of the ecological issues pertaining to their distribution and a few factors affecting these remarkable organisms, as well as their diversity and how predator–prey dynamics could be explained.

Since then, the study of microbial communities has exploded. Microbial diversity is "larger than ever" and keeps on growing; spatio-temporal distributions of microbes can not only be described in great detail, but the underlying principles structuring their populations and communities are emerging. Genomes are being sequenced by the bucketload, and improved functional annotations, community structure-function analyses, genetic manipulations and other omics of single genomes, as well as of metagenomes, are uncovering novel functions to unknown gene sequences and the roles they play, from the cellular level all the way up to the ecosystem. We know much better the ecology of microbes, how communities are composed, how they fluctuate and what drives their changes; we also grasp nutrient flow between trophic levels and describe some specific interactions in great detail.

Symbiosis at large, as first proposed by Anton de Bary as "a phenomenon in which dissimilar organisms live together", has also greatly benefited from these advances. Yet, this book is on predation and one may ask about the connection between symbiosis, even in its broader sense, and predation. While predation between larger organisms is obvious, predatory interactions between microbes may be a lot more difficult to detect in the environment, or even to define. Is a ciliate phagocytosing a bacterium a predator in the same sense as myxobacteria lysing the colony of a nearby bacterium? Are these different than a *Bdellovibrio* and like organism (BALO) penetrating into the periplasm of another bacterium to feed on it, grow and replicate within it? Or are BALOs better described as parasitoids? To complicate this idea further, BALOs do not need their prey to be alive, as they can grow on dead or on reconstituted cells. So is it a scavenger? Continuing with this train of thought, in larger organisms, predatory features are well defined, i.e. anatomical and physiological adaptations for detecting, catching, killing and digesting prey. This leads

one to wonder about the "guts and claws" of predatory bacteria, be they myxobacteria or BALOs. Nevertheless, significant advances have been made to understand how bacterial predators detect, attach, kill, manipulate and exploit their bacterial prey. These understandings have made it possible to explore their potential as biocontrol agents of deleterious bacteria. One such application is therapy of antibiotic-resistant pathogens in humans and other animals, to provide part of a solution to the expending spread of antibiotic resistance.

The ability of predators to cull bacterial populations, such as specific pathogens or general biomass, renders them attractive for numerous applications. To rationally and wisely apply and exploit this potential, a good knowledge of their ecology is necessary. In this monograph, we tried this blend: bringing together applications and potential along with ecological knowledge of predatory bacteria. Towards this end, the chapter by Sester, Korp and Nett discusses secondary metabolites produced by predatory bacteria, focusing on myxobacteria and BALOs; Furness, Whitworth and Zwarycz detail predatory interactions and dynamics of myxobacteria with their prey at the population and biochemical level. Herencias, Salgado and Prieto explore industrial applications of BALOs, including their "domestication" for use as "cell crackers" and as *in-situ* modifiers of microbially produced biochemicals; Najnine, Cao and Cai describe the application of BALOs as biocontrol agents in aquaculture, and how they reduce pathogen loads, while Jurkevitch addresses BALO population dynamics and their role in wastewater treatment. By summarizing what environmental factors affect BALO predation, including prey effects, as well as physical and chemical variables, Im, Bäcker and Mitchell provide explanatory power to observed behaviours and "dos and don'ts" for applications. Finally, Kuppardt-Kirmse and Chatzinotas remind us that bacterial predators are not immune to themselves being eaten by other predators, and they present the principles of microbial intraguild predation and how this affects predatory networks.

It is the hope of all the authors included in this monograph that these chapters and the information provided within will stimulate young and older scientists alike to entangle the intricate dance between predator and prey at the microbe scale and enjoy the study of these remarkable interactions.

Enjoy!

R. J. Mitchell, A Personal Touch

It is my hope that for many young scientists, like it was for me when I read one of Edouard's articles many years ago, you will be fascinated by and captivated with predatory bacteria and that this passion will grow into a career.

Rehovot, Israel — Edouard Jurkevitch
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Contents

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About the Editors

Edouard Jurkevitch studied Soil and Water Sciences for his first and second degree and Microbiology for his third degree, all at the Hebrew University of Jerusalem (HUJI). He obtained his Ph.D. in Agricultural Microbiology in 1992 for a thesis on ecological and physiological roles of bacterial siderophores. He was a postdoctoral fellow for three years at CNRS in France, where he studied nodule formation in legumes. In 1995, he joined HUJI's Faculty of Agriculture in Rehovot. He has a deep interest in microbial ecology, reflected in his research topics: predatory interactions between bacteria from cellular processes to community interactions; the relationship between feeding strategies in flies and their gut symbionts; and soil microbial forensics, a cutting-edge application of microbial ecology.

Robert J. Mitchell followed his heart and traveled to South Korea, where in 2004 he received his Ph.D. in Environmental Sciences from the Gwangju Institute of Science and Technology (GIST) studying environmental toxicity sensing using bacterial bioreporters. After two postdoctoral fellowships studying oral pathogens and fermentations/bioenergy production, first at Harvard University and then at the Korea Institute of Science and Technology (KIST), respectively, he joined the Ulsan National Institute of Science and Technology where he continued to pursue his career as a professor. His lab has meshed all of his previous experiences to delve deeper into the fields of applied microbiology and pathobiotechnlogy, with a heavy emphasis given towards understanding predatory bacteria.

Predatory Interactions Between Myxobacteria and Their Prey

Eleanor Furness, David E. Whitworth, and Allison Zwarycz

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1 Introduction

In this chapter we explore what is currently known about myxobacterial predation. We provide a general introduction to myxobacterial biology, describing their diversity, distribution and social biology, before considering their predatory behaviour in more detail. Ecological factors affecting predation will be discussed, and rationalised with our current understanding of the predatory mechanisms employed by myxobacteria. We also highlight important gaps in our current knowledge of the ecology of myxobacterial predation.

The predatory strategy exemplified by myxobacteria is communal, involving secretion of predatory material into the shared environment; it has thus been described as group attack, or 'wolf-pack' predation.

2 Wolf-Pack Predation

2.1 The Many Strategies of Microbial Predation

Predatory microbes have evolved to exploit several distinct predatory strategies, which have been recently categorised depending on how predator cells encounter their prey, whether they attach to prey cells, and the molecular mechanisms by which prey cells are killed and consumed (Perez et al. 2016). In some cases the predatory

strategy is not easy to define: it can be difficult to discriminate between strategies (for instance whether predation requires contact between predator and prey cells, or whether cells just need to get very close), and some predators may employ multiple strategies simultaneously. The strategy employed by a predatory organism can even change depending on the prey organism being consumed or on prevailing predator/prey abundance (Perez et al. 2016).

Epibiotic predators attach to prey cells and deliver toxins and hydrolases into the prey cell through specialised secretion systems. The predators remain adhered to the outside of the prey cell while consuming its digested contents, and this predatory strategy is employed by genera including *Vampirovibrio* and *Micavibrio* (Soo et al. 2015; Wang et al. 2011). However, epibiotic predation can exhibit features that are usually associated with other predatory strategies. For instance, the epibiotic predator *Stenotrophomonas maltophilia* secretes diffusible antibiotics, while *Ensifer adhaerens* can attack prey as groups (Jurkevitch and Davidov 2007; Perez et al. 2016).

Endobiotic (or direct invasion) predators have a bi-phasic life-cycle. In attack phase, they hunt for susceptible prey and in growth phase they attach to the prey cell, force their way inside it and replicate within the host cell's cytoplasm or periplasm (Guerrero et al. 1986).

Prokaryotes that use a *group attack* strategy work cooperatively to lyse prey cells, either through the secretion of toxins and digestive enzymes into the extracellular space, or by direct contact with the prey (Perez et al. 2016; Velicer and Mendes-Soares 2009). Because the nutrients released by prey lysis are not privatised or ring-fenced by the predator, non-secreting predators and non-predatory bystanders can also benefit from the released nutrients (Mendes-Soares and Velicer 2013; Whitworth 2011).

2.2 *Myxobacteria Are Group Attackers that Employ a Wolf-Pack Mechanism*

A subset of group attack predators use a wolf-pack strategy, in which the predators use social gliding motility to move alongside prey, allowing subsequent contact-dependent lysis of prey cells (McBride and Zusman 1996; Pan et al. 2013). This is the strategy adopted by members of the myxobacteria, and it has also been described for species of the *Herpetosiphon* genus and *Lysobacter* strains (Livingstone et al. 2018b; Pan et al. 2017; Seccareccia et al. 2015).

As paradigms of the group attack wolf-pack strategy, myxobacteria are thought to require a minimum number of attacking cells (a quorum) in order to lyse prey, and require contact with prey for successful predation (McBride and Zusman 1996; Pan et al. 2013; Rosenberg et al. 1977). These are key defining features for distinguishing between the different strategies and sub-strategies of predation, and yet both features are contentious aspects of myxobacterial predation. For instance, myxobacterial

predators seem capable of killing at a distance through the secretion of diffusible secondary metabolites and outer membrane vesicles (Berleman and Kirby 2009; Evans et al. 2012; Findlay 2016; Xiao et al. 2011), while there is microscopic evidence of single myxobacterial cells being able to lyse prey (Berleman and Kirby 2009; McBride and Zusman 1996; Shilo 1970).

Myxobacteria cannot swim through liquid media, but can swarm slowly over surfaces through gliding motility (Mauriello et al. 2010; Munoz-Dorado et al. 2016; Nan and Zusman 2011). Thus their hunt for prey is considered social, as their motility is social. Without attachment of predator cells to prey cells, there must be transfer of toxins and enzymes from predator to prey through the environment. This predatory strategy therefore is likely to require life on a surface rather than in liquid, to avoid the dilution of secreted toxins/enzymes and ensuring that nutrients released from lysed prey do not get diluted below vital concentrations (Whitworth 2011). A related feature of group attack predation is that since predatory cells do not attach to or invade specific prey, instead secreting cocktails of antimicrobial substances into the extracellular space, they can consequently kill a very broad range of prey organisms (Livingstone et al. 2017; Morgan et al. 2010).

However, while wolf-pack predator prey range is broad, it is also patchy (Livingstone et al. 2017; Morgan et al. 2010), with patterns of prey susceptibility and predatory activity not congruous with phylogeny (of prey or predator). This suggests that both predatory activity and prey resistance are a consequence of multiple genes that are actively evolving – an archetypal microbial arms race that is highly specific to the particular strain of predator and the strain of prey being considered. Nevertheless, even when considering a single prey, the manifestation of predation can vary significantly depending on ecological variables. For instance, while wolf-pack behaviour is observed when prey cells are sparse, with small groups of myxobacteria surrounding prey cells, myxobacteria can also successfully prey upon dense colonies of prey cells (Berleman et al. 2008; Perez et al. 2011, 2014). The term ‘frontal attack’ is preferred for those situations rather than ‘wolf-pack’ (Perez et al. 2016), even though the predatory mechanism employed is likely the same.

Group attack predation by definition requires a group to attack, and it has been proposed that prey killing requires a group because communal secretion is required to reach extracellular concentrations of metabolites/enzymes high enough to trigger prey lysis (Rosenberg et al. 1977; Whitworth 2011). Such cooperativity is a hallmark of most aspects of myxobacterial biology (Whitworth 2008), so to contextualise myxobacterial predation we need to understand their socio-biology; the opportunities it creates, but also the vulnerabilities it exposes.

3 Myxobacterial Cooperativity

3.1 The Myxobacterial Life-Cycle Is Inherently Cooperative

Myxobacteria are facultatively multicellular – individual cells are viable entities in their own right, however, at higher densities cells increasingly interact with each other and new population-level behaviours emerge. Myxobacterial communities feed together through cooperative predation, but myxobacteria also respond to starvation as a community. When starved, a population of *M. xanthus* cells initially aggregates into raised mounds (Kuner and Kaiser 1982). Some cells within the nascent mounds are destined to autolyse, providing fuel for the surviving minority of cells to differentiate into dormant myxospores (Lee et al. 2012). Presumably, such a cooperative behaviour has evolved so that starvation causes myxobacteria to produce a population of myxospores. Therefore when food becomes available again, rather than an individual germinant, a population of germinants are released, – able to immediately start feeding efficiently as a population (Munoz-Dorado et al. 2016). Myxobacteria also cooperate when using motility machinery to move around their environment (Mauriello et al. 2010), when growing vegetatively they can share membrane damage (Vassallo and Wall 2016), charitably supporting less-able individuals between cells and sometimes culling them via outer membrane exchange of toxins (Vassallo et al. 2017). Thus every phase of the myxobacterial life-cycle is inherently cooperative (Fig. 1).

Motility *M. xanthus* can move across a surface by gliding motility, using one or both of two different motility engines (Li et al. 2005; Youderian et al. 2003; Youderian and Hartzell 2006). Each engine works better or worse under different environmental conditions, for instance on different percentage agar plates (Hillesland et al. 2007; Spormann 1999). The two engines are denoted A-motility (for adventurous motility, observed for single cells), and S-motility (for social-motility, requiring cell-cell contact). Myxobacterial cells are rod-shaped and their movement is characterised by gliding in the direction of their long-axis, with periodic reversals of direction (Kaiser and Warrick 2011; Wu et al. 2009). Movement leaves behind trails of slime, and other cells preferentially travel along pre-existing slime-trails or channels within the slime, giving rise to population-level patterns of motility (Berleman et al. 2016; Gloag et al. 2016; Stevens 2000). The engine for S-motility is type-IV pilus extension and retraction (Wu and Kaiser 1995). Pili are extended from the leading pole of a moving cell, and when a pilus tip adheres to EPS (exopolysaccharide) on the surface on another 'target' cell, pilus retraction is triggered, causing the moving cell to pull itself towards the target cell (Li et al. 2003). This engine thus requires cells to be close enough to touch each other with their pili, and S-motility is thought to help myxobacteria maintain population cohesion when migrating outwards during colony growth (Balagam and Igoshin 2015; Gloag et al. 2016; Kaiser and Warrick 2011).

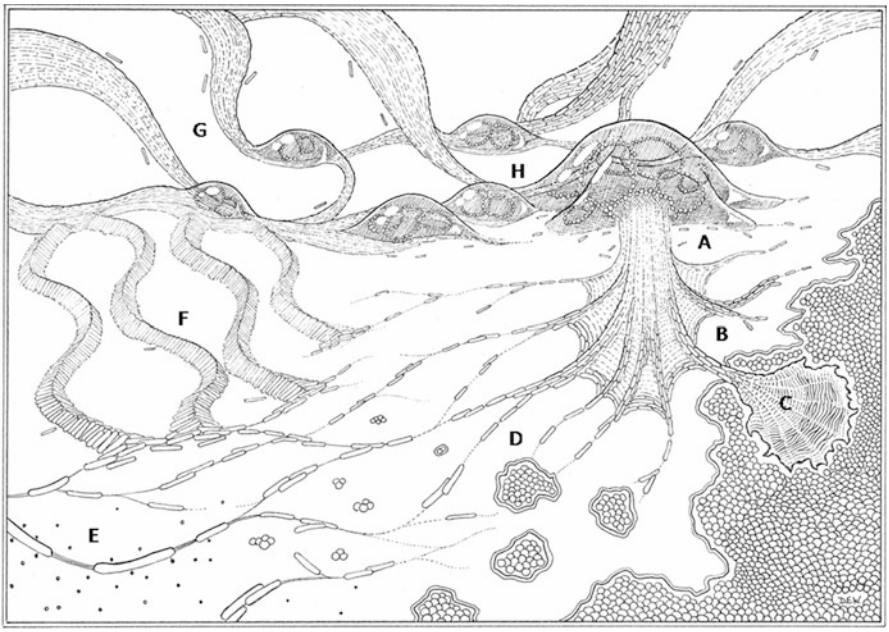

Fig. 1 The life-cycle of predatory myxobacteria. Spores within a fruiting body sense the availability of nutrients and germinate, swarming outwards in search of prey (**a**). As myxobacteria approach a prey colony, they can make a frontal attack (**b**), with predator cells penetrating the prey colony and rippling as they consume the prey (**c**). Alternatively, small groups of predatory cells move between patches of prey along slime-trails in a 'wolf-pack' mode of predation (**d**). Killing of prey is due to the secretion of toxins, enzymes and outer membrane vesicles (**e**). When all prey has been consumed and predatory cells are starving, they secrete A-signal and associate into rippling ridges of aligned cells (**f**). As starvation proceeds, ripples develop into streams of cells, which collide and form small, motile aggregates (**g**). Aggregates become progressively larger until the form a static fruiting body, within which cells differentiate into spores (**h**)

Cell-Cell Transport Adjacent *M. xanthus* cells are able to engage in a phenomenon called outer membrane exchange (OME). Cells belonging to the same TraA-mediated compatibility class are able to transiently fuse outer membranes, and exchange material between themselves (Pathak and Wall 2012; Pathak et al. 2012). This material can include membrane components, and this arguably allows distribution of membrane damage across all cells in a population and in doing so charitably enables individual cells to overcome otherwise fatal membrane damage (Vassallo et al. 2015; Vassallo and Wall 2016; Whitworth 2017). However, a similar, but surprisingly uncharitable phenomenon is also potentially used by *M. xanthus* to kill 'less-fit' siblings, with delivery of toxins via type VI secretion systems (Troselj et al. 2018). OME also enables exchange of toxins, killing non-kin that don't have the required anti-toxin, potentially promoting clonality of the population (Vassallo et al. 2017), although whether this mechanism operates in nature is unclear (Wielgoss et al. 2018).

Aggregation and Fruiting When a population of myxobacteria becomes starved, cells initially aggregate to form raised mounds. These mounds can migrate, split and merge, before stabilising into static fruiting bodies containing myxospores (Curtis et al. 2007b; Xie et al. 2011). Aggregation is preceded by a phenomenon called rippling, in which cells align side-by-side into ridges, which move backwards and forwards reflecting off one another (Igoshin et al. 2001; Welch and Kaiser 2001). Rippling is associated with a higher reversal frequency than during vegetative growth, but as starvation continues, reversal is suppressed and cells aggregate into motile streams of cells, whose 'collision' seems to nucleate aggregate formation (Cotter et al. 2017; Holmes et al. 2010). The aggregation phase of the life-cycle is regulated by a complicated gene-regulatory network and co-ordination of the population's behaviour is achieved through the exchange of two main inter-cellular signals (Kaiser 2004).

Intercellular Signalling The first signal (A-signal) is a mixture of peptides, proteases and the amino acids they generate (Kuspa et al. 1992a, b). It is believed to act as a quorum-signal, with the amount of A-signal indicative of the number of cells present and how starved they are (Kaplan and Plamann 1996). A-signalling leads in turn to the production of C-signal, a later signal of aggregation. C-signal is exchanged on cell-cell contact between cells in the population, with levels of C-signalling increasing with cell density as aggregation proceeds and cells find themselves in ever more intimate association with one another (Ellehauge et al. 1998; Sogaard-Andersen et al. 2003). C-signalling seems to be responsible for the increase and then decrease in reversal frequency exhibited during development, through regulation of the Frz chemosensory system (Igoshin et al. 2004; Jelsbak and Sogaard-Andersen 1999). It should be noted however, that there are conflicting models of how the C-signal (the CsgA protein) actually acts, whether it is a protein that docks with a receptor, or an enzymatic activity (Boynton and Shimkets 2015; Konovalova et al. 2012; Rolbetzki et al. 2008).

Differentiation At the high cell-densities found within fruiting bodies, C-signalling is high enough to trigger differentiation into myxospores. The peptidoglycan of cells is remodelled, changing the cells from rods into spherical spores, spore coats proteins are expressed and a polysaccharide coat is produced, encapsulating the myxospore (Bui et al. 2009; Dahl et al. 2007; Muller et al. 2010). However, spore formation is not the only example of differentiation during fruiting. Some cells are left outside fruiting bodies, and these 'peripheral rods' are thought to act as scouts for the availability of prey (O'Connor and Zusman 1991). In addition, more than 90% of cells entering the nascent fruiting body do not end up as myxospores, instead they are destined to lyse (Lee et al. 2012). Fruiting body sporulation is a process that takes days, yet is triggered by starvation, and autolysis of the majority of cells is likely required to provide surviving cells with the energy and nutrients needed to finish the developmental process (Wireman and Dworkin 1977).

Perils of Cooperation Cooperative societies are vulnerable to exploitation by non-cooperative individuals, and this is true of myxobacterial societies as much as

it is for human and other animal groups (Fiegna and Velicer 2003; Travisano and Velicer 2004). A myxobacterial mutant, whose genotype results in it not undergoing developmental lysis, will increase its proportion within a cooperative population. The fitness advantage of such 'cheating' is greatest when the cheat is a small minority of the population. When a population is predominantly composed of cheats, then it can face extinction, as not enough autolysis occurs to fuel sporulation (Fiegna and Velicer 2003; Velicer et al. 2000). Mechanisms have evolved to purge populations of cheats and to reduce the burden of cheaters on a population (Travisano and Velicer 2004; Velicer 2005). Various aspects of the myxobacterial cycle may have evolved as cheater-resistance mechanisms (Travisano and Velicer 2004; Velicer 2005). For instance, the amino acids of A-signal are particularly costly to synthesise, and their secretion as an early starvation signal may entice cheats/competitor strains to grow on the A-signal, alerting cooperative secretor cells to abort development (Whitworth 2015), while population bottlenecks enhance selection against sub-populations that contain cheats (Brockhurst 2007).

3.2 Cooperativity During Predation

Unlike epibiotic and endobiotic predatory strategies, group attack is reportedly a highly cooperative process. Members of the population secrete toxins and digestive enzymes into the surrounding milieu, and prey lysis releases nutrients into the same space. Thus predatory activity appears cooperative as it happens in a public space, which all members of the population can contribute to, and take from (Perez et al. 2016; Velicer and Mendes-Soares 2009).

In their now classic experiment, Rosenberg et al. (1977) studied growth of *M. xanthus* at different cell densities in shaken cultures. Cells were provided with either casein (protein), or hydrolysed casein as sole carbon and energy source. They found that the per cell growth rate was faster at higher cell densities when growing on casein, but was not density-dependent on hydrolysed casein. Their interpretation was that the rate-limiting step in *M. xanthus* growth was hydrolysis of casein into peptides and amino acids for cellular uptake. Providing pre-hydrolysed casein allowed every cell to grow at its maximum growth rate, but when provided with casein, the amount of liberated peptides and amino acids was dependent on secreted proteases. When more cells secreted protease, there was a disproportionate increase in the amount of available peptides/amino acids, allowing each cell to grow faster (Rosenberg et al. 1977). This lead to the proposal that myxobacterial predation is cooperative, although the unnatural system employed by the Rosenberg et al., experiment makes the extrapolation to predation on a surface debatable (Marshall and Whitworth 2019).

Nevertheless, myxobacterial predation also exhibits other features that rely on cooperation. As cells migrate through a prey colony they ripple (Fig. 2), as they do during starvation-induced aggregation (Berleman et al. 2006). The purpose of rippling during predation, if any, is unclear. It is possible that ripple formation is

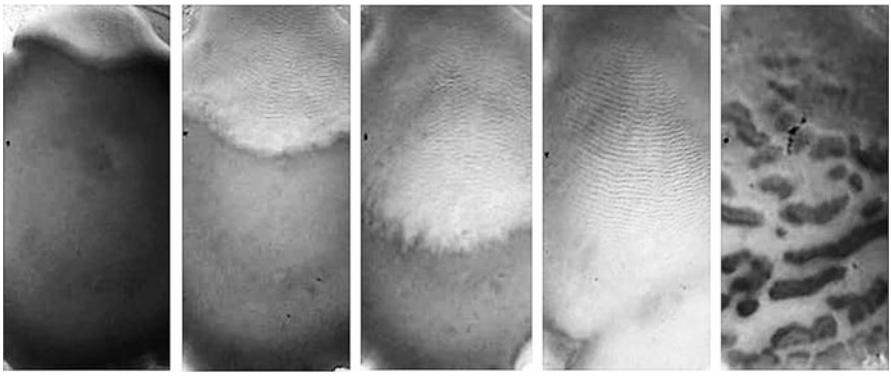

Fig. 2 Predation time-lapse. From left (day 1) to right (day 5), a myxobacterial isolate can be seen (top of the first image) progressively consuming a colony of *Escherichia coli* prey (darker mass comprising most of the initial image). With each day, the myxobacterial colony extends further into the prey, exhibiting rippling behaviour in the middle three images. Finally, upon complete prey depletion, fruiting bodies are left in the wake of predation (final image)

merely a behaviour that emerges when a population of cells has an increased reversal frequency. Increasing reversal frequency results in cells migrating less per unit time, and may be a beneficial adaptation during feeding (Zhang et al. 2012). Some authors argue that rippling is a predatory behaviour (predataxis), but whether rippling increases the efficiency of predation, or is merely coincident with predation remains to be seen (Berleman et al. 2006, 2008; Berleman and Kirby 2009; Zhang et al. 2012).

Another cooperative behaviour associated with predation is multicellular development (Berleman and Kirby 2007). Fruiting body formation is usually studied in monoculture by plating myxobacteria onto starvation medium. However, in experiments where spots of *M. xanthus* were plated alongside prey, fruiting was observed when the myxobacteria moved from an area of prey abundance to regions of relatively scarce prey. Conversely, encountering more prey impeded fruiting body formation. Thus fruiting body formation can be initiated independently of starvation, seemingly driven by interactions between the predator and prey (Berleman and Kirby 2007).

As with any other cooperative trait, myxobacterial predation is presumably open to exploitation by cheating genotypes. Cheats that did not secrete enzymes/toxins would presumably be fitter than secretor genotypes when at a minority, however would be incapable of predation when in pure culture. During wolf-pack mode predation, a small pack size would create a genetic bottleneck that could help purify the population of cheating genotypes (Brockhurst 2007). In small packs, the presence of a non-secretor would make a pack less competitive than a similar sized pack lacking cheats, whereas in larger packs the presence of a cheat would impose a negligible fitness penalty. If packs are generally small in the wild, then they will also amplify the effects of cheaters in subsequent stages of the life-cycle. For instance, if a hunting pack with five members contained a non-lysing developmental cheat, the

resulting population would become 20% cheat, and when undergoing development would be at a significant disadvantage compared to a population seeded from a pack of five cooperators.

While cheating during development has been investigated thoroughly (Velicer and Vos 2009), and predation has implications for cheater control throughout the life-cycle, predatory cheating *per se* remains to be demonstrated. It is also not clear whether there is a difference in behaviour towards cheating kin and non-kin competitors. Does clonal expansion foster cooperation with neighbouring cells as a general strategy, or is kin discrimination used to restrict cooperation to relatives? How closely related do kin have to be before they are considered kin to cooperate with, rather than competition? How often do myxobacteria encounter each other in the soil, and how different are they? Before even attempting to answer such questions, we first need to understand myxobacterial diversity and ecology.

4 Myxobacterial Diversity and Ecology

Myxobacteria (order Myxococcales) are diverse, abundant and widely distributed (Dawid 2000). Pure cultures of myxobacteria have typically been isolated by taking advantage of their ability to grow on either paper or prey organisms. They have consequently been generally classified into two (overlapping) functional groups: the cellulolytic and bacteriolytic myxobacteria, a grouping which broadly correlates with the formal taxonomy of the order.

4.1 Myxobacterial Taxonomy

The order Myxococcales, along with their close relatives the sulphate and sulphur reducing bacteria, belong to the class Deltaproteobacteria (Shimkets and Woese 1992). Presently, the myxobacteria are divided into three sub-orders, the basal Cystobacterineae (which includes the single most studied myxobacterial species, *M. xanthus*), and its two sister taxa Sorangiineae and Nannocystineae. Within the three sub-orders, eight families, around 30 genera, and nearly 60 species have been validly described to date (Garcia et al. 2010; Mohr 2018; Shimkets et al. 2006). This current taxonomic classification will inevitably expand – just 25 years ago only 2 sub-orders, 4 families, 12 genera and 38 species were known (Mohr 2018).

The majority of phylogenetic studies have examined myxobacteria from terrestrial environments (Dawid 2000; Garcia et al. 2010; Reichenbach 1999; Sproer et al. 1999), however, recent studies have included marine myxobacterial cultures and/or DNA sequences (Brinkhoff et al. 2012; Iizuka et al. 1998; Jiang et al. 2010). Marine myxobacteria are evolutionarily divergent from terrestrial species (Jiang et al. 2010), and an exclusively marine myxobacteria cluster (MMC) was recently found to be phylogenetically distinct from the three currently recognised suborders (Brinkhoff

et al. 2012). Nevertheless, it is not just the MMC that contains marine or salt tolerant myxobacteria; the Nannocystineae suborder (particularly the genera *Plesiocystis* and *Haliangium*) contain several halotolerant and halophilic bacteria (Albataineh and Stevens 2018; Garcia et al. 2011; Wrótniak-Drzewiecka et al. 2015).

It seems certain that as more exotic environments are sampled for myxobacteria, further novel clades within the order will be discovered, exposing the true diversity of predatory myxobacteria in the environment (Mohr et al. 2017, 2018).

4.2 The Environmental Distribution of Myxobacteria

Myxobacteria are virtually ubiquitous, but are most numerous in temperate and tropical nutrient-rich soils. They have been found in samples taken from all over the world, from a wide range of environments, including Antarctica, seawater and marine sediments, hot springs, glaciers, animal dung, sand, seed and leaf surfaces, cave wall biofilms, and from the fruiting bodies of several basidiomycete fungi (Dawid 2000; Iizuka et al. 2006; Powell et al. 2015; Reichenbach 1993, 1999; Zhang et al. 2005).

As well as being widely distributed, the myxobacteria are abundant. Zhou et al. (2014) analysed a variety of terrestrial soils and found that the total proportion of myxobacteria in the soil bacterial community ranged from 0.4% to a substantial 4.5%. In one Chinese soil the species diversity of the myxobacteria was second only to the actinobacteria, and at 4.1% of the total rRNA signal, were the fifth most prevalent family of bacteria (Zhou et al. 2014).

Of terrestrial myxobacterial bacteriolytic species, *Corallococus* spp. and *Myxococcus* spp. tend to dominate isolate collections (Charousova et al. 2017; Livingstone et al. 2017; Mohr et al. 2016; Zhang et al. 2013). For example, in a set of 113 bacteriolytic myxobacterial strains isolated from the UK, 70% were *Corallococus* spp. and 24% were *Myxococcus* spp. (Livingstone et al. 2017).

Far fewer estuarine/marine myxobacteria have been successfully isolated to date, with just five species validated taxonomically (Garcia et al. 2018), and six draft genome sequences available (Gemperlein et al. 2018). However, metagenomic sequencing has begun to provide insights into the ecological presence and significance of marine myxobacteria. The distribution and abundance of myxobacteria in marine sediment samples was assessed by Brinkhoff et al. (2012). Marine myxobacteria were detected in almost all of the samples and constituted up to 13% of the total bacterial 16S rRNA genes in North Sea surface sediment samples. This high proportion contrasts with the lower abundance found in samples from other regions, which ranged from 0.01% to 0.71% (Brinkhoff et al. 2012).

As myxobacteria have evolved to thrive in very different environments, we need to be mindful that the mechanisms of predation that they employ in one niche may be specialisations that aren't required for predation by myxobacteria living in other ecological niches.

5 The Ecology of Myxobacterial Predation

5.1 Arms Races at the Micro-scale

It is not yet known whether all myxobacteria are predatory, however it seems likely that it is a common feature of the order; predators have been isolated from all three myxobacterial sub-orders, including representatives from the *Chondromyces*, *Corallococcus*, *Enhygromyxa*, *Myxococcus*, *Plesiocystis*, *Pyxidicoccus, Racemicystis*, *Sorangium* and *Stigmatella* genera (Amiri Moghaddam et al. 2016, 2018; Awal et al. 2016; Livingstone et al. 2017; Perez et al. 2016).

Myxobacteria can prey upon a range of micro-organisms, including fungi and Gram-negative as well as Gram-positive species of bacteria (Findlay 2016; Livingstone et al. 2017; Morgan et al. 2010). Documented soil-dwelling organisms that myxobacteria can prey upon include *Arthrobacter globiformus, Bacillus* spp., *Curtobacterium citreum, Cytophaga johnsonae, Comamonas testosteroni, Fusarium roseum, Pseudomonas* spp., *Rhizobium vitis, Sinorhizobium* spp., *Sphingobium yanoikuyae* and *Xanthomonas* spp. (Bull et al. 2002; Morgan et al. 2010). While prey range is extremely broad, it is also patchy, with predatory strains exhibiting idiosyncratic patterns of activity against prey (Livingstone et al. 2017; Morgan et al. 2010). A lack of correlation between predatory activity/prey susceptibility and phylogeny (of both predator and prey organisms), implies that individual strains have evolved bespoke predatory and resistance mechanisms facilitated by horizontal gene acquisition – manifestations of an ongoing microbial arms-race (Livingstone et al. 2017).

Participation in the microbial arms race also seems necessary for the maintenance of predatory activity, as the predatory ability of *M. xanthus* diminishes in laboratory strains (Velicer and Stredwick 2002). The model myxobacterium *M. xanthus* DK1622 is ranked as a particularly poor predator compared to freshly isolated *Myxococcus* spp. strains (Livingstone et al. 2017), a situation also observed when comparing the predatory activity of *Corallococcus* spp. type strains with that of wild isolates (Livingstone et al. 2018c).

It would be expected that due to the diverse range of prey that they can consume, myxobacterial species could have a profound influence on the composition of microbial ecosystems. By introducing labelled biomass carbon in the form of *Escherichia coli* prey into soil, flow through the microbial food web could be traced (Lueders et al. 2006). In their recent study, Zhang and Lueders (2017) added the ^{13}C-labelled prey organisms *Pseudomonas putida* and *A. globiformis* to microcosms of soil and found that just a select few species of Myxococcales assimilated ^{13}C from *A. globiformis* but a far greater diversity of myxobacteria incorporated ^{13}C from *P. putida*, and at a faster rate. Thus, the available prey in an ecosystem dictates which myxobacteria can prey upon them. This implies a degree of specialism by some myxobacterial taxa, while others seem to act as more generalist predators. Specialists were also observed among the generalists when the prey range of isolated myxobacteria was assessed, with some strains exhibiting exceptional predatory

activity, but only against specific prey species (Livingstone et al. 2017; Morgan et al. 2010).

The prey range exhibited by predators is not just a consequence of them specialising to hunt particular prey; it is also a consequence of prey evolving defence mechanisms which work more or less well against different predatory strains. A variety of general protective mechanisms serve to protect would-be prey. Biofilm formation provides a physical barrier against predation (Hall-Stoodley et al. 2004), while other defensive strategies such as dormancy (DePas et al. 2014), toxin release, retreating quickly, and surface masking (Matz and Kjelleberg 2005) are also employed. *E. coli* prey responds to the presence of *M. xanthus* by both up-regulating and down-regulating hundreds of genes, in particular those encoding ribosome and lipopolysaccharide synthetic pathways, suggesting these may be the molecular targets of predation (Livingstone et al. 2018a).

Some prey species also exhibit specialised defence mechanisms against predation by *M. xanthus*. Production of exopolysaccharide galactoglucan by *Sinorhizobium meliloti* makes the colony resistant to frontal attack, while *Streptomyces coelicolor* induces production of the antibiotic actinorhodin when attacked (Perez et al. 2011, 2014). *Bacillus subtilis* responds to predation by producing the secondary metabolite bacillaene and/or by producing megastructures which are resistant to predation (Muller et al. 2014, 2015, 2016). Nevertheless, the prey response to predation is generally poorly understood and further exploration is needed to understand the targets of predatory attack and mechanisms that can convey resistance.

In addition to preying upon their soil co-inhabitants, myxobacteria are also able to prey broadly upon organisms more typically associated with the hospital environment, including *E. coli, Pseudomonas aeruginosa, Klebsiella pneumoniae, Proteus mirabilis, Staphylococcus aureus, Staphylococcus epidermidis, Enterococcus faecalis* and *C. albicans* (Livingstone et al. 2017). Globally, governments, health care authorities and the scientific community are responding to the threat of a possible return to a pre-antibiotic era, brought about by the spread of antimicrobial resistance (Moore et al. 2017). Amongst those bacterial pathogens of highest concern are the so called "ESKAPE", nosocomial pathogens resistant to many standard antibiotics, including *Enterococcus faecium, S. aureus, K. pneumoniae, Acinetobacter baumannii, P. aeruginosa* and *Enterobacter* spp. (Rice 2008). As known predators of several ESKAPE organisms, and likely predators of the others, myxobacteria are receiving increased research attention as prospective sources of novel antimicrobials.

5.2 Ecological Determinants of Predation

Cell Density In addition to the intrinsic activity of the predator and the susceptibility of its prey, many ecological factors are known to affect the efficiency of myxobacterial predation (Hillesland et al. 2007). Density of predator is thought to be critically important, as high concentrations of secreted hydrolytic enzymes are

required to provide enough hydrolysis product (for example amino acids from digested protein) to support cellular growth (Rosenberg et al. 1977). Predatory behaviour is also affected by prey density. In experimental evolution experiments, under conditions where prey were sparse, predators evolved increased search rates, although the increase in foraging proficiency was accompanied by a reduction in the ability to produce fruiting bodies during starvation (Hillesland et al. 2009). The ability to find prey is determined not only by the density of prey patches, but also by the solidity of the medium (Hillesland et al. 2007), with hard agar supporting greater predatory efficiency. Hard agar is known to be a preferred substrate for A-motility, whereas S-motility favours softer surfaces (Shi and Zusman 1993b).

Motility The importance of A-motility during predation was also demonstrated in experiments using single mutants with defects in A-motility genes or S-motility genes and double mutants lacking both A- and S-motility (Pham et al. 2005). Even though A- and S-motility systems work synergistically, with wild-type colonies spreading faster than the sum of individual A+S− and A−S+ cells (Mauriello et al. 2010), the A-motility system appears to be essential for predation. Using assays of swarm size expansion through a lawn of prey, mutations in A-motility reduced predatory efficiency by 95% compared to wild-type. However, the effect was not due purely to defects in motility, as swarm expansion in the absence of prey was reduced by only 40–60% of wild-type (Pham et al. 2005). In contrast, mutants in S-motility exhibited reductions in swarm size that were independent of the presence of prey. Predatory activity was completely abolished in double mutants lacking both motility systems (Pham et al. 2005).

Luring While the ability to move over a surface is required for a predator to move towards prey, myxobacterial predators may also attract prey to themselves (Shi and Zusman 1993a). This could be a response to the secretion of the early starvation A-signal by the myxobacteria, which the prey could perceive as food (Findlay 2016). Consistent with this suggestion, movement toward *M. xanthus* was considerably reduced in prey containing deletions in *tsr* or *tar*, genes needed for chemotactic responses to free serine and aspartate, respectively (Shi and Zusman 1993a). Similarly, mutants in A-signalling and other early developmental genes exhibit reduced predatory efficiency (Pham et al. 2005), however mutations in late developmental genes did not. Together such evidence suggests that the myxobacteria population is poised between predation when prey is available and development when prey is not. Conditions that reduce initiation of development tend to also enhance predation, while conditions detrimental to predation also stimulate development. This could be just a consequence of prey being a nutrient source, however genetic mutations and exogenous signalling compounds also seem to affect the poise between predation/fruiting (Lloyd and Whitworth 2017; Pham et al. 2005), implying integrated genetic regulation underlying the predation/development choice.

Sensitivity to Prey Myxobacterial genomes encode a plethora of regulatory proteins, particularly those belonging to two-component system (TCS) signal transduction pathways (Whitworth and Cock 2008a; Whitworth 2012, 2015). Most of the

characterised TCSs of myxobacteria are involved in the developmental programme (Whitworth and Cock 2008b), however some are known to respond to nutrient levels and other physicochemical stimuli (Kimura et al. 2001; Ueki and Inouye 2002; Whitworth et al. 2008). Many genes that were originally identified as developmental genes, because mutating them blocked development or initiated premature development, have since been shown to act as sensors of nutrients and presumably therefore 'prey sensors' (Diodati et al. 2008). Internal 'cellular' starvation within the cell is signalled by the stringent response (via production of the alarmone (p)ppGpp), while sensors of 'population' starvation include genes involved with A-signalling, such as the TCS proteins AsgD, SpdR and the Che3 chemosensory system proteins (Diodati et al. 2008). Disrupting such 'early' developmental genes (including those regulating A-signalling), tends to have more deleterious effects on predation than disrupting 'late' developmental genes, such as those regulating C-signalling (Pham et al. 2005). Many early developmental nutrient sensor genes act by inhibiting fruiting in the presence of nutrients, and disrupting them leads to fruiting body formation despite the presence of nutrients (Diodati et al. 2008). This probably explains why nutrient sensor genes cause significant reductions in predatory activity when deleted, as population behaviour is unbalanced, towards fruiting and away from predation.

Insensitivity to Prey There are a huge number of regulatory genes in the *M. xanthus* genome, and when starvation is sensed, an extremely complex gene-regulatory programme is activated. Therefore, it would be expected that *M. xanthus* should also be highly sensitive to the presence of prey, with the sensation of prey leading to differential regulation of many aspects of predatory behaviour. Nevertheless, *M. xanthus* does not appear to respond in this way. Transcriptome sequencing showed that when *M. xanthus* was mixed with live *E. coli* cells, only 12 of its 7300 genes were induced more than twofold in response, while 40% of the prey's genes exhibited significant changes in gene expression (Livingstone et al. 2018a). However, when *M. xanthus* was exposed to pre-killed prey 1319 of its genes were differentially expressed. *M. xanthus* therefore seems highly sensitive to the presence of nutrients, but when those nutrients are locked away within a prey cell, it does not perceive those prey cells as food *per se* (Livingstone et al. 2018a). This is consistent with experiments which observed the behaviour of *M. xanthus* in the presence of prey; an *M. xanthus* swarm will expand in all directions equally, even when spotted next to a colony of prey (Berleman et al. 2006). Yet, while *M. xanthus* remains apparently unaffected by the presence of whole prey cells in some experiments, there is evidence it may respond to indirect physicochemical indicators of prey presence, for example by elasticotaxis.

Elasticotaxis Stanier originally described myxobacteria directing their movement in response to elastic forces, which he termed elasticotaxis (Stanier 1942). As an agar surface was put under tension, *Corallococcus exiguus* grew rapidly perpendicularly to the applied force, but much slower in parallel to the force (Stanier 1942). In 1983, Dworkin noted the A-motility dependent directed movement of *M. xanthus* swarms in response to a physical force produced by glass/latex beads and suggested that elasticotaxis was responsible (Dworkin 1983). Using mutants of A- and/or

S-motility, it was later shown that elasticotaxis depends entirely on A-motility, whereas S-motility was not required (Fontes and Kaiser 1999). It has been suggested that elasticotaxis could be a method of locating prey, consistent with its requirement for A-motility. An elastic surface, generated from the secretion of exopolysaccharide polymers by bacterial colonies growing in the soil, would be deformed by a 'force' exerted by the presence of prey cells. This would provoke an elasticotactic response from myxobacteria nearby, increasing the chance that the predators would encounter the bacterial colony (Dworkin 1983; Fontes and Kaiser 1999).

Osmotic Strength Myxobacteria may also use osmotic pressure as another indirect mechanism for detecting prey. When exposed to live *E. coli* prey, the only three genes up-regulated more than fourfold in *M. xanthus* DK1622 were members of the *kdp* regulon (Livingstone et al. 2018a), which is best known for responding to changes in osmotic strength (Ballal et al. 2007). The *kdp* regulon has been shown to be induced by osmotic stress in *M. xanthus* (Livingstone et al. 2018a), leading to the proposal that in the wild the *kdp* regulon might be induced by the presence of prey indirectly, via changes in osmolarity.

Regulated Motility Myxobacteria cells move backwards and forwards in the direction of their long axis, and net migration is achieved by altering the frequency of cellular reversals. Reversal frequency is regulated by chemosensory signal transduction systems (such as the Frz and Dif systems), which reverse the polarity of both the A- and S-motility engines (Li et al. 2005). McBride and Zusman (1996) examined the behaviour of both wild-type and *frz* mutant *M. xanthus* cells in response to *E. coli* prey and found that wild-type responded to prey upon physical contact, remaining in a colony of prey until all the prey was depleted. In contrast, *frz* mutants only digested some of the prey, abandoning the colony before the prey source was exhausted (McBride and Zusman 1996). Kearns and Shimkets (1998) found that cellular reversals by *M. xanthus* could be suppressed by increasing concentrations of phosphatidylethanolamine (PE), suggesting that lipids acted as chemoattractants. The current model of fatty acid directed motility suggests that PE released by killed prey acts as an attractant (this time through the Dif chemosensory pathway), maintaining the position of the predator within the prey colony and driving recruitment of additional myxobacterial cells (Bonner and Shimkets 2006).

Eavesdropping In order to orchestrate quorum-requiring behaviours, many bacteria signal to each other that they are present, through the secretion of quorum signalling (QS) chemicals (Miller and Bassler 2001). A major class of QS molecules commonly used by Gram-negative bacteria are the N-acyl homoserine lactones (AHLs). *M. xanthus* does not make AHLs itself, but AHLs are produced by many of its potential prey organisms, including *Serratia marcescens*, *Pseudomonas syringae* and *P. aeruginosa*. Addition of AHLs was found to delay sporulation of vegetative cells and to stimulate germination of myxospores, tipping the balance of predatory/developmental behaviour of the population towards predation (Lloyd and Whitworth 2017). Additionally, AHLs stimulated the predatory activity and expansion rates of *M. xanthus* colonies. Therefore, it seems likely that in the wild myxobacteria use QS

molecules as indicators of the presence of nearby prey, essentially eavesdropping on prey conversations (Lloyd and Whitworth 2017).

5.3 Population Structure

Predatory activity isn't just affected by the predator and prey. Wolf-pack predation involves secreting toxins and digestive enzymes into a public space, and the nutrients released from lysed prey also accumulate in that public space. This means that bystanders can affect predation, usually by taking released nutrients for themselves. Such bystanders thus act as competitors, and potentially include different species from the prey and predator, non-secreting kin of the predators, or even predation-resistant kin of the prey.

Non-secreting kin of predators can be considered as cheats as they do not incur the metabolic costs of secreting predatory metabolites/enzymes and would therefore out-compete cooperatively secreting kin. However, at high proportions cheats would presumably harm the population, as there would potentially not be enough secretion to kill prey. Cheats can cause extinction of the whole population, while some populations can tolerate large proportions of cheats, and other populations purge themselves of cheats (Fiegna and Velicer 2003). Various mechanisms to reduce the impact of cheaters can evolve quickly, but the molecular basis of such policing mechanisms are usually hard to define (Amherd et al. 2018; Manhes and Velicer 2011; Travisano and Velicer 2004; Velicer and Vos 2009).

Even genetically similar isolates of *M. xanthus* display antagonistic behaviours towards one another when mixed to create chimeric populations, suggesting that sibling lineages rapidly diverge from one another and that it is possible to discriminate between kin (Rendueles et al. 2015b). As might be expected, antagonisms upon chimerism were stronger between isolates originating from distant samples, than between those that were taken from centimeters apart (Vos and Velicer 2009). But close proximity cannot guarantee cooperation between related strains, as non-cooperating genotypes can emerge by mutation even from within genetically cooperative clonal populations (Velicer et al. 2000). Social antagonism potentially reduces the risk of exploitation by cheating or defecting genotypes, as it inhibits free mixing of genotypes, yet natural populations are often found to be chimeras of strains possessing different social genotypes (Rendueles et al. 2015a).

Non-kin competitors will be frequently encountered in the complex community that resides in soil. Unless the released lysis products of killed prey are somehow privatised by the predators, they would be available for uptake by any and all competitors to fuel growth. Myxobacteria grow very slowly even under optimised laboratory conditions, with a doubling time of 3 h being typical in rich medium for *M. xanthus*. Therefore, many microbes can outgrow myxobacteria, and getting rid of contaminating organisms is a consistent problem when isolating myxobacteria from soil samples. It is quite possible that even predation-susceptible microbes could parasitise myxobacterial populations by outgrowing them, with parasitism even

more likely for predation-resistant organisms. Perhaps this explains the residual contamination found in cultures of many myxobacterial type strains lodged in culture collections across the world.

Many of the ecological determinants of predatory success are dependent on the mode of predation, and the molecular mechanisms of predation need to be considered if we want to develop a mechanistic rather than phenomenological understanding of wolf-pack predation.

6 Mechanisms of 'Wolf-Pack' Predation

6.1 Toxic Secretions

Although individual *M. xanthus* cells have been shown to consume *E. coli*, multicellular 'wolf-pack' predation is usually observed (McBride and Zusman 1996). Wolf-pack predation relies on the cooperative release of hydrolytic enzymes and secondary metabolites, which lyse prey and provide nutrients to be shared amongst the predators. Multicellular predation provides many benefits to the community compared to single-cell predation, such as higher levels of secreted hydrolytic enzymes and more cells for contact-mediated killing. Secreted factors are able to migrate, allowing killing to be contact-independent and accessing niches that the producing cells cannot physically reach. Myxobacteria appear to prefer hard surfaces when searching for prey, but show no difference in killing efficiency on different surface types (Hillesland et al. 2007). Nor does *M. xanthus* respond transcriptionally when presented with live *E. coli* cells (Livingstone et al. 2018a), suggesting that *M. xanthus* constitutively secretes its repertoire of lytic factors, regardless of environment, prey presence/absence and/or the nature of that prey.

6.2 Secondary Metabolites

The secondary metabolites produced by bacteria have many diverse roles, including communication (eg. AHLs), nutrient uptake (siderophores), light protection (carotenoids), as well as virulence, predation, and protection from competitors/predators (Nett (2019), and Chapters of this book by Sester et al. "Secondary Metabolism of Predatory Bacteria", and Im et al. "Environmental and Biotic Factors Impacting the Activities of *Bdellovibrio bacteriovorus*"). Myxobacteria are of particular interest with regard to secondary metabolites because they produce a massive range of bioactive compounds. Most myxobacteria produce several metabolites and the complement of metabolites produced can vary substantially between isolates from within the same species (Livingstone et al. 2018c). In 2010, 67 structures of myxobacterial metabolites had been described, with a further 500 chemical derivatives (Garcia et al. 2009; Weissman and Muller 2009; Weissman and Muller 2010).

In 2016, the list had increased by 42 (total of 109), illustrating the increasing pace of bioactives discovery (Herrmann et al. 2017). The described metabolites are mostly novel compounds belonging to a variety of structural classes, with several compounds existing as multiple structural variants.

The link between antibiotic biosynthesis and predatory activity has been long considered (Berleman and Kirby 2009; Korp et al. 2016; Xiao et al. 2011). Interestingly, myxobacteria seem to produce these bioactive compounds during their exponential growth suggesting that a fitness benefit is provided during bacterial feeding and cell division by these compounds. This differs remarkably from the bioactive compounds of *Streptomyces,* for example, which are mostly produced during nutrient limitation (Findlay 2016). Examples of antibiotics from myxobacteria include althiomycin, enhygrolide A, corallopyronins, cystobactamids, gulmirecins, myxopyronins and myxovirescin (Korp et al. 2016; Muddala et al. 2017).

Many secondary metabolite biosynthetic gene clusters (BGCs) are cryptic – it is not known under what conditions they are expressed, and therefore it can only be predicted what metabolites they produce. Nevertheless, tools for metabolite prediction are available and it has become clear that there exists the genetic information for tens of metabolites encoded in the large genomes of each myxobacterium (Livingstone et al. 2018c; Panter et al. 2018; Schneiker et al. 2007).

While it is known that myxobacteria secrete diverse metabolites, many with antimicrobial activity, it is not yet clear whether myxobacterial strains have evolved to secrete a particular antimicrobial which confers a selective advantage during predation on a particular prey organism, or whether they have evolved to generally secrete a cocktail of functionally redundant metabolites. Potentially the secreted metabolites are just toxic by-products of metabolism against which producing organisms have evolved resistance, with the producers then evolving to feed on the nutrients resulting from the killing of surrounding organisms (for more details on secondary metabolites of predatory bacteria, see Chapter "Secondary Metabolism of Predatory Bacteria" by Sester et al.).

6.3 Outer Membrane Vesicles (OMVs)

Extracellular vesicles are shed by the outermost membrane of all cells and organelles. Those produced by Gram-negative bacteria are called OMVs and are pinched-off portions of the outer membrane (OM) primarily containing OM and periplasmic proteins (Beveridge 1999). In essence, they are miniature copies of their producing cell, without the ability to synthesise or replicate. They are spherical in shape and differ in size, based on the producing species, but those of Gram-negative bacteria are usually 50–200 nm. While they appear to be produced ubiquitously by membranes, some OMVs have evolved additional properties, promoting their involvement in specific biological processes. Such roles for OMVs include predation, antibiotic resistance, bacteriophage resistance, and for pathogenic organisms host

cell modulation, coagulation, virulence factor delivery, adhesion and invasion (Kulp and Kuehn 2010).

Both soluble and membrane-soluble biomolecules can be packaged into vesicles, and OMVs isolated from different species contain a variety of packaged molecules, including DNA, toxins, antibiotics, resistance proteins, RNA, secondary metabolites and other compounds (Dauros-Singorenko et al. 2018; Evans et al. 2012; Kim et al. 2015; Schwechheimer and Kuehn 2015; Whitworth 2018). The secretion of predatory material within OMVs is advantageous for several reasons. Firstly, within themselves, OMVs maintain a highly concentrated dose of lytic factors, even at a distance from the producing cell. Diffusible factors naturally become increasingly more diluted through distance and time, however packaging them within an OMV means that while the OMVs become sparser with time, the concentration of material within the OMV is maintained. Secondly, the relatively small size of OMVs allows them to access spaces whole cells cannot, while reducing their rate of diffusion compared to soluble proteins/molecules. Finally, OMVs protect their contents from degradative enzymes, whether secreted by prey or by other predators.

The composition and quantity of OMVs produced has been shown to depend on media conditions, envelope stress, community structure, temperature, pH, and salt concentration (Katsui et al. 1982; Kulp and Kuehn 2010; McBroom and Kuehn 2007). Although little is known about the molecular mechanisms of OMV formation and release, several (non-exclusive) formation models have been proposed: stress-induced release, constitutive release, and targeted release (Kulp and Kuehn 2010; Mashburn-Warren and Whiteley 2006; McBroom and Kuehn 2007). Regardless of which mechanism prevails, it seems that there is an initial accumulation of lytic factors/DNA/misfolded proteins and OMV-forming compounds, vesiculation begins as the OM bulges to form the OMV, and the inner membrane (IM) and peptidoglycan (PG) also start to bulge and restructure. In some cases, the IM also forms the inner leaflet of OMVs, restructuring with the OM and PG, although this has not been observed in myxobacteria to date. As the OM bulge becomes more spherical, cargo molecules are packaged into the vesicle. Finally, the budding vesicle pinches off from the cell and migrates away from the producing cell (Fig. 3). There is evidence of active inclusion/exclusion of proteins into vesicles, although the molecular determinants of targeting have yet to be defined (Haurat et al. 2011).

The OMVs secreted by predators have important roles in myxobacterial predation, but prey organisms can also produce OMVs of their own, which help the prey resist attack by predators. For instance, OMVs can protect producing cells by binding and neutralizing the toxic secretions of predatory cells (Grenier et al. 1995; Manning and Kuehn 2011), while envelope material damaged by predatory attack could be actively eliminated from the cell by packing it into shed OMVs (McBroom and Kuehn 2007).

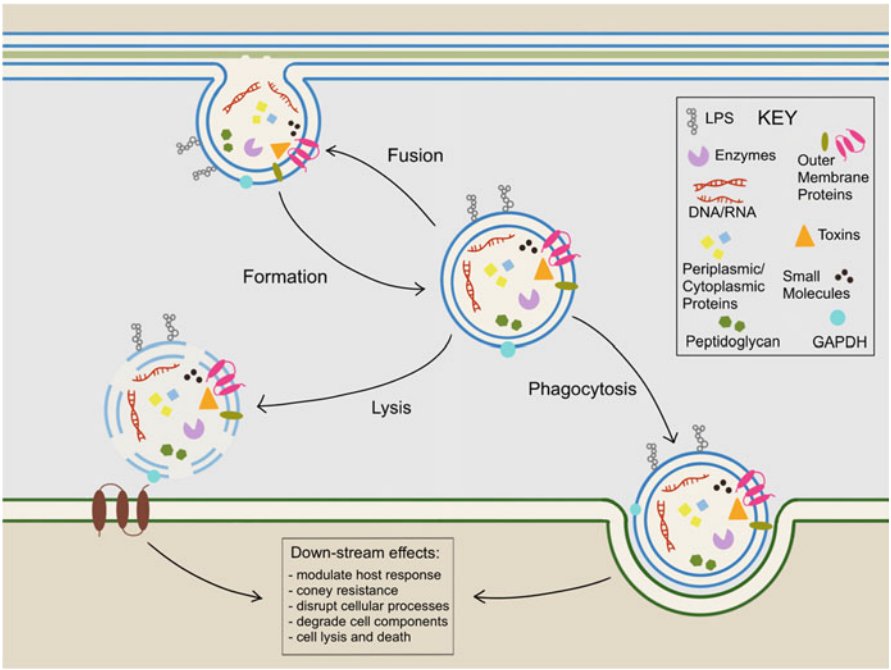

Fig. 3 OMV production, components and interaction with cells. OMVs are formed as the OM bulges, packaging a variety of components including peptidoglycan; OM, periplasmic and cytoplasmic proteins; DNA and RNA; enzymes and toxins; small molecules and lipopolysaccharide (LPS); with GAPDH found inside and on the surface of vesicles. Once produced, vesicles can fuse with self, kin or other Gram-negative cells. Alternatively, vesicles can bind to OM receptors (through GAPDH and other OM proteins), which can stimulate vesicle lysis. Some cells will phagocytose whole vesicles. In these cases, the vesicle can either be lysed by the target cell or fuse with the phagosome. Regardless of the incorporation mechanism, vesicles have several potentially dangerous downstream effects, including modulating host responses, disrupting cellular processes such as protein synthesis, degrading cellular components, and ultimately causing cell lysis and death

6.4 OMV-Mediated Predation

OMV-mediated attack can occur in several different manners, dependent on the target cell-type. They can be engulfed by cells, adhere to cells, fuse with cells, or lyse in the vicinity of cells. All of these actions can be spontaneous or directed, depending on the presence of particular chemical signals and/or receptors on the OMV and on the surface of the target cell. OMVs are more likely to fuse with the OM of Gram-negative cells, delivering their contents into the recipient cell's periplasmic space. However, OMVs tend to lyse in the vicinity of Gram-positive cells instead. For instance, the OMVs of *P. aeruginosa* adhere to the cell wall of Gram-positive bacteria, whereas they fuse with the OM of Gram-negative bacteria (Kadurugamuwa and Beveridge 1996). The OMVs of pathogenic microbes are also well-known for

delivering degradative enzymes into eukaryotic cells, for instance during infection (Amano et al. 2010; Kadurugamuwa and Beveridge 1996; Li et al. 1998). In fact, the inclusion of lytic enzymes appears to be a common feature of OMV proteomes studied (Amano et al. 2010; Bai et al. 2014; Kwon et al. 2009; Lee et al. 2009; Whitworth and Morgan 2015).

It should not therefore be surprising that OMVs are intrinsically predatory, including those produced by bacteria that do not have a predatory lifestyle. The first description of predatory activity in OMVs was for *P. aeruginosa* OMVs (Kadurugamuwa and Beveridge 1996), however the first description of predatory activity in the OMV produced by a predatory organism (*M. xanthus*), did not follow for more than a decade (Evans et al. 2012). The OMVs of *M. xanthus* show similar morphology to those of other microorganisms, with the occasional observation of tethering of vesicles to the OM, and chains of OMVs (Evans et al. 2012; Palsdottir et al. 2009; Remis et al. 2014; Wei et al. 2014).

Like the OMVs from other organisms, the predatory activity of *M. xanthus* OMVs is likely due to the large numbers of peptidases, phosphatases, lipases, and other packaged enzymes present in the OMVs (Berleman et al. 2014; Kahnt et al. 2010; Whitworth et al. 2015). Although several studies have investigated the secreted, OM and OMV proteomes of *M. xanthus*, differences in the profiles and methods used have prevented the formation of a definitive list of OMV components. In addition to digestive enzymes, several secondary metabolites have also been found in *M. xanthus* OMVs, including DKxanthenes, cittilin A, myxalamids, myxochelins, and myxovirescin A (Berleman et al. 2014; Evans et al. 2012).

Killing of prey *E. coli* and *P. aeruginosa* cells was observed in the presence of OMVs, but also on addition of OMV-free supernatant. OMVs and supernatant applied together to prey cells had an additive predatory effect, which may be due to lysis of OMVs and release of their contents into the supernatant prior to fractionation, active components/molecules unique to either fraction, or because supernatant components stimulate activity of OMVs (Evans et al. 2012). Killing of prey cells has been proposed to require fusion of the OMV membrane with the prey cell OM, as OMVs lysed by French-pressing lost predatory activity, and toxicity of intact OMVs was enhanced by the addition of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a protein known to fuse membranes together (Evans et al. 2012).

6.5 GAPDH-Assisted Predation

GAPDH is a moonlighting protein – a metabolic enzyme that has additional non-metabolic roles (Copley 2012; Kainulainen and Korhonen 2014; Wang et al. 2014). Many moonlighting proteins of pathogens have secondary functions in infectivity and adhesion, promoting cytotoxicity. In *M. xanthus* GAPDH is found in the extracellular matrix, cytoplasm, OMVs and culture supernatant and it has been proposed to work synergistically with OMVs, promoting delivery of OMVs to target cells (Curtis et al. 2007a; Evans et al. 2012; Whitworth and Morgan 2015;

Whitworth et al. 2015). The factors which mediate adhesion or fusion of myxobacterial OMVs to target cells are currently undefined, but it is probable that GAPDH plays a role in facilitating fusion, and possibly also initial molecular recognition of target membranes.

GAPDH is found in all domains of life, occasionally present in several isoforms and in various sub-cellular locations. In organisms that perform glycolysis/gluconeogenesis, GAPDH catalyses the interconversion of glyceraldehyde-3-phosphate and 1,3-bisphosphoglycerate in the cytoplasm. Until the mid-1980s, GAPDH was known as a housekeeping enzyme, functioning only in glycolysis/gluconeogenesis and used classically as a cytoplasmic marker. However, in 1985, GAPDH was shown to induce fusion of artificial phospholipid vesicles (Morero et al. 1985). Soon after, researchers showed that GAPDH is present on the surface of both *Schistosoma mansoni* and group A streptococci (Goudot-Crozel et al. 1989; Pancholi and Fischetti 1992). GAPDH can be differently localized depending on culture conditions; changes in pH induce secretion of GAPDH in *Streptococcus gordonii*, and trigger cell surface localisation in *Lactobacillus crispatus* (Antikainen et al. 2007; Nelson et al. 2001). Since these initial findings, GAPDH has been reported in the extracellular matrix, on the outer surface or in secreted vesicles of at least 79 bacterial, 12 parasites, 4 yeast and 2 fungal species (our unpublished observations).

Many of the moonlighting functions of GAPDH involve the fusion of membranes and vesicles. In eukaryotic cells, nuclear functions involve GAPDH recruitment to the nucleus during S-phase, where GAPDH aids in nuclear membrane fusion through phosphatidylserine binding (Kaneda et al. 1997; Sirover 2005). GAPDH is involved in intracellular membrane trafficking (endoplasmic reticulum to Golgi), promoting fusion of endoplasmic-derived vesicles with Golgi membranes. Intracellular membrane trafficking requires cytoskeletal changes, and GAPDH also promotes tubulin association into microtubules and microtubule bundling (Tisdale 2001, 2002). In the brain GAPDH forms a complex with pre-synaptic vesicles and stimulates loading of the vesicles with the neurotransmitter glutamate. This activity requires GAPDH's metabolic activity, in concert with that of phosphoglycerate kinase (PGK) to generate ATP at the vesicle surface (Ikemoto et al. 2003). In macrophages, GAPDH interacts and forms a complex with transferrin, which is then internalised through the formation of endocytic vesicles (Raje et al. 2007). An analogous function has also been observed in *S. aureus* (Modun and Williams 1999).

The most commonly described moonlighting function of GAPDH is promoting adhesion between membranes and biopolymers. In some bacteria, surface export of GAPDH (and subsequent adhesion) are necessary for virulence (Jin et al. 2011), while in the probiotic bacteria *Lactobacillus plantarum* and *E. coli* Nissle 1917, GAPDH is directly responsible for adhesion to host cells, enhancing colonization of intestinal mucosa (Aguilera et al. 2014; Kinoshita et al. 2008). Colonization through adhesion appears to be a universal mechanism in bacteria, suggesting it might also play a role in prey adhesion (by both myxobacterial cells and OMVs). *Saccharomyces cerevisiae* even appears to use GAPDH as an antimicrobial peptide to defend itself against microbial predation (Branco et al. 2014).

GAPDH is found in the extracellular matrix, culture supernatant, cytoplasm and OMVs of *M. xanthus* (Curtis et al. 2007b; Evans et al. 2012; Whitworth et al. 2015). Yet the *gapA* gene which encodes GAPDH encodes no signal sequence for extracytoplasmic targeting. However, GAPDH is encoded in an operon with genes for PGK, triose phosphate isomerase (TPI) and SecG – a component of the general secretory pathway. This operon structure is conserved in all public myxobacterial genomes sequenced to date, and 50 genomes that we have sequenced. SecG is not an essential protein, serving an auxiliary role in the secretory apparatus (Flower et al. 2000). Nevertheless, it is able to facilitate the export of proteins that lack effective signal sequences (Belin et al. 2015), and we hypothesise that SecG is encoded in the same operon as GAPDH as it is involved in the secretion of GAPDH. Indeed, both TPI and PGK enzymes have also been shown to localise to the cell surface in some organisms and are suggested to be adhesins (Trost et al. 2005; Wang et al. 2016). It is therefore likely that the GAPDH operon acts as an adhesion package with its own devoted secretion regulator SecG.

7 Perspectives

7.1 Gaps in Ecological Understanding

Our understanding of the ecology of myxobacterial predation is still sadly lacking. The position of myxobacteria in ecosystem foodchains is only just beginning to become clear. Moreover, the relative abundance, variety and susceptibility of prey, and the resulting predator-prey population dynamics have not been studied in natural contexts. Consequently, we have little idea to what extent myxobacterial predators affect the flow of nutrients through soil nutrient cycles. Fundamental questions remain to be answered: how do myxobacterial predators affect natural microbiomes, and with what dynamics? On what timescale does the predator-prey arms race give rise to predator-prey cycling? Such questions are certainly answerable, for instance through mesocosm experiments and pulse-chase labelling. Encouragingly though, there have been significant advances made in understanding the relationships between relatedness and cooperation/antagonism of myxobacteria living in close proximity with one another, and in ecological factors that are likely to affect predatory success in the wild.

Myxobacterial predation is one of the exemplars of bacterial cooperativity, however the evidence for cooperativity is limited, and many mechanistic features of wolf-pack predation appear to have evolved to maximise selfishness during feeding (Marshall and Whitworth 2019). Whether predation is truly cooperative or not has important consequences for understanding the mechanisms at play during predation, and the evolution of predatory activity and individuality by myxobacterial lineages. Can prey killing happen at a distance, mediated by diffusible public goods, or does it require predator-prey cell-cell contact?

Prey range is another crucial parameter affecting the ecological success of individual predators. What dictates prey susceptibility and how does it evolve? Are the pan-genomes of prey open, allowing the horizontal acquisition of predation-resistance genes? Certainly, the pan-genomes of the predators seem to be open (Livingstone et al. 2018c), to allow for genetic exchange and adoption of novel predatory molecules. In a natural context, do predator-prey populations cycle, or are the population evolutionary dynamics more parasite-like? Perhaps there is positive selection for myxobacteria that are less lethal, giving rise to stable population dynamics, with the myxobacteria 'farming' susceptible prey. Have predators and their prey living in the same niche been selected for stable co-habitation and sub-maximal lethality of the predator? Or instead of farming do myxobacteria adopt a 'slash and burn' approach, destroying all susceptible prey in an area and then lying dormant waiting for regrowth of the bacterial fauna? Perhaps they prefer to be itinerant, pillaging an area of its fauna and then moving on to another pristine area.

7.2 Gaps in Mechanistic Understanding

Similarly, there are many fundamental aspects of predation that are not mechanistically understood, complicated by the apparent uniqueness of each pair-wise encounter between particular predators and prey. In a small number of cases, the molecules responsible for prey killing, and resistance to predation have been identified, but in most cases it is not clear, and probably a mixture of multiple enzymes and/or metabolites are involved. Do myxobacteria evolve to secrete a cocktail of lytic factors, and can we learn general rules about the nature of the active molecules? Do they tend to be metabolites or enzymes? Which enzymes are most important in determining prey range and what are their substrate specificities?

After prey have been lysed what happens to the released nutrients? The myxobacterial secretome includes a large number of metabolic enzymes. Which catabolic pathways operate outside the cell? Which metabolites are taken up by the predator and do they include macromolecules? How does extracellular metabolism differ from intracellular metabolism? Which nutrients are growth-limiting and can monomeric molecules be incorporated into predator biomass without passing through central intermediary metabolism? Systems approaches using pulse/chase experiments with labelled prey should provide the answers, but how generalisable will the findings be?

There is conflicting evidence whether myxobacteria can sense (directly or indirectly) the presence of prey. The ability to respond to prey would allow sophisticated decision-making during predation, potentially manifesting as prey preference, choice between alternative predatory strategies (for example frontal attack versus infiltration or wolf-pack predation), conditional cooperation with kin, or even manipulation of prey biology. OMVs have been found to contain microRNAs which are able to affect the biology of recipient cells. In a predation context, this could take the form of disrupting prey quorum signalling, or promoting biofilm

degradation. It is extremely likely that regulatory RNAs will be targeted into prey by myxobacterial OMVs (Whitworth 2018), but which RNAs and what their resulting effect on host biology might be, can only be guessed at currently.

A set of 'core' predatory genes has been identified which tend to be conserved amongst predators and absent from non-predators (Pasternak et al. 2013), and it is therefore certain there will be additional predatory genes that are core for myxobacterial predators. However, the plasticity of the predatory phenotype, which differs widely even between very similar myxobacteria, implies the presence of a large set of 'accessory' predatory genes. Such a proposal is supported by pan-genome analyses, which indicate that two members of the same myxobacterial genus typically share less than 50% of each other's genes. In addition, there are likely to be many factors involved in predation, like GAPDH, which modulate the efficiency of core predatory proteins/genes, potentially in a prey-dependent fashion. Such phenomena make establishing causal relationships between the presence/absence of accessory/modulatory genes and predatory activity very difficult, but a worthwhile challenge for the future.

7.3 Potential for Exploitation

As professionally antimicrobial organisms, there is great potential for the use of predators and predator-derived/inspired products for the advantage of humanity.

Living cells of the predatory bacteria *Bdellovibrio* spp. have recently been shown to protect animals from pathogen challenge (Tyson and Sockett 2017; Willis et al. 2016). The low growth temperatures (usually up to 32 °C) required by myxobacteria makes their use as living antibiotics unlikely. However, myxobacterial OMVs have been shown to kill pathogenic bacteria (Evans et al. 2012; Schulz et al. 2018) and exhibit good biocompatability with human cells (Schulz et al. 2018), suggesting they could see use as multivalent antibiotics in some clinical contexts. There also exists the opportunity to engineer OMVs to tailor their delivery of bespoke cargo to particular target organisms/membranes and enhancing their toxicity (Baker et al. 2014; Schulz et al. 2018).

The potential for exploiting myxobacterial predators in horti/agri-culture has been investigated, and myxobacteria are shown to inhibit a variety of plant-pathogenic organisms (Bull et al. 2002; Hocking and Cook 1972; Taylor and Draughon 2001; Yun 2014). Applying myxobacteria to agricultural soil is an attractive alternative to fumigation with fungicides, however the resulting success of such an approach is limited by our current lack of knowledge regarding the relationships between predators and prey in the soil. For instance, partitioning of prey biomass into microbial predators seems to be dependent on prey species, but also on the soil sub-compartment being considered (Zhang and Lueders 2017).

There are clearly considerable potential advantages for humanity if we can rationally exploit myxobacterial predation, however success in such ventures requires continued increases in our understanding of their molecular ecology,

building on the rudimentary, but swiftly growing knowledge base we have introduced above.

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The Ecology of *Bdellovibrio* and Like Organisms in Wastewater Treatment Plants

Edouard Jurkevitch

Contents

1 Introduction

Bdellovibrio and like organisms (BALOs) are obligate predators of other bacteria. They have an absolute requirement for Gram negative prey in order to replicate and complete their life cycle. This peculiar life style profoundly affects their physiology and cellular biology and defines their ecology. We only partially know the molecular, physiological and structural features enabling this unique life style, and even less about their interactions with, and their effects on, microbial communities and trophic networks in the environment. Nonetheless, thanks to huge technological advances in molecular biology, including molecular ecology and biological computing over the past 15 years, significant strides have been made, providing novel understanding, yielding new concepts and approaches which make it possible to start bridging between cellular features and ecological outputs.

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This chapter summarizes the knowledge on BALOs in wastewater treatment plants (WWTPs). Other microbial predators are active in wastewater, mainly protists and phages, and other bacterial predators of bacteria like Myxobacteria (for more details see Chapters by Kuppardt-Kirmse and Chatzinotas "Intraguild Predation: Predatory Networks at the Microbial Scale" and Furness et al. "Predatory Interactions Between Myxobacteria and Their Prey"). These won't be treated here. Wastewater treatment plants offer "real life" conditions where complex, but mostly limited to the microbial scale, trophic interactions and high microbial diversity combine to provide a highly valuable output for the environment in general and for human communities in particular. Yet, WWTPs are also tractable and controlled engineered environments that can be manipulated and mimicked at various scales, providing unique opportunities to uncover and investigate ecological phenomena at the microbial dimension. In this chapter, we summarize what is known of predatory interactions between bacteria in WWTPs. We will then suggest how novel approaches may bring us closer to understanding their roles in water purification. This, in turn, may help improve WWTP operations by increasing ecological stability, remove pathogens, and provide alternatives to their costly implementation in low and medium income countries (LMICs).

2 The Biology of BALOs

The aim of this section is to provide the reader with some basic knowledge on the phylogeny, and life cycle of BALOs.

2.1 BALO Phylogeny, Distribution in the Environment, and Prey Range

BALO phylogeny and distribution. BALOs belong to the Proteobacteria. Until recently, most were affiliated to the δ-proteobacteria (Rotem et al. 2014). The discovery and isolation of novel bacterial strains has led to a reconsideration of their phylogeny, and to the creation of the class Oligoflexia that includes them (Hahn et al. 2017). Within the Oligoflexia, BALOs form the orders Bdellovibrionales and Bacteriovoracales. The former includes the family Bdellovibrionaceae and the latter the families Bacteriovoracaceae and the Halobacteriovoraceae (Koval et al. 2015; Hahn et al. 2017). In addition, a new family of predators, the Pseudobacteriovoracaceae of which only the type strain *Pseudobacteriovorax antillogorgiicola* is known is placed in the order Oligoflexales (McCauley et al. 2015; Hahn et al. 2017). As *P. antillogorgiicola* was isolated from a gorgonian octocoral on marine agar, it is not an obligate predator. Its predation mode (epibiotic or periplasmic, see below) is not known.

The only genus within the Bdellovibrionaceae is *Bdellovibrio* (Davidov and Jurkevitch 2004), of which two species have been defined: *B. bacteriovorus* and *B. exovorus*. *B. bacteriovorus* is found in soil, and associated with plant roots (Klein and Casida Jr. 1967; Uematsu 1980; Jurkevitch et al. 2000; Oyedara et al. 2016), and in freshwater, e.g. rivers and lakes (Davidov and Jurkevitch 2004; Hobley et al. 2012a; Li and Williams 2015). Both *B. bacteriovorus* and *B. exovorus* are found in wastewater, the latter has so far only been found in this habitat (Chanyi et al. 2013). *B. bacteriovorus* is a periplasmic predator: it invades the space between the cytoplasmic membrane and the outer membrane of its prey. In contrast, *B. exovorus* is epibiotic, meaning it remains attached to the outer side of its prey, feeding on it from the outside (Koval et al. 2012; Chanyi et al. 2013).

Bacteriovoracales are all periplasmic predators. *Bacteriovorax stolpii* is a freshwater (including wastewater) and soil bacterium within the Bacteriovoracaceae, a monophyletic offshoot of the Bdellovibrionaceae (Koval et al. 2015). *Peredibacter starrii* is also found in freshwater and soil environments (Davidov and Jurkevitch 2004). As the family name Peredibacteraceae is deemed illegitimate, *P. starrii* is classified as a genus within the Bacteriovoracaceae (LPSN Bacterio.net, http://www.bacterio.net/peredibacteraceae.html, May 13, 2019). Lately, it was found that *Peredibacter* sp. was the most abundant BALO predator in the upper layers of perialpine lakes while Bdellovibrionaceae and Bacteriovoracaceae were proportionally more abundant at greater depths (Paix et al. 2019). The second recognized family in the order Bacteriovoracales is Halobacteriovoraceae. Two species have been defined, i.e. *H. marinus* and *H. litoralis*. They form different clusters which preferentially populate estuarine or marine waters, and are apparently selected by salinity levels (Pineiro et al. 2013). They have not been found in freshwater (Koval et al. 2015). A few isolates have been retrieved from salt lakes (Pineiro et al. 2004).

Finally, a few isolates classify to the α-proteobacteria. These are *Micavibrio aeruginosavorus* and *M. admirandus*, both epibiotic predators isolated from wastewater and soil (Lambina et al. 1982, 1983; Davidov et al. 2006a). *Micavibrio* forms a deep branch lineage, sister to the Rhodospirillales but distinct from any other major α-proteobacterial groups (Davidov et al. 2006b; Wang et al. 2011).

The known BALO taxa have only been rarely isolated from or detected in terrestrial animals, including humans (Schwudke et al. 2001; Kikuchi et al. 2009; Iebba et al. 2013) but they appear to be more readily associated with aquatic animals (Kelley and Williams 1992; Wen et al. 2009; Cao et al. 2012, 2015; Richards et al. 2012; Welsh et al. 2015).

Prey Range BALOs have so far been shown to exclusively prey on Gram negative bacteria, both in the planktonic, suspended phase as well as in biofilms (Kadouri et al. 2005, 2007). Moreover, they can destroy the biofilm matrix of Gram positive bacteria without consuming the cells (Im et al. 2018). BALOs are usually isolated and tested for prey range with bacterial strains from laboratory collections, and these may originate from various source (Chanyi et al. 2013; Cao et al. 2015; Enos et al. 2018). However, when tested for prey range using strains isolated from the same environment the BALO came from, the predators appeared to prefer these

co-locating strains, possibly as a result of selection for locally prevailing conditions (Rice et al. 1998; Pineiro et al. 2004). Differences in prey range are also observed between strains of BALO predators belonging to the same species (Jurkevitch et al. 2000; Li et al. 2011). Conversely, different prey strains belonging to the same species are differentially "palatable" to a particular BALO (Jurkevitch et al. 2000; Dashiff et al. 2011; Li et al. 2011). BALOs prey equally well on pathogenic and commensal bacterial strains, as well as on bacteria resistant to antimicrobials (Dashiff et al. 2011; Kadouri et al. 2013).

So far, no universal prey, even for a specific habitat, has been found to be consistently more efficient at "baiting" BALOs. This includes *Vibrio haemolyticus* P5, which has been extensively used for isolating marine BALOs (Schoeffield and Williams 1990). As many strains can be used as prey in the laboratory, and since many prey may not be culturable (Rinke et al. 2013), the true prey range of BALOs under natural conditions is still not known. Importantly, and as known today, BALO phylogeny, prey range and prey phylogeny are unlinked.

2.2 *Essentials of the BALOs' Predatory Life Cycle*

As mentioned above, BALOs exhibit a periplasmic or an epibiotic predatory life style. These appear to be fixed, and to not depend upon the prey (Chanyi et al. 2013).

BALOs actively and rapidly swim during a so-called attack phase (AP) using a single, polar flagellum, in search of prey cells. They possess chemotaxis systems, which they use to detect amino acids (LaMarre et al. 1977), high bacterial biomass (Chauhan and Williams 2006) and to a small extend, prey cells (Lambert et al. 2003). As of today, little is known on the biology of the epibiotic predators beyond their phylogeny and the visual description of their life cycle. Upon encounter, epibiotic predators attach to the prey's cell wall, consume the prey content from the outside, to leave an empty cell, and grow by binary division. During predation, vesicle-like and remnants of lipid structures can be observed within and outside the prey cell (unpublished). Some genetic details are available, showing that epibiotic predators have significantly smaller genomes than periplasmic BALOs, encoding for up to half of the total secreted proteins found in periplasmic BALOs; they generate energy through glycolysis and the tricarboxylic acid cycle and lack biosynthetic pathways for essential amino acids, vitamins, and precursors, similarly to periplasmic predators (Pasternak et al. 2014). In both periplasmic and epibiotic predators, gene expression is largely altered between the AP and the growth phase (GP), with contrasting expression of motility and search genes and chromosome replication, translation, transcription, energy production and cell division genes (Wang et al. 2011; Karunker et al. 2013). For further detail see (Lambert et al. 2010; Wang et al. 2011; Karunker et al. 2013; Pasternak et al. 2013, 2014).

The life cycle of periplasmic predators is known in much finer details. Some of its main features are presented here. Periplasmic BALOs have absolute requirements for type IVa and type IVb pili for prey invasion, as well as for gliding motility (Evans

et al. 2007; Mahmoud and Koval 2010; Avidan et al. 2017; Duncan et al. 2019). In order to enter the prey, the predator makes a hole in the prey's cell wall, and squeezes through it (Kuru et al. 2017). All the while, the prey's peptidoglycan is extensively remodelled by specific peptidoglycan endopeptidases to prevent invasion by additional predators, as shown in *B. bacteriovorus* (Lerner et al. 2012), and the predator prevents self-inflicted damage to its own cell wall by using a protective protein (Lambert et al. 2015). The re-shaped prey cell which now contains the predator is called a bdelloplast. Homologous genes for the cell wall modifying machinery were found in the periplasmic *H. marinus* but they are absent from either of the epibiotic predators *M. aeruginosavorus* and *B. exovorus* (Pasternak et al. 2014). The sequence of events starting with penetration defines a transition phase (TP) characterized by a specific pattern of gene expression (Rotem et al. 2015). The TP is followed by the GP, which is promoted by an as yet undefined soluble prey cell fraction. It is thought that this two-step sensing strategy enables the predator to evaluate prey quality (Rotem et al. 2015). During GP, the prey's macromolecules are sequentially degraded by different types of hydrolytic enzymes (Dori-Bachash et al. 2008; Karunker et al. 2013; Im et al. 2018). The predatory cell grows as an aseptate filament containing multiple nucleoids, with chromosome replication starting at the onset of the growth phase. The final length of the filament depends upon the size of the prey cell, and sets the number of replications, progeny and cycle duration (Kessel and Shilo 1976; Makowski et al. 2019). GP is sustained by a soluble prey cell-derived signal, the depletion of which leads to growth arrest and to cell division (Ruby and Rittenberg 1983). Strikingly, division is a synchronous, multi-site septation process that can yield an odd- or an even number of progeny (Fenton 2010; Makowski et al. 2019). Cell division is not associated with chromosome replication as this terminates shortly before septation (Makowski et al. 2019). Finally, focal lysis of the bdelloplast creates pores through which flagellated AP progeny cells are released (Fenton et al. 2010).

An intriguing aspect of BALOs' cell cycle is the spontaneous appearance of host-independent (H-I) derivatives that grow in rich medium in the absence of prey (Barel and Jurkevitch 2001; Roschanski et al. 2011). H-I mutants can retain predatory activity, *de facto* being facultative predators, but very few strains have been isolated from the wild (Hobley et al. 2012a). Primary saprophytic H-I mutants require prey cell extract for growth, while secondary axenic H-I mutants can robustly grow on rich media (Roschanski et al. 2011). While the mutations responsible for these phenotypes have been mapped (Cotter and Thomashow 1992; Roschanski et al. 2011), their relation to the observed phenotypes is not understood. Moreover, H-I mutants appear to be not as effective predators as wild-type strains are and genetic revertants have not been observed. Altogether, how they survive in nature, i.e. what niche they occupy, is enigmatic. The secondary messenger cyclic-di-GMP was shown to play a role in the transition for wild-type to H-I, as mutations in specific diguanylate cyclases differentially prevented wild-type or H-I growth (Hobley et al. 2012b). Cyclic-di-GMP is often involved in regulating phenotype change in bacteria (Jenal et al. 2017) and its signaling networks may be very developed in *B. bacteriovorus* (Rotem et al. 2016). So far, no H-I mutants have been retrieved

from epibiotic predators. H-I mutants are powerful tools for investigating BALO genetics, as mutants in essential predatory functions which are thus lethal in the wild-type strain are viable due to the non-obligate character of H-I variants (Medina et al. 2008; Duncan et al. 2019). An illustration of the life cycle of periplamic, epibiotic and H-I variant BALOs is shown in Fig. 1.

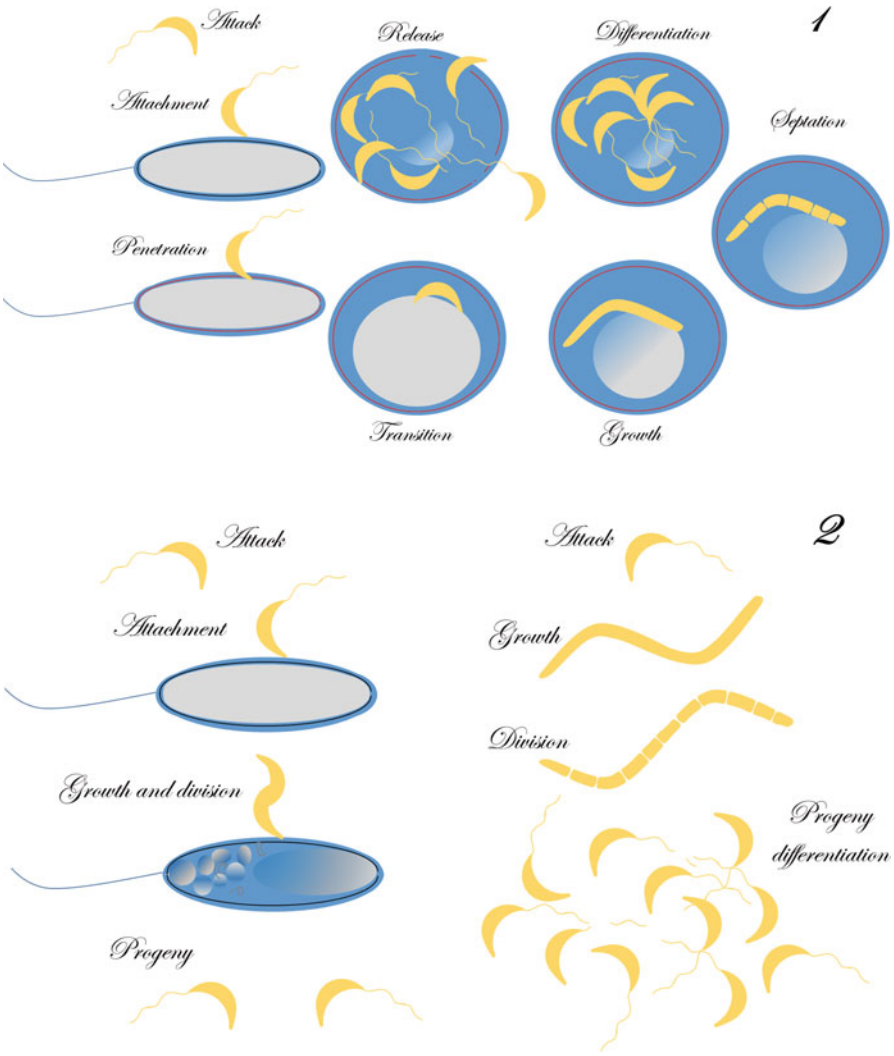

Fig. 1 The life cycle of periplasmic BALOs (panel 1), of epibiotic BALOs (left) and of host-independent variants (right) (panel 2). The internal black line represents the peptidoglycan; the red line, the peptidoglycan processed by the predator upon invasion – see main text for details. Vesicle-like bodies and membrane-like remnants are often visible in prey of epibiotic BALOs (unpublished data)

3 BALO Population Dynamics

Here, we explore various aspects of BALO dynamics in controlled laboratory microcosms. These points will be made relevant in the section dedicated to BALOs in WWTPs.

BALOs have generation times of 2.5–4 h on *E. coli*-sized prey, depending on predator, prey strain, and conditions, and yield 3–6 progeny per prey (Fenton et al. 2010). Growth in liquid cultures in Erlenmeyer flasks, microtiter plates or else are classically started with a high concentration of prey and predatory populations lower by orders of magnitude. Classically, prey and predators are tracked using dilution plating, counting prey and predator with colony forming units (CFU), and plaque forming units (PFU), per milliliter, respectively (Jurkevitch 2012). The development of specific 16S rRNA-gene targeted primers now enables determining predators (and prey) without relying on plate counts (Zheng et al. 2008; Van Essche et al. 2009). Furthermore, BALOs engineered to express fluorescent proteins can also be conveniently tracked and quantified (Mukherjee et al. 2016; Sathyamoorthy et al. 2019).

Within 24–30 h, depending on predator and prey strains, and temperature, the inverse composition is achieved, i.e. high and low predator and prey populations, respectively (Sathyamoorthy et al. 2019). In closed vessels, damping predator-prey oscillations can occur (Afinogenova et al. 1977). Few studies have used open vessels to study BALO predatory dynamics, showing that an oscillating predator-prey regime can be achieved dependent upon prey density, dilution rate or nutrient concentration, with stable oscillations achieved at high prey density (Varon 1979). (Whitby 1977) showed that in the system examined (*B. bacteriovorus* 6-5-S and *Aquaspirillum serpens*), dilution rates of 0.1–0.3 sustained stable oscillations for periods of up to 1 month, and a maximum growth rate of 0.45 was measured. At a lower dilution rate (0.05) a stable equilibrium was established, and at higher rates, the predator was washed out, becoming extinct.

Prey biofilms are efficiently preyed upon, and destroyed (Kadouri and O'Toole 2005; Kadouri et al. 2007; Kadouri and Tran 2013). So far, no prey that a BALO can exploit has been shown to be resistant to predation when grown as biofilm vs. as suspended cells (Kadouri and O'Toole 2005; Kadouri et al. 2007; Dashiff et al. 2011; Kadouri and Tran 2013). Although many studies have been conducted to compare prey survival in biofilm vs planktonic growth (Chanyi et al. 2016; Feng et al. 2016; Dharani et al. 2017; Sun et al. 2017) most use the colorimetric crystal violet method that only shows the proportion by which the biofilm has been reduced. Only few have used a comparative metric (cell counts) which can reveal differences in sensitivity to predation between life styles. These few studies however, showed that with *E. coli*, *Acinetobacter baumannii* and *Enterobacter gergoviae* biofilm cells were significantly more resistant to *B. bacteriovorus* predation than their planktonic counter parts (Kadouri and O'Toole 2005; Dashiff et al. 2011). However, both phenotypes of P*seudomonas aeruginosa* and *Klebsiella pneumoniae* prey were equally sensitive to *M. aeruginosavorus* and to *B. bacteriovorus,* respectively (Kadouri et al. 2007; Dashiff et al. 2011), suggesting that both the predator and

the prey play a role in determining the outcome of predation under these conditions. Prey sensitivity can be measured by the predator's growth rate and/or by the remaining living cells of the prey population in a predatory culture. This can vary by orders of magnitude between strains (Dashiff et al. 2011). Remaining cells are resistant to predation, albeit resistance is transient, i.e. plastic, and disappears as the population grows back when nutrients are present (Shemesh and Jurkevitch 2004). While it may be hypothesized that when existing, the differential sensitivity to predation between biofilm and planktonic fractions may stem from intrinsic differences in resistance to predator attachment/penetration due to changes in prey physiology or from effects of the biofilm matrix, the phenomenon is not understood in neither growth phenotypes. Additionally, the predator may also actively affect prey susceptibility, as knockout mutations in nuclease genes in *B. bacteriovorus* resulted in increased predation in biofilms (Lambert and Sockett 2013).

Under natural conditions, communities are complex, and multiple predators and prey may encounter each other. This issue has barely been researched. Results of experiments conducted with a mixture of prey and a single predator showed that *B. bacteriovorus* reduces prey in multispecies cultures as efficiently as in single-species cultures, in suspended cells and in biofilms (Loozen et al. 2015; Im et al. 2017). The predator also exhibits prey preferences which can be expressed as differences in remaining prey levels, and faster attachment to a preferred prey (Rogosky et al. 2006).

Finally, temporal predatory dynamics are largely affected by spatial structure. (Hol et al. 2016) grew dual cultures of *B. bacteriovorus* and *E. coli* as prey in a micro-chip array composed of connected patches or in a large patch of the total same volume. The prey population drastically declined in the latter, but both the predator and the prey persisted in the former. (Dattner et al. 2017) further showed that in soil, the spatial heterogeneity of the soil matrix enabled co-existence of a viable, slowly declining *B. bacteriovorus* population over a week (the time frame of the experiment) possibly by providing refuge to a *Burkholderia stabilis* prey. Under such settings, organic and inorganic particles may act as decoy particles which may further affect predator-prey dynamics (Wilkinson 2001; Hobley et al. 2006). For details on predator-prey dynamics and modelling, and for multi-level predatory interactions and community stability, see the Chapter by Kuppardt-Kirmse and Chatzinotas "Intraguild Predation: Predatory Networks at the Microbial Scale".

4 *BALOs in Wastewater Treatment Plants (WWTPs)*

4.1 A Primer on Wastewater Treatment

The basic function of the wastewater treatment plant (WWTP) is to speed up the natural processes by which water purifies itself. A first main goal is to reduce biological oxygen demand (BOD) to low levels (within a few tens of mg per litre) in the effluent released to the environment. BOD is a measure of the amount of

oxygen required for microbial metabolism of organic compounds and of ammonia in water. The second main goal is to drastically limit the pathogens present in the effluent as to reduce risks of contamination. Additional goals, which are not always part of WWT include the removal of nutrients (the influent WW is nutrient-rich) to curtail the deleterious effects of increased high nitrogen and high phosphorus concentrations in the environment, such as the eutrophication of aquatic ecosystems, and the removal of inorganic and synthetic organic chemicals, including contaminants of emerging concern, such as pharmaceuticals, hormones and pesticides. Such requirements necessitate further treatment steps and more sophisticated processes upon the classical goals of the WWTP. Lately, due the rapid increase in antibiotic resistance in the clinic and in the community (Berendonk et al. 2015), the role of WWTP in spreading antimicrobial resistance (AMR) and antibiotic resistance genes (ARG) through selection and genetic exchanges between microorganisms has become a major topic of investigation. Dealing with these various demands, which usually are not part of the basic scheme of a WWT, is a challenging task.

The "Classical" WWTP Wastewater (WW) is collected through the sewage system, flows to the WWTP where it enters primary treatment as influent, often preceded by a preliminary step to remove large floating objects. Primary treatment removes coarse solids by settling, which can be complemented by a sedimentation step for finer particles. The influent then flows to secondary treatment which constitutes the "heart" of the WWTP, where the organic matter is broken down, removing 90% or more of it. The secondary reactor is essentially a microbial digester where myriad biochemical reactions and interactions are maintained by the most diverse microbial community found in man-made systems. Recent studies have shown that microbial communities include over 2000 operational taxonomic units (OTUs, a similarity-based grouping of sequence reads of the same allele, usually the 16S rRNA gene in bacteria) of bacteria and 1000 OTUs of micro-eukaryotes (Semblante et al. 2017; Cohen et al. 2019). These, along with archaea, remove organic carbon and nitrogen to CO_2 and ammonium, respectively. Further oxidation of the latter through nitrification yields nitrate. As of today, the most commonly applied secondary treatment is the conventional activated sludge (CAS) process. It is the main focus of this chapter. However, as membrane bioreactors are becoming increasingly popular, the application of BALOs in these systems is also reviewed below. CAS is effected by a suspended growth process in which microbes colonize a mixed liquor consisting of water and of suspended organic matter (flocs). The mixture requires oxygen which is provided by mechanical means or by the injection of pressurized air. Alternatively, wastewater is treated by flowing along with air through a trickling filter (so-called attached growth processes) made of minerals (slag, stones) or plastic materials. Trickling filters provide a large, aerated surface area upon which mixed microbial biofilms develop. In both the suspended and the trickling filter approaches, organic matter mineralization not only supports a large microbial diversity but also a large biomass, called the sludge. In an additional step, the sludge may be separated from the effluent by settling in a clarifier basin. In suspended growth processes, the activated sludge is returned in part to the secondary

reactor, enabling continuous operation. Although the suspended sludge is well mixed, the organic flocs, and the water liquor are hosts to sympatrically-segregated bacterial and micro-eukaryotic populations. They also differ in dynamics, as the microbial composition of the liquor fluctuate more strongly and more rapidly than that of the flocs (Cohen et al. 2019). A drawback of this technology are the costs incurred by the need for aeration of the reactor, and often more important, for the disposal and/or treatment of excess sludge for downstream applications, to the extent of up to 50% or more of total operational expenses (Wendland and Ozoguz 2005). Accordingly, improvements upon the existing technologies and practices that can lead to a decrease in operational cost e.g. through sludge reduction, are sorely needed.

Various technologies have been developed to replace or to complement activated sludge-based processes. They include land treatment, constructed wetlands, anaerobic digestion, membrane-based filters and others which are out of the scope of this review.

Things WWTPs Don't Do – Or Don't Do Too Well In addition to degradable organic matter, WW carries refractory contaminants that are only partially, poorly or not degraded in WWTPs (Kümmerer et al. 2018). Among them, pharmaceutically active compounds, personal care products, artificial sweeteners, and endocrine disrupting chemicals are found in the influent and in treated effluents at concentrations ranging from $ng.L^{-1}$ to $\mu g.L^{-1}$ (Tran et al. 2018). A major source of concern are antibiotics and other antimicrobial compounds. Antibiotics are used at large scales in medicine and agriculture. Fifty to ninety percent of the consumed antibiotics or their degradation products are excreted and thus discharged into the environment where they may deleteriously affect aquatic ecosystems (Kümmerer 2009). The large distribution of these compounds at detectable concentrations may be an important factor driving the increase in antibiotic resistance (AR) that finally also impacts upon the clinic (Berendonk et al. 2015). Although discharged concentrations of antibiotics are well below the minimal inhibitory concentration (MIC), they still can drive selection for increased resistance (Negri et al. 2002; Gullberg et al. 2011, 2014). Moreover, mixtures of compounds e.g. antibiotics and heavy metals, or other chemicals can further lower minimal selective concentrations, enhancing multidrug resistance (Gullberg et al. 2014). Accordingly, WWTPs are environments that may promote selection for AR. It has recently been shown that selection also occurs at very low antibiotic concentrations in the complex microbial communities found in WW (Murray et al. 2018). Mobile genetic elements such as plasmids, conjugative transposons and integrons may facilitate the horizontal dissemination of antibiotic resistance genes between bacterial species (von Wintersdorff et al. 2016). The distribution of mobile elements and of the ARGs carried by them largely varies between habitats (Gatica et al. 2019), between regions and even within a WWTP, as seen between sludge and effluent (Gatica et al. 2016) suggesting that dissemination of and selection for ARGs involve complex biotic and abiotic (and their interactions) factors. One such factor can be predation of ARG-carrying bacteria. In that case, ARGs are certainly digested, as the rest of the DNA is (Monnappa et al. 2013).

4.2 *Tracking Predator-Prey Interactions* In-Situ

Tracking BALO predator-prey interactions in pure culture, i.e. the growth of a predatory strain and the concomitant consumption (and then decrease) of a prey is rather straightforward. Population sizes of the predator and of the prey can be measured by dilution plating, counting colonies of the prey and plaques of the predator (Jurkevitch 2012). Optical density also comes in handy: as BALOs cells are small, they absorb little light, enabling one to follow the decrease in optical density of the prey culture as their cells are lysed by the predator (Jurkevitch 2012). As mentioned above, the ability to express fluorescent proteins in *B. bacteriovorus* has made direct tracking of the growth of the predator possible and precise (Mukherjee et al. 2016; Sathyamoorthy et al. 2019).

Natural and other complex samples can be addressed by high throughput sequencing. The large number of reads obtained per sample uncovers non-dominant populations such as BALOs, the population sizes of which can then be estimated in terms of relative abundance. The rapid expansion of the application of these technologies has already yielded an understanding that BALOs are ubiquitous in WWT, and that they may play a significant role in bacterial turnover, at least under some conditions and microhabitats as described below. Sequencing also exposes the diversity of BALOs, including hitherto uncultured ones (Kandel et al. 2014). The first studies with quantitative PCR targeting BALOs aimed at assessing the specificity of the primers but they also showed that in a seawater sample (Zheng et al. 2008) and in a freshwater sample (Van Essche et al. 2009), BALO concentration was higher by two orders of magnitude than that detected by plaque counts, providing the first evidence for much larger BALO abundances than previously thought.

4.3 *Wastewater BALO Communities and Their Dynamics*

From Then Onward *Bdellovibrio bacteriovorus* was the first BALO to be isolated, from soil (Stolp and Petzold 1962). As the interest in predatory bacteria grew, they were searched for in various environments, including wastewater (sludge) (Dias and Baht 1965). In this first study, which of course relied on the isolation and the counting of plaques on specific prey (*Pseudomonas fluorescens, Salmonella paratyphi*), BALOs appeared to be an extremely rare type of bacterium in wastewater, averaging less than 300 cells per ml for the highest counts, and they also seemingly were unaffected by sludge processes. It was concluded that they were not active during sludge treatment. Few studies followed; three studies by (Staples and Fry 1973) and (Fry and Staples 1974, 1976) showed that larger numbers of *Bdellovibrio* spp. were present in all the WWTPs examined, and that their numbers increased between inflow and effluent. However, the BALO predators still

constituted at most 0.01% of the total heterotrophic bacterial community. BALOs were not retrieved from settling sludge, arguably because they cannot withstand anaerobic conditions (Fry and Staples 1976). The largest concentration was measured in effluents where BALOs reached 0.2% of the cultured bacteria (about 10^5. pfu.ml^{-1} and 5.10^7 cfu.ml^{-1}, respectively). However, the authors concluded that the predators (then called 'parasites') did not reduce the number of bacteria spilled into the river, the temperature of which (8-13 °C) was shown to prevent their growth.

As wastewater often contains toxic compounds, the sensitivity of BALOs to pollutants was explored. (Varon and Shilo 1981; Cho et al. 2019) showed that the growth *B. bacteriovorus* was strongly reduced in the presence of organic and inorganic chemicals, including heavy metals. Most of the compounds affecting the predators also affected the prey but a few (cadmium, copper, sodium laureth sulfate) were more potent on the predators. Similar results were obtained by (Markelova 2002; Cho et al. 2019) who showed prey and predator inhibition by 0.1% and 0.01% urea and phenol respectively. However, survival was higher when predators were associated with biofilms, which contained higher proportions of bdelloplasts, suggesting that the predator was shielded (Sanchez-Amat and Torrella 1990).

Thus, BALOs were thought to represent a rather minor fraction of the wastewater bacterial community, susceptible to environmental insults. As with environmental and ecological microbiology at large, culture-independent, DNA-based technologies proved to be a game-changer for asserting BALOs and their function in this environment.

In contrast to the results discussed above, not all pollutants appear to have a similar effect on BALOs: (Chen et al. 2014) using denaturing gradient gel electrophoresis (DGGE), a sequence-based analytic method, found *B. bacteriovorus* to be an important component of the microbial community of a combined photoreactor and a packed bed bioreactor used for the removal of the triphenylmethane dye ethyl violet. The microbial degraders appeared to be various *Ralstonia, Stenotrophomonas, Comamonas* and *Delftia,* with the three later species known to be potential BALO preys (Chanyi et al. 2013). A limitation of these findings is that DGGE cannot provide reliable assessments of relative or absolute abundance.

The developments of BALO-targeted (quantitative)PCR and 16S rRNA-gene sequencing enabled (Kandel et al. 2014) to use culture-independent approaches to quantify BALOs in zero discharge systems (ZDS) in which fish are grown at high density (Shnel et al. 2002; Cytryn et al. 2005). ZDSs are closed water systems. They usually include a nitrification loop (e.g. a trickling filter), a denitrification and an organic matter digester loop, complemented with a sulphide-removal reactor such as a fluidized bed reactor, resulting in the main water contaminants being converted to gases (Shnel et al. 2002; Cytryn et al. 2005). Aquaculture ZDSs sustain large fish yields and can use freshwater as well as seawater (Gelfand et al. 2003; Kandel et al. 2014).

BALOs and the general bacterial populations were analyzed by quantitative PCR (qPCR) over a 7-month period by targeting the Bdellovibrionales and the Bacteriovoracales with taxon-specific and general 16S rRNA gene primers, respectively. It was found that both families of predators co-existed in the different ZDS compartments, in fresh water-based systems as well as in seawater-based systems. Together, the two families of predators constituted 0.13–1.4% of the total Bacteria community. Thus, while BALOs are not a quantitatively major fraction of the community (as expected from obligate predators) they are not so-called "rare populations" (Albertsen et al. 2013). Their relative abundance was highest in the organic matter digester which also sustained the highest bacterial diversity, mostly composed of Gram negative taxa, suggesting a wide range of potential prey and direct coupling between predator and prey abundance. The samples were retrieved from the upper, largely aerobic part and thus whether BALOs can be found (and be active) in settling sludge remained unknown. Yet, and although they are considered aerobic, the presence of *cbb3*-type oxidases in their genomes suggests that BALOs may colonize oxygen-limited environments such as the upper layers of sediments, where they have previously been found (Williams 1988). (Kadouri and Tran 2013) showed that predatory bacteria preyed upon biofilms in low oxygen conditions but not on planktonic cells. The BALOs were however, not able to prey on biofilms under anoxic conditions. This contradicts a finding by (Monnappa et al. 2013) who found predation albeit limited, under completely anoxic conditions as long as nitrate was present in the medium. Although BALOs do not have *bona fide* nitrate reductase genes except for *Micavibrio aeruginosavorus* (Rendulic et al. 2004; Pasternak et al. 2014), they do include a number of nitrite reductases in their genomes. At least one (Bd2203 in *B. bacteriovorus* HD100) shows homology to nitrate reductases, thereby possibly explaining these results. Noteworthy, facultative predators (mostly Myxococcales) were highly abundant in the systems. As they are Gram negative, they may fall prey to BALOs; the occurrence of such interactions would suggest complex intraguild predation (IGP) networks at the microbial level, including not only phages, and protists, but also facultative and obligate bacterial predators (for more details on IGP, see the Chapter by Kuppardt-Kirmse and Chatzinotas "Intraguild Predation: Predatory Networks at the Microbial Scale").

4.3.1 BALOs in Advanced WWT Technologies

Effluents from activated sludge bioreactors can be further treated by microfiltration (MF) systems to remove particulate matter, increasing quality, with MF substituting the sludge setting unit and enabling total retention of the suspended solids (Bai and Leow 2002). MF membranes however, foul over time as particulate matter, including microbial cells adhere to them, causing a rapid and continuous reduction of permeation flux with time. In order to test the potential of BALOs to prevent MF membrane fouling, the outcome of predation of an *E. coli* suspension was evaluated by measuring flux parameters, with *B. bacteriovorus* predators alone (Kim et al. 2013), in combination with a flocculant (aluminum sulfate, alum) or along with

powdered activated carbon (PAC), a material reducing adsorption (Kim et al. 2014). The predator alone treatment efficiently sustained higher membrane fluxes than controls without predators. However, predation led to increased irreversible membrane biofouling, most probably caused by the accumulation of prey cell debris, resulting in pore blockage. The addition of chemical amendments – especially alum- to the predators further increased fluxes over controls, and reduced irreversible fouling. Another lab study was carried out to measure the effect of adding *B. bacteriovorus* to the membrane filtration process of activated sludge. It used a dead-end reactor with suspended solids of 3–3.5 $g.l^{-1}$ and a COD of 730–780 mg. l^{-1}, also finding improvements in fluxes (Yılmaz et al. 2014). In summary, BALOs may prove to be a worthwhile additional improvement to ease clogging in microfiltration-based devices.

A series of studies examined various wastewater treatment line architectures containing aerobic, microaerobic or anaerobic side reactors coupled to membrane bioreactors. More specifically, the addition of one or more external microaerobic or anoxic reactors in the return sludge loop of a conventional activated sludge process reduced sludge in large proportions (Semblante et al. 2014). The data supported the idea that the proliferation of slow-growing nitrifiers in the main aerobic sequencing batch reactor, and of hydrolysers and of fermenters causing sludge autolysis in the external oxygen-deficient reactors resulted in sludge reduction (Semblante et al. 2017).

Along those lines, the effects of treatments like hydraulic retention times, side-stream ratio, packing carriers, and ultrasonication on sludge reduction and dewaterability, and pollutant (nitrogen, phosphorus, COD) removal, in the different settings were measured (Cheng et al. 2017, 2018; Zheng et al. 2019). Different combinations of architectures and treatments realized significant improvements over controls (i.e. systems lacking side reactors or packing carriers etc). As an example, micro-aerobic conditions in some treatments favored sludge reduction by enriching for hydrolytic and fermentative bacteria, generating abundant substrates for hydrolysis, bringing about the disintegration of sludge floc structure and contributing to the breakdown of both refractory and biodegradable compounds (Cheng et al. 2018). As another example, packing carriers and ultrasonication applied in an membrane bioreactor (MBR) with an anaerobic side-stream reactor (ASSR-MBR) enriched for hydrolytic bacteria reduced the deterioration of sludge performance caused by a low temperature (Zheng et al. 2019).

In direct relevance to this chapter, it was observed that *Bdellovbrio* were present under all conditions tested but some led to significant increases in their abundance, with *Bdellovibrio* populations constituting up to a few percent of the total bacterial community. Microaerobic conditions and high retention times (in some of these systems, hydraulic and solid retention times are similar (Cheng et al. 2017)) promoted high BALO populations, which reached 1.5% of the total Bacteria population; a low side stream ratio or the presence of packing carriers in the ASSR-MBR also significantly increased the BALO community (Cheng et al. 2017; Zheng et al. 2019), albeit to lower levels. Based on these correlative results, it was suggested that along with the hydrolytic populations mentioned above, BALOs contribute to sludge

reduction, possibly by the predators affecting turnover of hydrolytic Gram negative populations through predation. Similar results were obtained with other processes aiming at activated sludge reduction based on the insertion of a micro-aerobic or an anoxic tank upstream to an anoxic/aerobic unit containing a feedback loop to both units. These architectures led to an increased abundance of the facultative predators Myxobacteria in studies by both (Zhou et al. 2014) and (Semblante et al. 2017) and in this latter case, also of BALOs. It should be noted that in the Zhou et al. (2014) study, sequencing was performed with the 454 Roche technology which, while enabling long reads, produced relatively low numbers of sequences (a few thousands) per sample. Also worthwhile mentioning, in all the surveyed studies, *Micavibrio* were absent from the data. *Micavibrio* strains seem to have a rather restricted prey range compared to most other BALOs (Davidov et al. 2006a; Kadouri et al. 2007; Dashiff et al. 2011), and this property (if true) may restrict their distribution. Nonetheless, a *Micavibrio*-like bacterium was detected in a sludge incubation experiment in which 13C-labeled bicarbonate was used to monitor the flow of carbon from uncultured nitrifiers to heterotrophs (Dolinšek et al. 2013). The predator was discovered by separating the heavier 13C-labelled nucleic acids, followed by 16S rRNA gene sequencing, and further localized by fluorescent *in-situ hybridization* (FISH). It was shown to attach to (and seemingly prey on) nitrite-oxidizing sublineage I *Nitrospira* but not to sublineage II *Nitrospira* in sludge flocs, suggesting a highly specific interaction.

Additional studies experimented with manipulating sludge processes by directly inoculating BALOs into the mixed liquor, demonstrating that BALOs can indeed affect sludge. In their study, (Yu et al. 2017) showed that sludge biolysis increased with the concentration of the introduced predators. It appeared that BALOs promoted bacterial cell lysis resulting in increased sludge disintegration which generally correlated with sludge dewaterability, results that support the role of BALOs in sludge processing. Microscopic observations suggested that BALO-treated flocs were smaller, and had a more porous structure with less connective filaments. Further, the physical state of WW flocs can be manipulated by operational conditions to achieve changes in output parameters (e.g sludge settling time). Under high hydraulic selection pressure that brings about washout of slow settling particles, compact granules containing self-immobilized bacteria in extracellular polymeric substances (EPS) are selected for (Feng et al. 2017; Szabó et al. 2017). The redox status of granules may shift from anaerobic/anoxic in the internal core to aerobic in the granule's outer layer (de Kreuk et al. 2005). Recently, both Szabó et al. (2017) and Feng et al. (2017) have shown that BALOs populate the granules, inhabiting specific locations within them (Szabó et al. 2017), and altering the structure of their microbial populations (Feng et al. 2017). By applying FISH targeting specific taxa, Szabó et al. (2017) precisely mapped the distribution of various species onto the granules. Bacteria associated with the external layers were also shown to have relatively low retention times suggesting easier washout caused by erosion than internally located microorganisms. *Bdellovibrio* were found in the inner parts of the granules (Fig. 2) where they actually increased in abundance during the course of the experiment. BALOs may withstand anaerobic conditions, and may even grow under such conditions (see above) but oxygen may still be able to reach these deeper

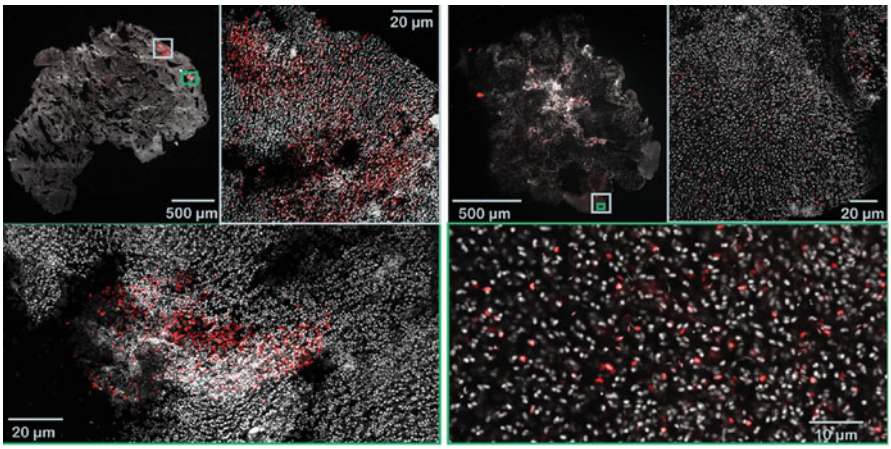

Fig. 2 FISH- confocal laser scanning microscopy images of *Bdellovibrio* in sludge granules. Cryosections of granules at ×200 magnification and at ×400 magnification. FISH probe BDE-535, according to Mahmoud et al. (2007). Grey, total cells (Syto 40); red stain, *Bdellovibrio*. (From Szabó et al. (2017) under the terms of the Creative Commons 4.0 International Licence)

regions (Szabó et al. 2017). One may speculate that predation of -relative to BALOs-large bacteria would increase oxygen diffusion by reducing demand and by creating larger channels. Studies by (Feng et al. 2016, 2017) add to the understanding of BALO-linked processes occurring in the suspended organic fraction in WWTPs. They isolated BALOs and Gram negative bacteria from WWTP, showing that almost all of the latter could be used as prey (Feng et al. 2016), as shown earlier in other aquatic habitats (Rice et al. 1998). Among these potential prey were *Bacteroidetes*, which are potentially major floc and granule hydrolyzers, and *Rhodocyclales*, both taxa that had hitherto not been tested as BALO prey. Inoculation of a BALO into suspensions of flocs or of granules, followed by community 16S rRNA gene sequencing showed that the selected predator strain significantly reduced the relative abundances of many taxa, including *Bacteroidetes* and *Rhodocyclales*, providing evidence for *in situ* predation of prey belonging to these genera (Feng et al. 2017). That said, indirect effects brought about by floc/granule structure breakdown due to predation of susceptible strains may release other bacteria to the suspension without predation, as shown by BALOs disrupting biofilms formed by Gram positive bacteria without preying on them (Im et al. 2018). In the Feng et al study (2017), predation led to a remarkable decrease in the floc and in the granule microbial biomass, and in viability by circa 50% and 50-fold, respectively. It can be remarked that Eukarya were also impacted by predation. This indirect effect of bacterial predation further shows the intricate interactions between the various types of predators present.

Treated wastewater is used to replenish natural habitats and for irrigation, while processed sludge can be used for energy and soil fertilization. Another, complementary approach for using residues of WWT is to develop their added value, for

example by producing microbial proteins from sludge, to yield high quality feed and possibly food. Matassa et al. (2016) aerobically converted sludge from a potato-processing plant into protein. The notable feature in relation to microbial predation was the very high proportion (30%) of 16S rRNA reads affiliated to *Bdellovibrio,* and the high bacterial diversity obtained in a sequencing batch reactor (SBR) operated at low selection pressure, i.e. at high solid retention time, in contrast to a continuous reactor with a short retention time. Explaining how such high relative abundance of predators can be sustained is difficult. One may think that the bacterial (prey) turnover is rapid, and or that a large part of the predatory *Bdellovibrio* population is actually not predatory but of the "host-independent type" living off the high protein content of the medium. Whether this or that, or any other hypothesis, is valid should be theoretically and empirically tested. If it could be shown that H-I variants grow and take over under the conditions prevailing in the Matassa et al. (2016) potato processing sludge experiment, the selection processes and mechanisms at play would certainly be worthwhile investigating. This would also show that H-I mutants are actually viable in nature and are not mutational dead ends. Another study explored the use of wastewater to produce polyhydroxyalkanoates (PHAs), which are carbon and energy storage compounds of many bacterial strains (Wijeyekoon et al. 2018). PHAs are fully biodegradable and possess thermoplastic properties that make them attractive natural replacements of petroleum-derived plastics. The community of a SBR with a long (4 day) solid retention time was dominated by PHA producing bacteria belonging to the *Proteobacteria* (73.0%, of which 84% were *Rhodocyclaceae*) and to the *Bacteroidetes* (25.2%, of which *Saprospiraceae* constituted 20.5%). These taxa are dominant in WWTP (Kandel et al. 2014; Semblante et al. 2017; Cohen et al. 2019) and may be preyed upon by BALOs (Feng et al. 2017). The third most abundant taxon in the reactor was *Bdellovibrio* (3.5%). *B. bacteriovorus* contains a poly(3-hydroxyalkanoate) depolymerase enabling it to consume medium chain length PHAs (Martínez et al. 2012), conferring energy and an ecological advantage to the predator (Martínez et al. 2013). For more details on biotechnological and industrial applications of BALOs, including applications relevant to PHA production, see the Chapter by Herencias et al. "Emerging Horizons for Industrial Applications of Predatory Bacteria".

Although still rather limited in scope and number, the studies presented in this and in the above sections indicate that natural BALO populations are an integral part of WWTP reactors and that in contrast to earlier findings, they react dynamically to operational and environmental changes. We would like to tentatively propose that relatively long solid retention times (which controls the concentration of bacteria throughout the system), and the addition of side reactor(s) that operate under various environments (anoxic, microaerobic, aerobic) to a main activated sludge unit promote bacterial diversity and a high abundance of hydrolysers (*Bacteroidetes* such as *Sphingobacteriales*) and of *Proteobacteria*, mainly *Rhodocyclales*, —that are potentially active in the degradation of organics, in phosphate accumulation and in denitrification-enriching for BALOs and possibly also, Myxobacteria. Hydrolysers, and predators that may prey upon them, may in turn promote floc reduction (Kandel

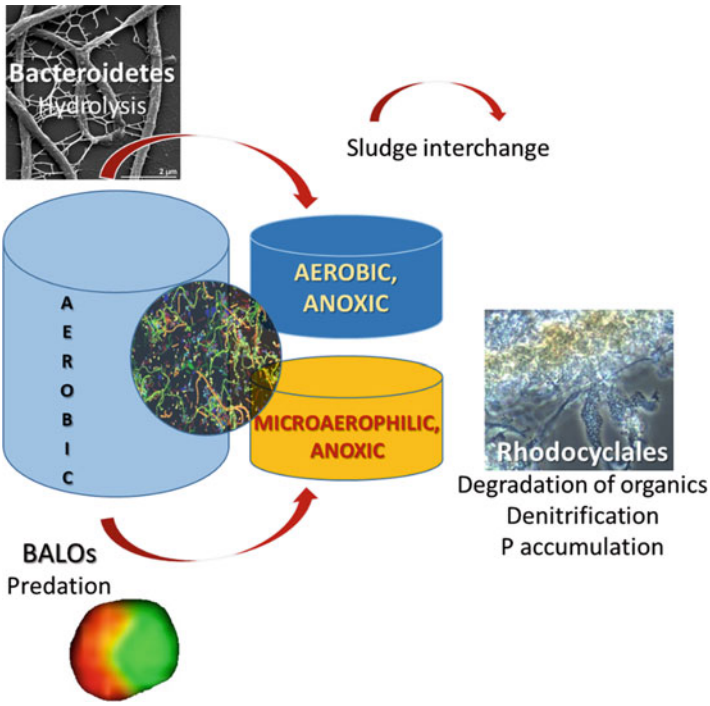

Fig. 3 Wastewater treatment schemes that may affect the concentration of predatory bacteria in the system. Side reactors with different operating conditions than the main reactor, and increased solid retention time may positively affect microbial diversity, also increasing Rhodocyclales and Bacteroidetes. The former may degrade organics, denitrification and P accumulation, the latter may increase hydrolysis of flocs, and in concert with increased predators that may also prey upon them, bring about sludge reduction

et al. 2014; Zhou et al. 2014; Cheng et al. 2017, 2018; Feng et al. 2017; Semblante et al. 2017; Wijeyekoon et al. 2018) (Fig. 3).

Two interesting observations will conclude this section. The study by Feng et al. (2017) included a transcriptomics (RNASeq) analysis of flocs and granules exposed or not to *B. bacteriovorus*. Although community composition and floc structure were altered by the inoculated predators (see above) the expressed functions were not, hinting that predation is either not discriminatory enough in term of taxonomic differences to impact upon the functionality of the community or, alternatively, that functional redundancy and compensation mechanisms are at play. The latter would somewhat be surprising as the large loss in cell viability (50-fold) and biomass (50%) engendered by predation may be defined as a large scale disruption, i.e. a situation thought to bring about functional disruptions. Another study examined the role of the second messenger cyclic di-GMP on the stability of aerobic granules in a sequencing batch reactor (Wan et al. 2013). Cyclic-di-GMP is a cellular signal that strongly affects bacterial phenotypes such as motility, biofilm formation, EPS and cellulose synthesis, virulence, and many other features (Jenal et al. 2017).

In *B. bacteriovorus*, different effectors of cyclic di-GMP metabolism differentially affect H-I formation by preventing it or by making it obligatory; they also affect gliding, progeny exit from the bdelloplast, and attachment to prey (Hobley et al. 2012b; Milner et al. 2014). The addition of manganese to the reactor brought about disintegration of granules, causing a significant decrease in cyclic-di-GMP cellular concentrations of the total bacterial community, leading to a decrease in EPS (Wan et al. 2013). A clone library (therefore restricted in size and coverage as compared high throughput sequencing) showed a high representation of *Bdellovibrio* 16S rRNA gene inserts in the control treatment (circa 4%). The manganese treated samples still had a rather high (circa 2%) but significantly reduced BALO population. Whether c-di-GMP metabolism plays a role in the association of *Bdellovibrio* with biofilms (granules are biofilm-like structures) is not known. As with the gene expression changes in the BALOs and in the prey populations in flocs, this interesting question remains to be further investigated.

5 Outlook: Basic Questions, Technological Bridges, and Applications

The information analyzed in this review unequivocally shows that BALOs are almost always present in WWTPs. They usually consist of low abundance but not rare populations and they can significantly increase in size, as a response to biotic and abiotic-driven changes in the environment, showing that BALOs are active members of microbial trophic networks. A key aim is to understand their "space" in the networks, i.e. their effects on the dynamics and stability of microbial ecosystems. This broad aim can be reduced to more focused (yet still broad) questions such as: what is the impact of BALO predation on the community structure and community components thereof; how qualitatively and quantitatively do BALOs directly (by predation) and indirectly (e.g. by breaking down flocs/biofilms) contribute to bacterial mortality and to nutrient release, i.e. to bacterial turnover; what is their relationship to other microbial predators, e.g. other bacterial predators like Myxobacteria, phages and protists? Such knowledge, which can be obtained from experiments under natural and under controlled conditions would be valuable both for theory and for applications. In order to decipher the role and impact of BALOs on the intricate microbial trophic networks of WWTPs, of other microbial ecosystems, and more globally on nutrient flow, precise quantitation and identification of predator and prey interaction dynamics is necessary. It should be remarked that since BALOs require a prey to grow, many BALOs may not be cultured in the laboratory as their prey may by themselves be unculturable. Fortunately, the sequencing revolution has been accompanied by other powerful advances in microbial community analysis, a few of which are presented here.

QPCR based on specific primers can reveal the sizes and fluctuations of specific BALO populations in absolute terms (Zheng et al. 2008; Van Essche et al. 2009) that can also be expressed as relative to the total bacterial population size if this is measured

using general primers targeting the 16S rRNA gene (Kandel et al. 2014). Thus, by combining high throughput sequencing and qPCR, it might be possible to track and identify predators and their dynamics in complex samples. Yet, a pertinent question remains: how can the predators' prey be identified so the impact of predation be quantified in detail? An approach is computational: by statistically correlating fluctuations/co-occurrence in terms of abundance of the Gram negative populations to those of the BALO populations, links may appear (Welsh et al. 2015). These can be characterized and quantified to uncover the potential prey range of the different predators, their impact on the prey population, and possible mechanisms underlying the revealed dynamics. However, empirical approaches that would directly detect such interactions *in situ* and confirm the computations are forcefully required. Methods could be developed based on existing technologies such as FISH for labelling predators and fluorescence activated cell sorting to obtain bacterial populations interacting with a labelled predator. Sorted samples could be sequenced to reveal the composition of the interacting populations. Emulsion, Paired Isolation and Concatenation (EPIC)-PCR makes use of emulsion PCR to isolate single cells which can be identified and linked to a chosen genomic feature (Spencer et al. 2016). It may therefore be possible to apply it to uncover direct interactions between cells, and obtain comprehensive identification of pairwise interactions between predators and prey. Predatory interactions can also be uncovered and analysed at the metabolic level using stable isotope probing. As shown by (Chauhan et al. 2009) and Dolinšek et al. (2013) using BALOs, nutrient flow from labelled prey to predators can be tracked to identify active predators. In the former case heterotrophic bacteria were labelled using a rich medium, in the latter, autotrophs were labelled with bicarbonate, both using ^{13}C. Predators preying upon these two metabolic classes were then identified by cloning the "heavy", ^{13}C-labelled DNA. Use of these approaches individually or in conjunction with each other will enable researchers to track and decipher complex interactions in natural ecosystems or in microcosms mimicking them; or in simplified settings aiming a describing in mechanistic details specific interactions between micro-predators and prey under various conditions (Johnke et al. 2017a, b).

These are but a few examples of novel technologies that could in the (hopefully near) future, help solve questions pertaining to the ecological theory of microbial predation, as an example, when using the IGP approach to decipher community interactions. Such approaches would also provide much needed data for modeling predator-prey interactions and understanding how simple, and more complex ecological systems stabilize. Applications could come all along. At the top of the list, WWT could greatly benefit, through the devise of approaches and technologies that improve the ecological stability of WWTPs, by reducing deleterious and operational disruptive fluctuations in community structure and by improving the efficiency of positive processes involving BALOs (e.g. sludge reduction). It might also become possible to envisage small scale, decentralized WWT systems that target microbial biomass, and more specifically pathogen and ARG reduction, helping to reduce their burden on LMICs.

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Intraguild Predation: Predatory Networks at the Microbial Scale

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Contents

1 Introduction

Microbes live and interact within diverse communities of organisms which are embedded in complex networks of competition, parasitism, and predation. Predatory interactions in microbial communities have been of central interest in microbial ecology and ecological theory (Cohen et al. 2019; Gao et al. 2019; Gause 1934; Karakoç et al. 2017; Miki and Jacquet 2008; Pernthaler 2005), and interactions within microbial foods webs have been recognized as crucial key drivers for energy fluxes and nutrient transfer and recycling (Azam et al. 1983; Clarholm 1985; Sherr and Sherr 2002). It is now well accepted that top-down control by predators is the most important factor for mortality in bacterial communities (Breitbart 2012; Sherr and Sherr 2002). However, we still do not fully understand how diverse these

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interactions are, to which extent they are affected by environmental changes and how temporal and spatial scales impact their dynamics and their contribution to ecosystem processes. In order to be able to predict the functioning of microbial communities in the context of global change, we need to fully address multispecies predator-prey interactions. This requires integrating research on eukaryotic micro-predators of microbes, i.e. protists, as well as viruses and predatory bacteria, with a specific focus on the combined effect of micro-predators with partially overlapping prey ranges (Johnke et al. 2014). Only then will we be able to comprehensively understand the relevance on top-down control as compared to a resource-driven bottom-up control in different environments and along different scales. One important, but rather neglected, aspect of multiple predation is whether predators which potentially share prey resources also trophically interact with each other, a process which is called intra-guild predation (IGP) (Polis et al. 1989). While IGP has been described and studied in many ecosystems for higher organisms (Arim and Marquet 2004; Vance-Chalcraft et al. 2007), there is still a considerable knowledge gap regarding its relevance to the microbial world. In this chapter, we therefore attempt to first briefly present the key micro-predator groups on the microscale (in particular the protists and viruses, but also bacterial predators), to introduce the basic concept of intraguild predation (IGP) and to summarize several microbial IGP studies. We finally provide an outlook touching on a few selected aspects and techniques which may be very useful in order to address microbial IGP in a context of basic and applied ecology.

2 Protists

Protists, single-celled eukaryotic microbes ranging mostly between less than 2 and 200 μm in size (Sherr and Sherr 2016), were probably the first microbes, if not even the first micro-predators, which were visualized and described by Antonie van Leeuwenhoek in the second half of the seventeenth century (Corliss 1975). Eukaryotic microbes were traditionally studied either as photoautotrophs (“algae”) or heterotrophs (“protozoa”) (Sherr and Sherr 2016). “Protozoa” were further separated into three broad categories according to morphology and locomotion, namely the ciliated, the amoeboid and the flagellated protists (Finlay and Esteban 2001). Protists are ubiquitously distributed and very abundant in the environment: 1 g of soil can contain between 10,000 and 100,000 individuals (Geisen and Bonkowski 2018), while several millions of phagotrophic protist cells per ml may be found in aquatic systems (Berninger et al. 1991). As a consequence, non-pigmented, phagotrophic protists constitute a highly relevant factor for the top-down control of bacteria in the environment.

Advances in particular in molecular biological and phylogenetic approaches have revolutionized the former rather simplistic morphology-based protist classification, diversity and evolution (Geisen et al. 2018) resulting in an eukaryotic tree of life which is mainly represented by single-cell protists taxa (Keeling and Burki 2019). As compared to prokaryotic communities, ribosomal RNA based techniques were

used with some delay for the study of free-living protistan communities in the environment. The first application of PCR-based fingerprinting (Van Hannen et al. 1999) and Sanger sequencing (López-García et al. 2001; Moon-van der Staay et al. 2001) indicated that, similar to the prokaryotes, a significant number of protists may have escaped description by culture-dependent techniques or direct observation. The emergence of amplicon high-throughput sequencing approaches provided even more exciting insights into the unprecedented diversity and distribution of protists in natural and engineered systems (Cohen et al. 2019; Geisen 2016; Lentendu et al. 2014; Massana et al. 2015; Singer et al. 2019).

In this chapter we focus on phagotrophic (i.e. predatory) protists that are capable to consume bacteria or other eukaryotic microbes. Many bacterivorous protists may in fact be facultative omnivores and grow on a wide range of other (micro) organisms including yeasts, fungi, protists or even nematodes (Geisen et al. 2018). While not further discussed here, we want to emphasize that the functional and ecological roles of unicellular protists are much more diverse, including parasites, symbionts, primary producers, fungal feeders, saprotrophs and mixotrophs (Adl and Gupta 2006; Caron et al. 2017; Geisen et al. 2018).

The fundamental importance of phagotrophic protists for microbial foods webs was first conceptualized in the paradigm of "the microbial loop" for aquatic ecosystems (Azam et al. 1983) and later on with some modifications also for the interactions of soil protists and plants (Bonkowski and Clarholm 2012; Clarholm 1985): by consuming bacteria, protists liberate nutrients (in particular nitrogen and phosphorus) otherwise immobilized in bacterial biomass and make them available for other organisms. In soils, additional auxiliary indirect effects on plant growth were postulated, resulting from predation-driven changes in the rhizosphere bacterial communities (Bonkowski and Clarholm 2012). The wide range of morphotypes, sizes and feeding modes (e.g. as filter, diffusion and raptorial-interception feeders) strongly indicate that phagotrophic protists should not be treated as one indistinguishable functional group (Fenchel 1987; Jürgens and Massana 2008; Montagnes et al. 2008). Despite being often considered as rather general predators in comparison to viruses and predatory bacteria, prey selectivity is very common and may also be one explanation for the coexistence of different phagotrophic protists. Selectivity largely depends on distinct traits of prey cells, including motility, cell surface characteristics, chemical cues, prey aggregation, prey concentration and cell size (Boenigk and Arndt 2002; Boenigk et al. 2002; Jakobsen and Tang 2002; Pernthaler 2005; Šimek and Chrzanowski 1992). Prey selectivity in combination with distinct bacterial anti-predation mechanisms (e.g. see Matz and Kjelleberg 2005) and competition among prey thus significantly affects the composition, diversity, evenness and function of the resulting prey community (Flues et al. 2017; Gao et al. 2019; Kurm et al. 2019; Salcher et al. 2016; Saleem et al. 2012; Sherr and Sherr 2002). Finally, diversity at both the protist predator and the bacterial prey level interactively determine trophic networks and microbial driven functions (Saleem et al. 2016).

3 Viruses

In the last two decades, convincing evidence has been presented that a second group of micro-predators, the viruses, complements the protists as primary agents of bacterial mortality. The so far accumulated evidence shows that viruses are the most abundant biological entities or at least as abundant as their hosts (Bergh et al. 1989). Average concentrations of viral particles in surface seawater reach up to 10 million per milliliter (Wommack and Colwell 2000), while virus-to-bacteria ratio (VBR) often ranges between 1 and 100 (Parikka et al. 2017; Wigington et al. 2016). In soil, viral abundances range between 10^3 and 10^9 per gram dry soil (Narr et al. 2017; Williamson et al. 2017) with VBR varying much more than for aquatic systems (Williamson et al. 2005). The number of viral particles in the human gut is in the range of 10^8 and 10^9 per gram of faeces (Hoyles et al. 2014; Kim et al. 2011).

The polyphyletic origins of viruses, the presence of viral genomes either as DNA or RNA in different configurations and the rapid "mosaic" nature of virus evolution has somewhat limited the description of environmental viral communities based on a single universally shared marker gene (however, see Adriaenssens and Cowan 2014). The advent of metagenomic tools and analysis pipelines has enabled us to characterize the diversity of environmental viral communities. Following the first study from marine communities in 2002 (Breitbart et al. 2002), several large-scale investigations in surface and deep-ocean marine ecosystems have been performed, significantly expanding our knowledge on marine viral communities (Brum and Sullivan 2015; Roux et al. 2016). Extending metagenomics studies to other systems such as the human oral cavity and gut (Norman Jason et al. 2015; Pride et al. 2012), soils, freshwater or subsurface systems (Brum et al. 2015; Cárcer et al. 2015; Emerson et al. 2018; Kallies et al. 2019; Paez-Espino et al. 2016) has greatly increased our knowledge on the diversity and biogeography of previously unknown viral communities.

Despite this remarkable progress in describing viral diversity, many aspects of viral ecology and virus-host interactions remain largely unknown. Viral life cycles can be classified into lytic, lysogenic, chronic, and pseudolysogenic ways of reproduction (Ackermann and DuBow 1987). The lytic cycle results in the daily lysis of 20–30% of the bacterial biomass in aquatic systems (Suttle 2005) and may even destroy up to 89% of the prokaryotic production in marine sediments (Danovaro et al. 2008). In contrast, during the lysogenic cycle, the genome of the (temperate) virus is integrated into the genome of the host as a prophage, which is then replicated along with the host. Carrying a prophage can be of advantage for the host cell during times of low growth rates or under unfavorable conditions (Breitbart et al. 2018) for instance due the suppression of non-essential host metabolic activities (Paul 2008). Different environmental signals can induce the lytic cycle again, triggering the production of new viral particles and the release of these particles after host lysis (Campbell 2006). The question of which type of virus-microbe interaction is prevalent in which ecosystems is currently unresolved and remains a matter of

controversy. Density-dependent models, such as the Kill-the-Winner model (Thingstad 2000) support a prevalent role of lytic viruses, while alternative models, such as the Piggyback-the-Winner model predict that lysogeny may be a dominant viral strategy at high host abundances (Coutinho et al. 2017; Knowles et al. 2016; Silveira and Rohwer 2016). As for protist-bacteria interactions, bacterial strains can develop resistance against viruses, resulting in coevolutionary arms-races (Stern and Sorek 2011).

Most of the increasing evidence for the impact of the viral component in the environment has been obtained from marine systems demonstrating that there are still significant gaps in the progress of viral ecology in other ecosystems. Lytic viruses contribute to marine biogeochemical cycles either as a "shunt" by redirecting carbon away from larger organisms towards other microorganisms or as a "shuttle" by creating lysates that sink to the bottom of the sea (Fuhrman 1999; Guidi et al. 2016). Most impressive are the consequences of virus-host interactions for the genetic landscapes and biogeochemical cycles: marine viruses constitute a reservoir of microbial metabolic properties, which can be shared among their microbial hosts (Dinsdale et al. 2008; Fancello et al. 2013; Hurwitz and Sullivan 2013). The transfer and the expression of genetic information, which modulates microbial metabolism through host metabolic genes or 'auxiliary metabolic genes' (AMGs) (Breitbart and Rohwer 2005; Enav et al. 2014; Graham et al. 2019; Lindell et al. 2005), has provided exciting evidence for the viral impacts on host fitness and various important large-scale biogeochemical processes (Breitbart and Rohwer 2005; Gao et al. 2016; Hurwitz and Sullivan 2013; Roux et al. 2016; Williamson et al. 2008). However, the presence and dynamics of AMGs potentially affecting critical microbial functions in soils under human pressure is so far unclear (Graham et al. 2019; Trubl et al. 2018).

Revealing and quantifying virus-host interactions both in situ and in the lab poses one major challenge in basic and applied viral ecology. Isolation of new marine viruses, single-cell metagenomics and virome studies have recently indicated that broad-host range viruses may be much more distributed in nature than previously assumed (Brum et al. 2016; Kauffman et al. 2018; Munson-McGee et al. 2018; Roux et al. 2016). Also, there is no consensus about what exactly constitutes a "broad" versus a "narrow" host range (de Jonge et al. 2019). One so far underestimated aspect may be the unresolved methodological biases during virus isolation (de Jonge et al. 2019). In addition, several ecological parameters such as host diversity, host density and quality have been described as relevant in determining virus host range in different environments (Dekel-Bird et al. 2015; Heineman Richard et al. 2008).

4 Predatory Bacteria

The third group of micro-predators includes predatory bacteria, which are either obligatory or facultative bacterivorous bacteria. Similar to viruses, and in contrast to protists, many predatory bacteria are smaller than their prey, thus requiring different

strategies in order to penetrate the prey cell or to lyse the prey (Pérez et al. 2016). Bacterial hunting strategies can be roughly separated into three general groups: (i) epibiotic predation in which the prey is consumed from the outside, (ii) endobiotic predation, which requires penetration of the host and (iii) group attack and killing in the extracellular environment (Pérez et al. 2016). However, several variations exist within each of the hunting strategies, while several bacteria apply so far undescribed strategies or strategies which do not fit in any of these groups (Gerphagnon et al. 2015; Martin 2002; Pérez et al. 2016). A common characteristic of most hunting strategies is the need for motility, either as flagellum motility or as slow gliding motility. The first predatory bacteria were described in the 40s (Beebe 1941), yet, they belong to the least studied micro-predators in environmental systems. Predatory bacteria vary in their prey range (Chen et al. 2011; Enos et al. 2018; Jurkevitch et al. 2000); however, the in situ prey preferences are mostly unknown, since studies on prey range are limited by the number of possible prey which are amenable to cultivation.

The significance of predatory bacteria in environmental systems remains understudied, although different predatory bacteria, such as members of the polyphyletic taxon *Bdellovibrio*-and like organisms (BALOs), have been reported to be widely distributed in marine, freshwater, and terrestrial ecosystems, and to represent a key factor for the mortality of prey in some ecosystems (Davidov et al. 2006; Kandel et al. 2014; Li and Williams 2015; Paix et al. 2019; Pineiro et al. 2007). Similar to the other two micro-predator groups, amplicon sequencing, DGGE-fingerprinting and PCR-free metatranscriptomics have contributed to a wide range of new information regarding either the diversity of this group (Li and Williams 2015; Paix et al. 2019), its relevance as highly abundant and active component of the soil food web (Petters et al. 2018), or its genomic equipment required to act as predators (Crossman et al. 2013; Pasternak et al. 2013).

The fact that some of the bacterial predators have a potentially restricted host range alike viruses has attracted significant interest in applying predatory bacteria as "living antibiotics" (Dwidar et al. 2012; Kadouri et al. 2013; Reardon 2015). Prey bacteria can develop resistance to predation by BALOs (Varon 1979), yet, ecological conditions will affect the expression of different types of resistance (Gallet et al. 2007, 2009). In addition, prey populations may exhibit transient phenotypic plasticity leading to increased resistance to predation (Shemesh and Jurkevitch 2004). However, as compared to the other two groups, there is still a significant gap in quantifying the extent to which predation by bacteria affects energy and nutrient fluxes, and ecological interactions within a complex microbial food web. For more details on BALOs, see the chapters by Jurkevitch and Im et al.

5 Intraguild Predation

5.1 Ecological Theory of Intraguild Predation

Intraguild predation (IGP) occurs when two species of the same guild that share a prey and thus potentially compete, also engage in a trophic interactions with each other (parasitism or predation) (Rosenheim et al. 1995), even if they acquire prey with different strategies (Polis et al. 1989). "Guilds" is used here in the broadest sense and includes all organisms in a community that share a common food source (Rosenheim et al. 1995).

IGP is widespread within many ecological communities and at all trophic levels (Arim and Marquet 2004; Holt and Polis 1997). It differs from classical predation and from competition, because potential competition is reduced and the predator directly profits from energetic benefits (Polis and Holt 1992). The impact of IGP on population dynamics and species distribution is more complex and can result in different outcomes including co-existence, alternative stable state or exclusion (Polis and Holt 1992); with IGP, vertical niche breath of consumers are increased thus resulting in increased complexity of the whole food web (Duffy et al. 2007). However, the potential effects of IGP on diversity and functioning in more diverse food webs have so far not gained the attention this fundamental question requires (Irigoien and de Roos 2011; Wang et al. 2019).

Polis et al. (1989) classified different IGP configurations with respect to symmetry and age structure. Asymmetric IGP occurs when one species (A) is always the intraguild predator of another (smaller) species (i.e. the intraguild prey, IG prey). Most predators consume prey in a particular size range regardless of that prey's trophic levels and are thus frequently omnivorous both on a resource and on a smaller consumer of that resource (Polis et al. 1989). Symmetric IGP occurs during mutual predation between A and B. In contrast to micro-organisms, age structure can be quite important in guilds of higher organisms, as seen in the frequent occurrence of age-dependent IGP (Polis et al. 1989). One widespread asymetric configuration, potentially relevant also on the microscale, is coincidental IGP: here, a larger consumer uses food resources which are colonized by or serve as hosts for smaller organisms (Polis et al. 1989).

Models predict that a three-species intraguild predation assemblage is stable if the intermediate predator is using the shared prey more effectively than the intraguild predator (Holt and Polis 1997). The consequences of IGP are, in particular, relevant in a context of biocontrol, which is a highly important ecosystem service provided by natural or introduced predators of pest species (Rosenheim et al. 1995). So far IGP has been shown to have positive, negative or neutral effects on prey suppression by natural or introduced predators (Boulanger et al. 2019; Rosenheim et al. 1995). A meta-analysis comprising 126 comparisons from marine and terrestrial ecosystems showed that the effects of IGP varied across ecosystems, and that the effect of an additional top or intermediate predator depended on the performance of these predators when alone. Adding a top predator could increase IGP, resulting in

decreased prey consumption (Vance-Chalcraft et al. 2007). The authors thus suggested that before augmenting a system with multiple predatory control agents it is reasonable to verify if IGP occurs; multiple control agents will more effectively control the target prey in the absence of IGP than a single control agent.

IGP can slow down the bottom-up energy flows between different trophic levels and release prey, which subsequently are able to transform more energy inflows into biomass (DeBruyn et al. 2007; Schneider et al. 2012; Wang et al. 2019); the increased biomass at lower trophic levels may in turn serve as resource for organisms at higher trophic levels (Wang et al. 2019). Simulations of complex food-web models showed that IGP increases species diversity, biomass and energy fluxes across trophic levels potentially due to an expanded vertical niche space with IGP; this in turn may decrease exploitative competition between species, thus creating more niches for species coexistence (Holt and Polis 1997; Wang et al. 2019).

5.2 *Microbial Intraguild Predation*

The theoretical concepts of intraguild predation (IGP) suggest two main factors which have far reaching implications for coexistence of IGP members and system stability (Holt and Polis 1997). First, the level of environmental productivity should determine the coexistence between IG prey and IG predator. Second, an IG prey being a superior competitor for the common resource prey is a necessary condition for coexistence. Microbial systems are very suitable to further identify conditions interacting with these factors in IGP, such how abiotic and biotic parameters affect growth of the prey, the competitive traits of IG prey and predator species, as well as defense traits of bacterial prey. In the following we will highlight several recent results with respect to microbial IGP. Most of the herein presented (mostly experimental) studies focused on different protist species as IG prey and IG predators, and bacteria or algae as the common resource, while some also examined the relationship between mixotrophic protists (as IG predator) and bacteria (as IG prey). In addition, we present the few studies involving interactions with or between so far neglected micro-predator groups.

5.3 *Microbial IGP Along Productivity Gradients*

Theory suggests that coexistence of IG prey and IG predator is most likely occurring at intermediate levels of environmental productivity (Holt and Polis 1997). Under low productivity conditions the competition for the prey dominates, which could favor the IG prey. In contrast, high productivity conditions favor the IG predator due to its higher abundance that can drive the IG prey to extinction.

Different experiments have confirmed the importance of intermediate productivity levels for coexistence. In microcosms with bacteria as the common resource and

two ciliates as IG prey (*Tetrahymena*) and IG predator (*Blepharisma*), resource enrichment resulted in a decline of IG prey abundances, whereas bacteria and IG predator abundances increased (Diehl and Feissel 2000, 2001). In agreement with theoretical prediction is also the exclusion of a ciliate IG predator (*Blepharisma*) by a ciliate IG prey (*Colpidium*) under low bacterial productivity. At increasing levels of bacterial productivity coexistence was observed (Morin 1999). Further resource enrichment supported only the IG predator (*Blepharisma*), while the two IG prey species (*Tetrahymena* sp. and *Colpidium* sp.) were depressed or excluded. Field studies from an eutrophic lake largely support these findings. Seasonal monitoring of the abundance and biomass of bacteria, heterotrophic nanoflagellates (HNF) and ciliates revealed a clear dominance of ciliates (IG predator) over HNF (IG prey) due to the strong grazing pressure of the IG predator (Kisand and Zingel 2000; Zingel et al. 2007). Lack of top-down regulation of the IG predator in this study site could explain this clear outcome. A theoretical model with a tritrophic food web motif, which is capable of switching between IGP and food chain motifs, confirmed the stabilizing effect of IGP. Stable systems changed with increasing nutrient availability to oscillatory dynamics although the transition occurred for linear food chains at lower nutrient levels than for IGP (Karnatak and Wollrab 2017).

However, there are also results that challenge the theoretical predictions. Incubating the IG predator *Ochromonas* and IG prey *Microcystis* at different nitrogen concentrations did not result in a decrease of IG prey abundances by the IG predator as expected at high nitrogen concentrations (Wilken et al. 2014). The authors assumed that intraspecific interferences at high IG predator densities reduced the grazing rates on the IG prey. This indicates that there are relevant predator traits influencing IGP that should be considered. Mesocosms consisting of copepods as top predators and complex experimental food webs comprising distinct IGP units showed that productivity had no influence on the abundance of IG prey at any case. The heterotrophic dinoflagellate *Oxyrrhis* for example acted as IG prey for the copepods and preyed on nanophytoplankton and persisted under high and low productivity to a similar extent (Ptacnik et al. 2004). The same outcome was observed for predation of picophytoplankton (the IG prey) by the mixotroph IG predator *Chrysochromulina*, indicating that the interconnection of several IG prey and IG predators in more complex food webs possibly allows a broader range of system productivity to maintain coexistence.

In a similar complex food web study, where only top predators were removed by filtration, the IG prey was less predated by the IG predator under enrichment conditions (Piwosz and Pernthaler 2011). Contrary to theoretical predictions, the abundance of IG prey cells in the vacuoles decreased when more resources were available for the bacterial prey. When only the top predator was removed more IG prey cells were found in the vacuoles of the omnivorous flagellates. IGP can under certain conditions be of advantage for IG predators even under low productivity settings, as shown in laboratory microcosms containing the ciliate *Colpidium* as IG prey and the freshwater ciliate *Tetrahymena vorax*, which exhibits inducible trophic polymorphism (Banerji and Morin 2009). *T. vorax* transformed into macrostomes

which could feed on the IG prey instead of on bacteria thus gaining a greater net benefit.

This overview of selected experimental and field studies as well as models on microbial IGP shows that the abundances of microbial IG members and their coexistence cannot easily be predicted and are not only regulated by the productivity even in relatively simple food webs. With increasing complexity of the food webs and of interconnections, IG prey and IG predators could coexist under a wider range of environmental productivity.

5.4 Trait-Mediated Microbial IGP

A necessary condition for stable coexistence of intraguild members is the presence of an IG prey that is superior in resource use than the IG predator (Holt and Polis 1997). The relevance of the competitive traits of the IG prey and predator species have therefore been in the focus of some experimental studies or model observations. Price and Morin (2009) showed that different initial densities of the two IG predators *Blepharisma* and *Tetrahymena vorax* did not establish alternative stable states, but that resource consumption was the primary cause for the failed persistence of *Tetrahymena* in the microcosms. As the latter is in its microstome form also acting as IG prey for both IG predators, its high rates of predation by *Blepharisma* (Price and Morin 2004) and the lack of superiority in resource consumption could explain this observation. It is also important to mention in this context that the assembly sequence determines if one IG predator is excluded or if both can coexist (Price and Morin 2004). However, under particular circumstances, the IG prey can form commensalistic relationships with the IG predator to overcome the smaller resource competitive strength of the IG prey. In a system consisting of two flagellates, one acting as common resource (phototrophic dinoflagellate *Scrippsiella trochoidea*), one acting as IG prey (dinoflagellate *Gyrodinium dominans*), and a ciliate IG predator (*Favella ehrenbergii*), the IG prey showed higher growth rates in the presence of the IG predator (Löder et al. 2014). The reason for this unexpected result was the behavior of the IG predator, which after taking up common resource cells, rejected and released a proportion of the caught cells in an immobilized status. This immobilized prey could subsequently be easily used by the IG prey as resource. Such commensalism might open an additional possibility for stable coexistence of IG members in spite of their competition (Löder et al. 2014).

Predators in environmental systems usually differ in their prey range. Kang and Wedekin (2013) studied in two models whether a generalist or specialist IG predator differently affected stability and coexistence. Although both models can have multiple stable states, the IGP model with the IG predator being a generalist tends to allow for coexistence of all three species. Unstable dynamics of IGP systems could also be stabilized by the addition of a top predator that feeds preferentially on the more efficient competitor for the common resource (Kratina et al. 2012).

Grazing pressure can result into (rapid) evolution of traits for microbial prey defense (Friman et al. 2014; Hiltunen and Becks 2014; Yoshida et al. 2003). These defense traits impact IGP systems, but can also be influenced by IGP. Friman et al. (2016) investigated the effects of IGP on the development of prey defense with *Pseudomonas fluorescens*. In the presence of an IG predator (*T. vorax*) *T. pyriformis* became the IG prey and the prey defense evolution of *P. fluorescens* was constrained. This study emphasizes that known predator-prey interactions might develop completely different in complex communities with multiple predators. Similarly, Hiltunen et al. (2014) could show that in separate experiments the common resource prey algae could evolve defenses against each predator (a rotifer and a flagellate) and both predator and prey settled into cycles of predator and prey abundance that are typical for eco-evolution. However, including both predators, i.e. the flagellate as IG prey and the rotifer as the IG predator, increased the food web complexity and impeded any prediction of the experimental outcomes (Hiltunen et al. 2014). In the absence of evolution the same complex food web developed temporal dynamics with abundances peaks that corresponded exactly to the theoretical predictions (Hiltunen et al. 2013).

A model investigating the effect of inducible defense on community dynamics in a two-predator system with the prerequisite that defense against one predator leads to vulnerability to the other predator revealed strong destabilization of the system due to a strong synchronizing effect (Velzen et al. 2018). The latter depended highly on the exchange rate between the phenotypes: a high rate had a negative impact on the stability while intermediate rates stabilized the system.

Defense mechanisms can also be induced in the IG prey (Kratina et al. 2010). Predation of the ciliate *Euplotes* (the IG prey) by a turbellarian flatworm induced morphological anti-predation changes; the IG prey species with the greater capability of inducing defensive morphotypes persisted longer under predation by the IG predator.

5.5 *Viruses and Predatory Bacteria – Underexplored Micro-predators in IGP*

As seen in the above examples, microbial IGP experiments have so far mostly used protists, however, other, less-studied members of food webs can also be involved in IGP. Viruses and bacterial predators are possible IG prey, or may be part of coincidental IGP (Holt and Polis 1997). However, up to now experimental or field studies are focusing only on pairwise interactions (e.g. virus and bacteria or virus and protist) or on the combined effects on the common resource (i.e. bacterial prey or host), neglecting potential interactions with an IGP configuration. Viruses are known to be preyed on or be inactivated by different protist groups, such as HNF and ciliates (Bouvy et al. 2011; Deng et al. 2014; Miki and Jacquet 2008; Pinheiro et al. 2007). These studies investigated the ability of protists to ingest and potentially inactivate

viruses but did not consider uptake of prokaryotic viruses as part of IGP food webs. Further experimental studies investigating the extent to which this IGP occurs and their impacts on complex food webs are necessary.

Miki and Yamamura (2005) postulated in a model that grazing of protists on viruses has a negative effect on the abundance of viruses and indirectly causes a reduction in bacterial species richness, which was called "kill the killer of the winner" hypothesis. However, several studies investigating the influence of protists presence on viruses found a stimulation effect on viral activity and proliferation (Berdjeb et al. 2011; Jacquet et al. 2007; Ram and Sime-Ngando 2008; Šimek et al. 2001; Weinbauer et al. 2007) which might be due to cascading effects from the grazing induced resource enrichment. A recent study offered an additional explanation for that observation: The phagocytic vesicles of protists were found to act as a reaction chamber for the infection of suitable bacterial hosts with viruses (Aijaz and Koudelka 2017). As not all bacteria that are engulfed by protists are digested, bacterial lysogens (i.e. bacteria containing a prophage) can be induced and the released viruses can subsequently infect co-ingested bacteria, which finally might result into a release of freshly produced viral particles. That means that protists can actively contribute to the dissemination of viruses.

Co-predation of bacteria by protists and viruses has also been shown to influence the evolution of defense mechanisms. Bacteria that grew in the presence of protist predators and lytic viruses exhibited less susceptibility to viruses (Örmälä-Odegrip et al. 2015) or diverged into defense specialists (Friman and Buckling 2013). These examples indicate that strong pairwise coevolution can persist even in complex food webs, if different selection results in evolutionary diversification of distinct defense mechanisms (Friman and Buckling 2013).

Few studies have investigated the combined predation of bacteria by BALO and viruses. Chen and Williams 2012 demonstrated that viruses and BALOs (*Bacteriovorax*) do not necessarily compete for their prey but can infect and successfully reproduce in the same bacterial cell of *Vibrio vulnificius*. An experiment including *V. vulnificius* as prey and the predatory bacterium *Halobacteriovorax* and a virus as predators showed that the combination of both predators lead to the greatest reduction of bacterial cells, confirming that BALOs along with viruses and protists are significant contributors to bacterial mortality (Chen et al. 2018). In another study, predation by either only viruses or by combination of viruses with flagellates was compared (Ram and Sime-Ngando 2014). Presence of virvirus and three bacterial strains as common resourceuses in the microcosm experiments resulted into low-diverse prey communities, in contrast to the high diversity observed when both, viruses and flagellates were present.

Clearly, studies investigating the combined impact of IGP on the bacterial preys as well as on the different possible IG preys are missing. Only one study investigated so far the relationship between three different predators with differing prey range, a ciliate protist, a BALO and a virus and three bacterial strains as common resource (Johnke et al. 2017a). A potential suppression of the more specialized predators (i.e. virus and BALO) by the more generalist ciliate, which was able to consume all three bacterial strains, allowed the coexistence of all species. IGP could be the reason

behind this observation where the ciliate would act as IG predator and the BALO and the virus would be the IG prey. In a related study the authors tested the general ability of different protists to grow on two BALO strains (Johnke et al. 2017b). These experiments showed for the first time, that BALOs may indeed serve as prey for protists.

6 Future Perspectives

The studies presented here demonstrate, that advancing our understanding of microbial food webs requires experiments focusing on the combined effects of interacting multiple predators, which differ in their prey range. Integrating multiple predator-prey interactions may better reflect multitrophic food webs, their evolutionary dynamics and their effects on ecosystems than additive pairwise experiments (Friman et al. 2016; Griffin et al. 2008; Philpott et al. 2012). However, microbial IGP studies integrating complex food webs with more complex network structures are rather rare, and have not yet covered all predatory microbial groups.

It has been generally recognized that IGP is not only of importance for basic ecological questions, but also for the application of biological control agents (Müller and Brodeur 2002; Rosenheim et al. 1995). Despite the growing number of studies on the environmental or biotechnological application of protists (Gao et al. 2019), BALOs (Dwidar et al. 2012) and viruses ("phage therapy" (Doss et al. 2017; Thiel 2004)) to either combat potentially pathogenic bacteria or to improve microbiome functions, IGP (including coincidental IGP) has so far been neglected as a factor potentially disrupting biological control or the promotion of plant beneficial functions.

Experimental systems with assembled microbial communities are excellent tools to address ecological and evolutionary questions related to multiple enemies and IGP under very controlled conditions and with high replication, and could thus complement field observations (Altermatt et al. 2015; De Roy et al. 2014; Saleem et al. 2012). Findings obtained from assembled laboratory systems cannot easily be extrapolated to natural ecosystems containing many more species, with potentially so far undescribed interactions. However, results from the micro-scale can provide hypotheses which can subsequently be further tested with other organism groups or under more natural conditions. There is in particular a need to extend the spatial scale of "closed" experimental systems, which lack several parameters that are often governing interactions in the field and colonization of patchy habitats. Protist microcosms have been shown to be able to link theory with patterns in metacommunities (Carrara et al. 2012; Livingston et al. 2017) and could be easily modified to include several IGP configurations. Assembling predator-prey systems in microfabricated landscapes, combined with e.g. live imaging provides exciting options to study interactions along gradients of chemical conditions, physical structures and landscape fragmentation at the microscale (Aleklett et al. 2018; Hol et al. 2016; Otten et al. 2012).

Detecting microbial interactions or microbial IGP networks within environmental communities or even in relatively species-poor assemblages remains a difficult task. Microbial ecologists are increasingly using PCR- and sequence based approaches, direct process measurements or single-cell analyses – alone or in combination – to reveal interactions and energy fluxes. For example, sequence association network analysis (or co-occurrence analysis) of multi-trophic microbial community sequencing data might help to detect predator-prey relationships in general and potentially also IGP relationships in real world samples, as long as they are dominant enough to be detected (Faust and Raes 2012; Lentendu et al. 2014). Though sequence based networks are not a definite proof of real biological interactions across trophic levels, they provide correlations (Chow et al. 2014; Needham et al. 2017) which can be further tested for their biological significance.

Flow cytometry allows the quantification of possible IGP partners on a single cell level and subsequent sorting for further single cell genomics (Lentendu et al. 2013; Petro et al. 2019) potentially enabling also evolutionary approaches. It is also feasible to isolate interacting predator-prey members such as virus-host pairs with viral tagging (Deng et al. 2012; Willner and Hugenholtz 2013) or protists that had taken up prey bacteria (Fu et al. 2003). Fluorescence in-situ hybridization (FISH) has already been successfully used to make IGP relationships visible and to identify involved microorganisms (Piwosz and Pernthaler 2011). There exist probes for different bacterial (Bouvier and del Giorgio 2003) and protist groups (Mangot et al. 2018; Piwosz and Pernthaler 2011; Zhan et al. 2018), for *Bdellovibrio* (Mahmoud et al. 2007) and also for viruses (Allers et al. 2013). Moreover, it is possible to detect and quantify which prey is engulfed in the vacuoles of protists as was shown in the work of Piwosz and Pernthaler (2011).

Different approaches are available to investigate trophic interaction with more detail via tracking energy and carbon fluxes between partners. Time-resolved DNA or RNA stable isotope probing (SIP) can be used to follow the carbon flow over different trophic levels, connectivities in food webs or micro-predator niche differentiation when labelled carbon resources or prey organisms are used (Chatzinotas et al. 2013; Kuppardt et al. 2010; Zhang and Lueders 2017). The combination with metaproteomics (i.e. protein-SIP) would provide the most direct link to functions by identifying proteins involved in metabolic process (Jehmlich et al. 2016). Finally, micro-scale spatial resolution of the contribution of individual cells to particular processes involving trophic interactions can be achieved by combining of FISH, SIP and nanoSIMS (nanoscale secondary ion mass spectrometry) (Musat et al. 2016).

To conclude, we envisage, that combining these so far mostly neglected methods in multi-scale experimental systems with microbial predators of differing prey range will reveal novel ecological and evolutionary implications of microbial IGP.

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Antibacterial Activities of *Bdellovibrio* and like Organisms in Aquaculture

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Contents

1 Introduction

Aquaculture is the cultivation of aquatic living organisms, especially fish, shellfish, crustaceans, molluscs and seaweed in natural or controlled freshwater or marine environments. With the development of economy and the improvement of living standards of growing population, demand for aquatic products in the world is rapidly rising. In the past few decades, aquaculture has increasingly contributed to the food production, supplying raw materials for industrial and pharmaceutical uses, as well as for ornamental fish trade. While continuing to rely on traditional fishing,

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aquaculture industry has been vigorously developed to make up for the lack of supply in the consumer market. Hence, it has quickly become one of the fastest growing and most auspicious industries for providing animal super molecules and food security to the planet population (Le 2010; De et al. 2014). Taking China as an example, its national aquatic product output was increased from 59,076,800 metric tons in 2012 to 690,012,500 metric tons in 2016, an increment of 16.82% (FSF 2018). It is expected that the growth of aquaculture industry will continue at an even faster pace in the coming future.

However, production of fish, shellfish and seafood is often disrupted by environmental pollution, resource allocation and unpredictable mortalities that are the results of negative interactions between aquatic organisms and pathogens (Cabello 2006). Disease outbreaks in aquaculture are more and more common, becoming a severe problem which affects both the economic development and the socio-economic status of the people involved in many countries. In fact, there are actually hundreds of diseases that can affect farmed organisms. A majority of them are caused by bacteria like *Aeromonas (Ae.) hydrophila* (Irianto and Austin 2002), *Bacillus* (*Ba.*) *cereus* (Liu et al. 2016), *Edwardsiella* (*Ed.*) *tarda* (Irianto and Austin 2002), *Flexibacter columnaris* (Wakabayashi 1991), *Pseudomonas* (*Ps.*) *fluorescens* (Wang 2010; Austin and Austin 2016; Zhang et al. (2009b), *Ps. aeruginosa* (Cai et al. 2009), various species of *Vibrio* (*V.*) (Cheng et al. 2008; Al-Sunaiher et al. 2010), to name just a few.

In freshwater aquaculture, *Aeromonas* is considered a major problem (Zmyslowska et al. 2009; Cao et al. 2010). In mariculture, vibriosis, as caused by a number of *Vibrio*, like *V. harveyi, V. parahaemolyticus, V. alginolyticus, V.* (*Listonella, Lis.*) *anguillarum,* and *V. vulnificus*, is a major threat (Chatterjee and Haldar 2012). Early Mortality Syndrome (EMS), also known as Acute Hepatopancreatic Necrosis Disease (AHPND), is a newly emerged disease in penaeid shrimp [*Litopenaeus* (*Lit.*) *vannamei*] aquaculture, which is caused by a unique strain of *V. parahaemolyticus* carrying a plasmid that contains toxin genes homologous to *Photorhabdus* insect-related toxins (Tran et al. 2013; De Schryver et al. 2014). Its mortality rates can reach as high as 100% within a few days after occurrence of the disease (Wang et al. 2018). In addition to bacterial diseases, there are also viral diseases such as White Spot syndrome (as caused by white spot syndrome virus, WSSV) and Taura syndrome (as caused by Taura syndrome virus, TSV) in shrimp (Bondad-Reantaso et al. 2005) and parasitic diseases (such as caused by protozoan ciliates, *Ichthyophthirius* sp., *Trichodina* sp.) (Bondad-Reantaso et al. 2005). Most if not all of them, regardless of bacterial or viral nature, are conditional pathogens that cause infections or disease outbreaks when environmental conditions are deteriorated (and thus their numbers are high) and/or cultured organisms are under stress (De Schryver and Vadstein 2014). Therefore, elimination of pathogens or potential pathogens, or a reduction of their numbers, would help reduce the chances of disease outbreaks.

Currently, three types of strategies are being deployed to control pathogens and to protect farmed organisms from diseases, viz., chemical, physical and biological means.

Chemically: to control pathogens/diseases, aquaculture entities frequently use chemicals or antibiotics to combat infections (Cabello 2006). Various studies have already pointed out the negative impacts, in that the use of chemicals and antibiotics in aquaculture could result "in the emergence of antibiotic-resistant bacteria in aquaculture environments, in the increase of antibiotic resistance in fish pathogens, in the transfer of these resistance determinants to bacteria of land animals and to human pathogens, and in alterations of the bacterial flora both in sediments and in the water column" (Cabello 2006). Growing global concerns about chemical and antibiotic negative effects makes it necessary to seek environmentally friendly alternatives for a sustainable aquaculture production.

Physically: UV and Ozone (Summerfelt 2003) and filtration (Wold et al. 2014) techniques are being used to treat water and to reduce microorganisms in some sections of aquaculture, in shrimp larviculture in particular.

Biologically: probiotics, prebiotics and their combination (synbiotics), bacteriophages and nonviable bacterial products are increasingly being employed to control microbes and to prevent diseases in aquaculture as well as to improve water quality (Pérez-Sánchez et al. 2018).

As a potentially new type of probiotics, the predatory bacteria *Bdellovbrio* and like organisms (BALOs) are increasingly being applied in aquaculture, especially in China. Here in this chapter, we will review relatively high quality documented studies to assess BALOs antibacterial activities related to aquaculture and to evaluate their application potentials in aquaculture.

2 Probiotics in Aquaculture

Probiotics are delineated as live, dead or components of microbial cells which confer health benefits, better growth performances, less stress responses or better general vigour on the host when administered in an adequate amount (Gatesoupe 1999).

The concept of probiotics in aquaculture is relatively new, but their applications have been gaining popularity due to the demand for a sustainable and environmentally friendly aquaculture (Gatesoupe 1999; Newaj-Fyzul et al. 2014).

Up to now, probiotics used in aquaculture included yeasts like *Debaryomyces* sp., *Phaffia* sp. and *Saccharomyces cerevisiae* (Irianto and Austin 2002), various *Bacillus* species (Del'Duca et al. 2013), denitrifying bacteria (Wang et al. 2018), photosynthetic bacteria like *Rhodobacter sphaeroides* (Wang 2011), as well as lactic acid bacteria like *Lactobacillus* (Aguilar-Macias et al. 2010), *Enterococcus faecium* (Swain et al. 2009), and *Carnobacterium* (Kim and Austin 2006). Even some specific strains of the following genera have also been evaluated as probiotics due to their potentially beneficial natures: *Ae. hydrophila* A3–51 (Irianto and Austin 2002), *Ps. fluorescens* (Hai et al. 2009), *Shewanella* (*Sh.*) sp. (García De La Banda et al. 2012; Tapia-Paniagua et al. 2012; Jiang et al. 2013), and even *V. fluvialis* (Alavandi et al. 2004) and *Vibrio* spp. (Thompson et al. 2010).

BALOs had been proposed as a bio-agent around 1990s in China (Qin 1987; Yang and Huang 1997) and are gaining momentums from the start of this century (Yang et al. 2004; Li et al. 2017).

3 *Bdellovibrio* and like Organisms (BALOs)

BALOs are a group of small (0.25 μm wide and up to 2 μm long), rapidly motile, aerobic, Gram-negative and obligate predatory bacteria that are capable of invading/surrounding other bacteria for growth, reproduction, and survival (Jurkevitch and Ramati 2000; Rotem et al. 2014; Stolp and Starr 1963). The first observation of this tiny and rapidly moving microorganism was made by Stolp and Petzold (1962).

Taxonomically, Koval et al. (2015) reclassified the then-existing BALOs of class Delta-proteobacteria into four families, i.e., (I) family *Bdellovibrionaceae* with *Bdellovibrio* (*Bd.*) *bacteriovorus* as type species and *Bd. exovorus* as another identified species, (II) family *Halobacteriovoraceae* with *Halobacteriovorax* (*Hal.*) *marinus* as type species and *Hal. litoralis* as another identified species, (III) family *Bacteriovoracaceae* with *Bacteriovorax* (*Bact.*) *stolpii* as type species, and (IV) family *Peredibacteraceae* with *Peredibacter starrii* as type species. In the same year (2015), McCauley et al. (2015) proposed within the order *Bdellovibrionales* a new family *Pseudobacteriovoracaceae* with a new genus *Pseudobacteriovorax* (*Pseudobacteriovorax antillogorgiicola* RKEM611^T as the type strain). Then in 2017, with more comprehensive and in-depth research, Hahn et al. (2017) reclassified BALOs taxonomy, with the establishment of a new order *Bacteriovoracales* to encompass families *Bacteriovoracaceae* (Davidov and Jurkevitch 2004) (genera *Bacteriovorax* and *Peredibacter*), and *Halobacteriovoraceae* (Koval et al. 2015), with *Bacteriovorax* as the type genus; an emendation of the existing order *Bdellovibrionales* (Garrity et al. 2005) to only include genera *Bdellovibrio*, *Micarvibrio*, and *Vampirivibrio*, as well as other unclassified BALOs, with *Bdellovibrio* as the type genus; a reclassification of the family *Pseudobacteriovoracaceae* in the order *Oligoflexiales*. All these three orders, viz., *Bdellovibrionales*, *Bacteriovoracales* and *Oligoflexiales*, are under the class *Oligoflexia* (Nakai et al. 2014). Thus, BALOs belong no more to the class *Delta-* or *Alpha- proteobacteria*.

Reproductionally, *Bd. bacteriovorus* is the best studied member of all (Sockett and Lambert 2004). Its fast swimming attack-phase cells interact with their preys, attaching to the prey cells, penetrating prey cell wall and stay in their periplasm (which is called periplasmic predation) (Pasternak et al. 2014). This stage is called growth (or periplasmic) stage. There, it grows and multiplies, ending in the lysis of prey cells and the release of bdellovibrio progenies (Abram et al. 1974; Rotem et al. 2014). For more details, please consult the Chapter by Jurkevitch on BALOs in wastewater.

Depending on the environmental conditions and prey hosts, completing a whole life cycle takes roughly 3–4 h (Nunez et al. 2005). Further discussion on

environmental factors and their impacts on predation is available in the chapter by Mitchell. Because of this unique prey-attack characteristic, BALOs have been proposed as living alternatives to chemical and antibacterial agents in environment and public health (Sockett and Lambert 2004; Rotem et al. 2014), or as a bio-agent for use to control pathogens in mariculture (Yang et al. 2004).

3.1 Natural Existence of BALOs in Aquatic/Aquaculture Habitats and the Guts of Cultured Organisms

BALOs are widely distributed in nature (Fry and Staples 1976; Williams et al. 1995; Cai et al. 2008).

To examine BALOs natural existence in freshwater habitat, Shi et al. (1987) collected water (or mud) samples from sea, lakes, rivers and ponds from 258 places in 31 cities and counties across Anhui, Jiangsu, Shandong provinces and Beijing from November 1979 through April 1985. They employed 5 hosts for the detection of BALOs in each sample, viz., *V. cholera* biotype El Tor, *Shigella* (*Shi.*) *flexneri*, *V. parahaemolyticus*, and *Escherichia* (*Es.*) *coli*. Out of totally 325 samples, 254 samples showed the presence of BALOs, amounting to a positive rate of 78.15%. Their densities ranged from 1 plaque forming unit (PFU) per mL (or g of mud) to 5.88×10^3 PFU per mL (or g of mud). Unfortunately, the authors did not correlate the positive rates with months or seasons so as to rule out the temperature effect, as it could impact BALOs presence in nature (Sutton and Besant 1994). Yu et al. (1994) then conducted a survey in Spring (March to April) of 1993 on five major rivers in Chengdu city, China. They used the following host strains for each sample, viz., *Es. coli* 8099, *Ps. aeruginisa* 10123, *Shi. flexneri* F2a.1180, *Salmonella* (*Sa.*) *typhimurium*, *Ba. subtilis* 8017, *Ba. cereus* 4001, *Staphylococcus* (*St.*) *aureus* 6538, and found BALOs presence in all five rivers with an average content of 2.1×10^4 PFU mL^{-1}, ranging from 4.0×10^2 PFU mL^{-1} to 1.0×10^6 PFU mL^{-1}. On the basis of plaque forming characteristics, the authors isolated 5 strains of BALOs and found all 5 strains could lyse *Es. coli* 8099, *Shi. flexneri* F2a.1180, *Sa. typhimurium*, 4 strains could lyse *Ps. aeruginosa* 10123 and Gram-positive *Ba. cereus* 4001, and 3 strains could lyse Gram-positive *St. aureus* 6538. These studies not only demonstrated the natural existence of BALOs in freshwater environments, even at relatively high densities in some habitats, but also revealed their different lytic characteristics.

With respect to marine habitat, Taylor et al. (1974) had recovered 13 strains of *Bdellovibrio* from sea water off the coast of Oahu, Hawaii and the abundance of *Bdellovibrio* was 121–194 PFU per liter of sea water. Williams et al. (1995) recovered *Bdellovibrio* from submerged surfaces and other aquatic habitats of Chesapeake Bay, i.e., water and sediment, oyster shell surface biofilms, zooplankton, and plants. More recently, Li et al. (2011) isolated two strains of BALOs, viz., BDH12 and BDHSH06, from sediment of Daya bay in Shenzhen of China using *Sh.*

putrefaciens strain 12 and *V. parahaemolyticus* strain SH06 as prey, respectively. These two strains may form a new genus within the family *Bacteriovoracaceae* on the basis of partial 16S rDNA sequence analysis.

Apart from naturally existing waters, BALOs are also widely distributed in various man-made waters, like aquaculture environments. For instance, Schoeffield and Williams (1990) recovered *Bdellovibrio* from the water of a brackish tidal pond and also from an aquarium saltwater tank using *V. parahaemolyticus* P-5 as host organism. Yang and Huang (1997) isolated 44 strains of BALOs from marine shrimp farms. Their further studies showed that these 44 different strains had different prey ranges. While most of them could lyse Gram-negative bacteria like *V. cholerae* non-01, *V. harveyii*, *V. parahaemolyticus*, *V. alginolyticus*, *V. fluvialis*, *V.* (*Lis.*) *anguillarum*, *Es. coli*, *Ps. aeruginosa*, some could even lyse Gram-positive bacteria *Ba. subtilis* and *St. aureus*. Chu and Zhu (2010) utilized *Ae. hydrophila* J-1 as prey organism and isolated 14 BALO strains from cultured cyprinoid fish ponds. Among them, strain BdC-1 could lyse 23 Gram-negative bacteria comprising three genera of fish pathogens (i.e., *Ae. hydrophipla*, *V. parahaemolyticus*, *V. alginolyticus*, *V. harveyii* and *Ed. tarta*) and one strain of *Es. coli*, but could not lyse *Ba. subtilis* and *St. aureus*. To further explore BALOs natural existence and diversities, Wen et al. (2009) used two PCR-based methods to type saltwater BALOs in shrimp mariculture systems. The number of culturable BALOs that lysed *V. alginolyticus* was found to be in the range of $10–10^3$ PFU mL^{-1} in the surface water samples using double-layer agar technique. Among 130 BALOs they isolated, five and four phylotypes were revealed by denaturing gradient gel electrophoresis targeting the 16S rDNA V3 region and amplified rDNA restriction analysis of the *Bacteriovoracaceae* specific 16S rDNA fragment, respectively. Their phylogenetic analysis further showed that all of the representative isolates were identified as *Bacteriovorax* spp., but separated into four different clusters in the family *Bacteriovoracaceae*. This finding demonstrated that the relatively large number of saltwater BALOs with diverse phylotypes was naturally present in shrimp mariculture environments and they might well play an important role in shrimp farming ecosystem.

Apart from their existence in various waters, BALOs are also naturally present on aquatic (wild or cultured) organisms or in their guts. Using double-agar-overlay technique with *V. parahaemolyticus* P-5 as host, Kelley and Williams (1992) recovered BALOs from the gills of all 31 samples of blue crab (*Callinectus sapidus*) from different geographical regions in Chesapeake Bay and seasons (4 seasons). Zhang et al. (2009c) recovered *Bdellovibrio* sp. Bdm4 from the gut of Eel (*Anguilla* spp.) using *Ae. hydrophila* as prey. Cao et al. (2007) isolated *Bdellovibrio* sp. BDF-H16 from the gut of gibel carp [*Carassius* (*Ca.*) *auratus gibelio*] using *Ae. sobria* as host. They later also isolated *Bd. bacteriovorus* strain F16 from sturgeon [*Acipenser* (*Ac.*) *baerii*] gut using a sturgeon-pathogenic *Ae. hydrophila* as prey (Cao et al. 2012). More recently, Han et al. (2015) used molecular typing techniques to study BALOs diversities in the intestine of spiny sea cucumber [*Apostichopus* (*Ap.*) *japonicas*] and found *Bdellovibrio* and *Bacteriovorax* were naturally present in the guts. On the basis of phylogenetic features, they suggested

that potentially five new BALOs species could be proposed, but no further identification has yet been done.

Until now, documented findings on the natural existence of BALOs in the guts of various aquatic organisms are relatively few. The reason for this, apart from very few studies performed on the various organisms in aquaculture, could be due to the combination of the following three factors, viz., the methods used for their studies, their relatively rarities in the guts and various environmental factors (see Sect. 3.2). Traditionally, we tend to use the culture dependent method, i.e., double-layer plating, to isolate and study BALOs, rather than more sensitive modern molecular methods. For the double-layer plating method, the number of BALOs in the guts needs to be sufficiently high enough to be grown, even when an appropriate/lysable host is used. Once they are below certain numbers, double-layer plating method might not be able to recover them as other dominant bacteria could well overgrow in the culture. This argument is supported by the finding of Zeng et al. (2017), who followed pacific white shrimp (*Lit. vannamei*) from larval stage (15 days post-hatching) to adult stage (75 days post-hatching) in order to investigate the intestinal microbiota at different culture stages. By high throughput sequencing that targeted the V4 region of 16S rRNA gene, they found that the abundance of *Bdellovibrio* in all shrimp intestine samples was relatively rare, with only 0.002%, while other microbes were much higher, i.e., *Candidatus_Xiphinematobacter* and *Propionigenium*, both 3.4%; *Synechococcus*, 2.7%; *Shewanella*, 1.3%; *Cetobacterium*, 1.1%; *Bacillus*, 0.9%; *Robiginitalea*, 0.7%; *Fusibacter*, 0.5%; *Arcobacter*, 0.5% and *Lactobacillus*, 0.04%.

The following two studies not only further confirm the natural existing of BALOs in shrimp guts, but surprisingly demonstrate a beneficial link between their abundance in guts and shrimp health or growth. The first study was done by Yang et al. (2016) who used Illumina sequencing to investigate the intestinal bacterial community composition of healthy and diseased juvenile shrimp *(Lit. vannamei*). They found that "the relative abundances of *Planococcaceae* and *Bacteriovoracaceae* families significantly decreased, while that of *Vibrionaceae* remarkably increased in diseased juvenile shrimp digestive tract in relation to healthy one". This indicated that higher abundances of BALOs in guts are linked with better shrimp health. The second study was performed by Xiong et al. (2017), who also employed high throughput sequencing to study the underlying ecological processes of gut microbiota among cohabitating retarded (slow grow), overgrown (fast grow) and normal (normal grow) shrimp (*Lit. vannamei*). They discovered that *Bdellovibrionaceae* was present in all shrimp groups, but highest in the overgrown ones. This means that higher abundances of BALOs in guts are linked to higher shrimp growth rates. The findings of these two studies are very similar to what we have already learnt in human as Iebba et al. (2013) revealed a higher prevalence and abundance of *Bd. bacteriovorus* in the human gut of healthy subjects, implying that BALOs do contribute to the health of various hosts, regardless of reared organisms or human.

3.2 *Some Environmental Factors that Affect BALOs Natural Existence*

As to the environmental factors that affect BALOs presence and/or quantities, and in turn affect their recovery rates in the laboratory, previous studies have revealed that BALOs diversity and abundance in aquatic and aquaculture environments depend on the factors such as water temperature, pH, salinity and seasons, types of habitats (like water surface, water column, sediment and body parts of aquatic animals), and many more. Fry and Staples (1976) noted the positive correlation between the quality of river water and the number of bdellovibrios, viz., bdellovibrios were present in all liquid phases of sewage river sediments and polluted river waters but not in some unpolluted river waters. Seasonal influence on the abundance of BALO recovery was noted by Sutton and Besant (1994), in that the abundance of bdellovibrios was correlated with water temperature and status of habitats during particular seasons of the year. They also found the differences in the vertical distribution of bdellovibrios in the water column among three different tropical marine habitats of the Great Barrier Reef in Australia. They revealed that the number of bdellovibrios was more in sub-surface water than bottom waters in summer, but the reverse occurred in winter while in midwater its presence was the least in all seasons of the year. Interestingly, an opposite finding was reported by Williams and Falkler (1984) who found no significant differences between the abundance of bdellovibrios recovered from several depths of the water column at a site in the Miles River. This discrepancy might be due to the presence of water stratification in Great Barrier Reef and not in Miles River.

Some studies revealed that BALOs are surface-associated organisms and their recovery numbers are several 100-fold higher from the surface water microlayer than from subsurface waters (Williams 1987). In fact, it has been suggested that bdellovibrios prefer to associate with surfaces as they could be recovered from the shell of oysters as well as the epibiota on other surfaces in the aquatic environment (Kelley et al. 1997; Williams et al. 1995). More recently, Zhang et al. (2016) determined the diversity of microorganism communities and the relationship between microbial communities and hosts in *Lit. vannamei* aquaculture water and environmental factors at Chenghu Lake, Kunshan City, China. They found that the abundance of the pathogenic bacterial genus *Flavobacterium* and probiotic bacterial genus *Bdellovibrio* correlated positively with pH, total nitrogen and chemical oxygen demand (COD), and negatively with water temperature and ammonia nitrogen (NH_3-N). This means that BALOs would be more in organic rich environments, a result that is consistent with the finding of Fry and Staples (1976).

3.3 Prey Ranges of BALOs for Aquaculture Purposes

Various studies, and our own experience, have demonstrated that different strains of BALOs possess very different lytic capabilities against their bacterial hosts, and thus showing very different ranges of prey spectrum (Table 1). Some have very wide prey ranges, covering many Gram-negative bacteria, and even some Gram-positive bacteria, while others have very narrow ranges, covering only few species or strains. For example, Kongrueng et al. (2017) showed that *Bacteriovorax* sp. isolate NBV3 displayed a widest prey range (13 out of 14 strains tested, ca. 92.86% lysis rate), lysing all 5 (AHPND)-causing strains of *V. parahaemolyticus* (viz., EMS_1S_2, VP12, 7.2 L3, PeP_{16}, 6.1 L3), 2 clinical Vp strains (PSU5666, PSU5668), 2 environmental Vp strains (PSU5147, PSU5150), *Es. coli, V. alginolyticus, V. cholera* and *V. vulnificus*, but could not lyse *St. aureus*. Isolate MBV6 had the narrowest prey spectrum (5 out of 14 strains, ca. 35.71% lysis rate). Meanwhile, isolates BV-A and MBV5 did not have the widest prey spectrums, but they could lyse Gram-positive *St. aureus*. Furthermore, Chu and Zhu (2010) also showed that out of 14 BALOs they isolated in total, an isolate, designated as *Bdellovibrio* BdC-1 (It is more appropriate to use the term BALO here, as molecular identifications were not performed), formed the largest plaque on the double-layer plates. This isolate had a widest prey range and could attack 24 out of 26 prey strains tested (i.e., 92.31% preys tested could be lysed). It lysed all strains of Gram-negative fish pathogens, viz., *Ae. hydrophila* J-1, Y-1, S-1, 1292, TPS30, HAE-1, X-1, NL-1, GML, BJ, AhS-2, AN-1, BX-50, MF-1, SF911212D, A7, LS-4, M13, W-1*; Ed. tarta* M1*; V. alginolyticus* HY-1*; V. harveyi* BK*; V. parahaemolyticus* HY-2, but not Gram-positive bacteria *Ba. subtilis* CGMCC1.884 and *St. aureus* CGMCC1.89 (Chinese General Microbiological Culture Collections, Beijing, China). Huang et al. (2010) also showed that *Bdellovibrio* strain 506 and strain 512 (again, the term BALO would be more appropriate here as molecular identifications were not performed), which were isolated from seawater, could attack 29 (93.55% lysis rate) and 24 (77.42% lysis rate) out of 31 pathogenic vibrios strains tested. At the low end, Cai et al. (2008) isolated 4 strains of BALOs, viz., BDW01, BDW02, BDW03 and BDW04, and found that they lysed only 15 (36.6%), 16 (39.0%), 27 (65.8%), 26 (63.4%) out of 41 vibrio strains tested, correspondingly. Clearly, these data illustrate the strain specificities in the lysis of various preys. Finding whether or not BALOs strain lysis specificities have any associations with their origins or taxonomic classification, requires much more work.

Another interesting point we noted is that if different species of hosts are used for isolation, BALOs thus obtained may display lysis preference towards that type of species. For example, Li et al. (2011) employed *Sh. putrefaciens* strain 12 and *V. parahaemolyticus* strain SH06 for isolation and obtained BDH12 and BDHSH06, respectively. Their lysis experiments showed that though both BALOs shared 68.4% (39 out of total 57 strains) of the strains as their common preys, BDHSH06 demonstrated a higher prey (36 out of 39 strains, 92.3% lysis rate) toward marine vibrios, while BDH12 showed a higher predatory ability (16 out of 18 strains,

Table 1 A list of various BALOs for aquaculture purposes and their prey ranges

BALOs strains	Sources	Prey host	Gram nature	Bacteria that are susceptible to relevant BALOs	References
BD04	Freshwater crab pond sediments	*Ae. hydrophila* B2	Negative	*Ae. hydrophila* B2; *Ed. tarda* B1; *Es. coli* C600	Zhou et al. (2011)
			Positive	*St. aureus*	
BdC-1	Freshwater fish ponds	*Ae. hydrophila* J-1	Negative	*Ae. hydrophila* J-1, Y-1, S-1, 1292, TPS30, HAE-1,X-1, NL-1, GML, BJ, AhS-2, AN-1, BX-50, MF-1, SF911212D, A7, LS-4, M13, W-1*; Ed. tarta* M1*; Es. coli* DH5α; *V. alginolyticus* HY-1*; V. harveyi* BK*; V. parahaemolyticus* HY-2	Chu and Zhu (2010)
Bacteriovorax sp.	Shrimp farm saltwater and sediments	4x AHPND causing strains (PSU5429, PSU5499, PSU5562, PSU5579)	Negative	*Es. coli; V. alginolyticus; V. cholerae; V. parahaemolyticus* (AHPND causing strains: PSU5429, PSU5499, PSU5562, PSU5579, EMS_1S_2, VP12, 7.2L3, PeP_{16}, 6.1L3*;* clinical strains: PSU5666, PSU5668 and environmental strains: PSU5147, PSU5150), *V. vulnificus*	Kongrueng et al. (2017)
			Positive	*St. aureus*	
Bd19-9899, Bd20-9899, Bd25-9899	Freshwater fish ponds and other waters	*Ae. hydrophila* SC9626, *Ae. punctata* 58-20-9, *Ps. fluorescens 56-12-10, V.* (*Lis.*) *anguillarum* E3-11	Negative	*Ae. hydrophila* SC9626, *Ae. punctata* 58-20-9, *Ps. fluorescens* 56-12-10*, Ps. stutzeri* 9899, *V. (Lis.) anguillarum* E3-11	Ma et al. (1999)
Bdh5221	Shrimp pond seawater	*Ps. stutzeri*	Negative	*Ae. hydrophila; Ed. tarda; Es. coli; Ps. fluorescens; V. alginolyticus; V.* (*Lis.*) *anguillarum; V. harveyi; V. parahaemolyticus*	Xie et al. (2007)
			Positive	*Ba. subtilis*; *St. aureus, Sarcina* sp.	

4.2, 5.1, 3N.3	Coastal seawater and sediments	*Es. coli* 21, AB90054	Negative	*Ae. hydrophila* 1.927, Sc-96-24, Ah9802120388; *Ps. fluorescens* ATCC10646; *Ps. putrefaciens* 0397; *V. alginolyticus* 1833; *V. cholerae* B0165; *V. harveyi* V-1-3120, B0150; *V.* (*Lis.*) *anguillarum* Van-DC12R90387; *V. parahemolyticus* 0394	Cheng et al. (2017)
			Positive	*St. aureus* B0125	
Bd-M1	Shrimp pond seawater and sediments	*V. parahaemolyticus* DX-1	Negative	*Ed. tarta* M1, M2, ET-1, ET-13, ET753; *V. alginolyticus* HY-1, Val; *V. harveyi* BK, Ocean-1; *V.* (*Lis.*) *anguillarum* E-3-11, M8-1; *V. parahaemolyticus* DX-1, DX-2, DX-3, DX-4, HY-2, Vp1, Vp2, 89001; *V. vulnificus* Vv-1, A1, A2	Chu et al. (2009)
BDH12, BDHSH06	Coastal seawater	*Sh. putrefaciens* strain 12, *V. parahaemolyticus* SH06	Negative	*Ae. salmonicida* 33; *Enterobacter salazakii* Bh07, Bh08; *Klebsiella oxytoca* 31; *Pantoea agglomerans* 30; *Ps. aeruginosa*; *Serratia ficaria* 15, 20; *Sh. putrefaciens* 12, 24, 27, 28, 34, 17,18, 35, 22, 29, 32; *V. alginolyticus* 1, 2, 3, 4, 10, 11, 13, 16, 19, 23, 1833; *V. cholerae* (non-01/0139) 6, 10-211, 11-114, 11-201, SWBC-A, SWBC-B; *V. fluvialis* Bh02, Bh03, Bh05, Bh11, Sh03, Sh0, Sh12, Sh13; *V. minicus* Bh10, Bh12,BH13, Bh15, Be08; *V. parahaemolyticus* 8, 9, 16, 15, 21, 25, 26, Vp plus, Vp minus, Sh06	Li et al. (2011)

(continued)

Table 1 (continued)

BALOs strains	Sources	Prey host	Gram nature	Bacteria that are susceptible to relevant BALOs	References
BDE-1	Coastal sediment	*Ba. subtilis* GIM1.136	Negative	*Klebsiella oxytoca* 31; *Ps. aeruginosa* 17, 22, 29, 32, 35; *Serratia ficaria* 20; *Sh. putrefaciens* 12, 27, 28, 34; *V. alginolyticus* 1, 4, 5, 10, 11, 16,19; *V. cholerae* (non-01/0139) 3, 14; *V. parahaemolyticus* 8, 9, 25	Li et al. (2018)
			Positive	*Enterococcus agglometans* 30	
BDW01, BDW02, BDW03, BDW04	Coastal sediment	*V. parahaemolyticus* (strain Vp minus)	Negative	*V. alginolyticus* 1, 2, 3, 4, 10, 11, 13, 16, 19; *V.* (*Lis.*) *anguillarum* Mvm; *V. cholerae* (non-01/0139) 6, SWBC-A, SWBC-B, 11-201, 11-114; *V. fluvialis* Bh02, Bh03, Bh05, Bh11, Sh03, Sh07, Sh12, Sh13; *V. hollisae* Be08; *V. minicus* Bh10, Bh12, Bh13, Bh15; *V. parahaemolyticus* 8, 9, 21, 25, 26, Sh06, Vp plus, Vp minus	Cai et al. (2008)
Bd. bacteriovorus Bd9301, Bd9302, Bd9305, Bd9306, Bd9308, Bd9311	Coastal seawater	*V.* (*Lis.*) *anguillarum* 89027	Negative	*Aeromonas* sp. 8903, 8946, 8930; *Es. coli; Plesiomonas* sp. 8910, 8917; *Ps. aeruginosa; V. alginolyticus* 8918, 8938; *V. cholerae*; *V. fluvialis* 8961, 8972, 8932; *V. harveyi* 8971; *V. (Lis.) anguillarum* 8927, 8962, 8988, LP9018, 8974, 8930; *V. metschnikovii*;	Yang and Huang (1997)

				V. parahaemolyticus; *Vibrio* sp. 8942,8943,8959, 8991	
			Positive	*Ba. subtilis*; *St. aureus*	
F16	Guts of sturgeon (*Ac. baerii*)	*Ae. hydrophila* S1 (sturgeon pathogen)	Negative	*Aeromonas* sp. ATCC7966, X1, W1-L, T3, R402L, RK1119, S1, 706C, 40142G, PK-T, XL2-T, LK-T, PL-R, S2-S	Cao et al. (2012)
Bd. bacteriovorus H16	Guts of sturgeon (*Ac. baerii*)	*Ae. hydrophila*	Negative	*Proteus* (*Pr.*) *mirabilis* strain ZL003, ZXS02, BYK64285, BYK64291; *Pr. vulgaris* strain TWN3; *Proteus* sp. strain ZL0057, BYK000419, BYK00098	Cao et al. (2014)
Bd. bacteriovorus H16	Guts of sturgeon (*Ac. baerii*)	*Ae. hydrophila*	Negative	*V. alginolyticus* BYK00019, BYK0834; *V. (Lis.) anguillarum* BYK0638; *V. cholerae* GYL, LD081008B-1; *V. harveyi* BYK00034, ZL0022; *V. parahaemolyticus* ZL0025, ZL0040; *V. vulnificus* BYK000965	Cao et al. (2015)

GIM denotes Guangdong Institute of Microbiology, Guangzhou, China

88.9% lysis rate) towards non-vibrio bacteria. Taking into account a similar finding that the BALOs in the Great Salt Lake preferentially prey upon bacteria isolated from the lake rather than bacterial isolates from ocean (Pineiro et al. 2004), and considering that partial 16S rDNA sequencing analysis showed BDH12 and BDHSH06 shared 99% sequence similarity (Li et al. 2011), we tend to believe that this preference could be the result of host adaptation. Once hosts are changed, they might well show different preferences after certain period of time. This is also supported by our own laboratory observations: when we change a BALOs' host, it initially needs 5–7 days or more for plaques to appear on the double-layer agar plates. After several rounds of subculturing, plaque formation usually takes much less time.

3.4 *Effect of BALOs on Fish or Shrimp Survivals in Challenge Tests*

To further confirm BALOs antibacterial activities and their potential applications in aquaculture, laboratory challenge tests are a step forward. Various laboratory challenge tests done so far have clearly proved that BALOs successfully protect tested fish or shrimp from pathogens attack, and improved their survival rates, with higher BALOs concentrations offering better protection efficiencies (Table 2).

Again, we took the work done by Kongrueng et al. (2017) as an example (Table 2). In the challenge test, it was divided into control and test groups, each with three subgroups. Control groups were subdivided into artificial sea water (ASW) only control, AHPND Vp-only control and *Bacteriovorax* sp. BV-A-only control, while test groups contained three different doses of BV-A groups, viz., 10^2, 10^4 and 10^6 PFU mL^{-1}. To start the test, shrimp AHPND pathogen Vp PSU5429 at a final concentration of 10^7 CFU (colony forming unit) mL^{-1}, was added to the AHPND Vp-only control and the three test groups that had already contained appropriate doses of BV-A. Fifteen minutes later, twenty whiteleg shrimp (*Lit. vannamei*) postlarvae (PL24) were added to each tank. The test was run for 7 days and shrimp mortalities were recorded daily. At the end of the 7-day test, over 90% of shrimp were dead in the AHPND Vp-only control, and 0% mortalities were recorded in ASW-only and BV-A-only controls. In the test groups, shrimp accumulative mortalities of 72.5, 62.5, and 47.5% were recorded in the subtest groups that contained BV-A at the final concentrations of 10^2, 10^4, and 10^6 PFU mL^{-1}, respectively. This result clearly demonstrated the protective effect of *Bacteriovorax* sp. BV-A on postlarval shrimp, with higher BV-A concentrations offering better protection efficiencies.

Most of the challenge tests done so far used the mode of bath challenge, viz., pathogens and BALOs as well as tested fish or shrimp were all added to the test tank waters, more or less simultaneously (Table 2). In this way, it gives BALOs time to act on the pathogens before the latter goes inside the fish/shrimp and causes

Table 2 Effect of BALOs on fish or shrimp survivals in challenge tests

BALOs strains	BALOs Final concentrations (PFU mL^{-1})	Ways of BALOs application	Test duration	Fish or shrimp tested	Species and doses in the challenge test	Fish or shrimp survival rates (%)	References
Bdellovibrio BD2082	0	BD2082 addition to waters and bath challenge simultaneously	6 days	Channel catfish (*Ictalurus punctatus*)	*Ae. hydrophila* S2027 at 10^7 CFU mL^{-1}	0	Zeng et al. (2004b)
	1×10^4					0	
	1×10^5					75	
	1×10^6					100	
	1×10^7					100	
	0	Pathogens dorsal muscle injection first, BD2082 addition to waters later			*Ae. hydrophila* S2027 at 10^7 CFU mL^{-1}	0	
	1×10^4					0	
	1×10^5					0	
	1×10^6					0	
	1×10^7					0	
BdC-1	0	BALOs addition and bath challenge simultaneously	14 days	Gibel carp (*Carassius auratus gibelio*)	*Ae. hydophila* J-1 at 5.0×10^8 CFU mL^{-1}	20	Chu and Zhu (2010)
	5×10^3					65	
	5×10^5					95	
Bd. bacteriovorus Bd-9-25922	0	BALOs addition and bath challenge simultaneously	11 days	Cyprinoid and grass carp (*Ctenopharyngodon idellus*)	*Ae. hydrophlia* at 10^8 CFU mL^{-1}	16.7	Yang et al. (2000)
	1×10^3					66.7	
	1×10^5					100	
Bd. bacteriovorus H16	0	BALOs addition and bath challenge simultaneously	7 days	Shrimp (*Penaeus vannamei*)	*V. cholerae* QH at 5×10^6 CFU mL^{-1}	0	Cao et al. (2015)
	5×10^3					47.7	
	1×10^4					63.3	
Bd. bacteriovorus H16	0	BALOs addition and bath challenge simultaneously	7 days	Shrimp (*Penaeus vannamei*)	*Pr. penneri* isolate NC at 5×10^6 CFU mL^{-1}	0	Cao et al. (2014)
	5×10^3					58.0	
	5×10^4					78.6	

(continued)

Table 2 (continued)

BALOs strains	BALOs Final concentrations (PFU mL^{-1})	Ways of BALOs application	Test duration	Fish or shrimp tested	Species and doses in the challenge test	Fish or shrimp survival rates (%)	References
[a]*Bdellovibrio* sp.	0	BALOs addition and bath challenge simultaneously	20 days	Crucian carp (*Ca. auratus*)	*Ae. hydrophila* at 10^5 CFU mL^{-1}	0	Huang et al. (2009)
	2mL[a]					70	
	4 mL[a]					100	
	8 mL[a]					100	
Bacteriovorax sp. BV-A	Control groups:	BV-A addition and bath challenge simultaneously	7 days	Postlarval shrimp (*Lit. vannamei*) (PL24)	AHPND Vp PSU5429 at 10^7 CFU mL^{-1}	100	Kongrueng et al. (2017)
	0 (ASW[b] only)					> 10	
	0 (AHPND Vp only)					100	
	1×10^6 (BV-A only)						
	Test groups:						
	1×10^2					27.5	
	1×10^4					37.5	
	1×10^6					52.5	

[a]BALOs concentration was not given
[b]2% artificial sea water

infections/diseases. Few were done by another way of challenge test, viz., muscle injection. Here, Zeng et al. (2004b) had carried out a challenge test by injecting pathogenic *Ae. hydrophila* S2027 into the dorsal muscle of channel catfish (*Ictalurus punctatus*), then instantly added BD2082 to the rearing waters (Table 2). They found that, compared to bathing challenge test that they had done simultaneously, all test fish died with no survival at all in the muscle injection challenge test at the end of the 6-day period. On the basis of this comparison, they concluded that BD2082 did not have curative effects and could be better used for prevention purposes. As pathogenic *Ae. hydrophila* S2027 and BD2082 are initially separated physically and bound to have a time lapse before the latter could predate the former, their conclusion looks not quite convincing scientifically. Nevertheless, it does indicate that BALOs should be at the infection/action sites earlier than the pathogens or potential pathogens, or at least at the same time or not too much later if they want to exert their protective roles.

This line of thinking was further supported by a study performed by Willis et al. (2016), who first injected into the hindbrain of zebrafish (*Danio rerio*) larvae with a lethal dose of *Shi. flexneri* M90T ($> 5 \times 0^3$ CFUs). Then, $1–2 \times 10^5$ PFUs of mCherry-*Bdellovibrio* was injected into the hindbrain ventricle of zebrafish larvae 30–90 min later. *Shigella* enumeration results demonstrated that zebrafish larvae injected with *Bdellovibrio* were able to control *Shigella* replication significantly better than those infected with *Shigella* alone. Moreover, *Bdellovibrio* could rescue zebrafish from lethal *Shigella* infection, increasing survival by ca. 35% at 72 h post injection.

3.5 *Effects of BALOs on Various Bacterial Numbers and Water Qualities*

Although most of the studies performed so far heavily relied on traditional culturing techniques to determine the effects of BALOs on the number of various bacteria, they did show that BALOs applications can indeed control the number of various bacteria, including total heterogenic bacteria counts, total vibrio counts, and/or some specific bacterial counts like *Edwardsiella* sp., at least for a certain period of time (Table 3). For an example, Wen et al. (2010) applied *Bacteriovorax* sp. strain DA5 (as identified with 16S rDNA sequencing by Wen et al. 2014) to the larviculture of white shrimp (*Lit. vannamei*) from nauplius stage ($N_{5–6}$) to mysis stage ($M_{1–2}$), and determined larval survival and metamorphosis rates, heterogenic bacterial and vibrio numbers (Table 4), as well as some water quality parameters (Table 3). At the end of the 9-day rearing test, they found that the high DA5 group significantly improved survival (20.83% vs. 10.42% in control and 9.09% in low DA5 group) and metamorphic rates (25% vs. 10% in control and 9.5% in low DA5 group) of mysis larvae (Table 5). When considering the reduction of bacteria by DA5, it was apparent that the amounts of heterotrophs and vibrios in rearing waters were reduced (a low DA5

Table 3 Effects of BALOs applications on various bacterial numbers and water quality

BALOs strains	BALOs final concentration (PFU mL^{-1})	Test duration/ ways of BALOs application	Reared organisms	Bacterial counts		Water quality parameters	References
				TCBC (%, or log CFU g^{-1}/mL^{-1})	TVC/TAC (%, or log CFU g^{-1}/ mL^{-1})		
Bd. bacteriovorus Bd2082	0	30 days / Bd2082 added to the test tanks filled with water from fish ponds	No fish	TCBC: decreased by 56.4[a]	Not given	Not given	(2004a)
	1.5×10^4			TCBC: decreased by 97.5[a]	Not given	Not given	
Bd. bacteriovorus	0	65 days / BALOs added to the test ponds	Grass carp (*Ctenopharyngodon idellus*)	TCBC: 6.62[b] grew to 6.77[b]	TAC: 6.38[b] grew to 6.58[b]	Compared with control, DO increased, NH_3-N, COD and sulfide contents decreased	Zhang et al. (2009a)
	50			TCBC: 6.63[b] down to 5.54[b]	TAC: 6.36[b] down to 5.43[b]		
	1×10^2			TCBC: 6.61[b] down to 6.49[b]	TAC: 6.41[b] down to 5.40[b]		
	1.5×10^2			TCBC: 6.65[b] down to 5.57[b]	TAC: 6.49[b] down to 5.41[b]		
Bd. bacteriovorus	0	7 days / BALOs added to the test ponds	Snakehead fish (*Ophiocephalus argus*)	Not given	TVC: increased by 0.21 ± 0.13[b]	Compared with control, NH_3-N and NO_2-N contents decreased ($p < 0.05$), DO increased ($p < 0.05$) and pH not changed	Li et al. (2008)
	75			Not given	TVC: decreased by 4.04 ± 0.62[b]		
	0				Not given	Not given	

Bdellovibrio sp. Bdm4		5 days / Bdm4 added to the test ponds	Crucian carp (*Ca. auratus*)	7.8 ± 0.07^{b} grew to 8.38 ± 0.07^{b} (*Edwardsiella* in gut)			Zhang et al. (2009c)
				$6.63 \pm 0{,}03^{b}$ grew to 7.03 ± 0.07^{b} (*Edwardsiella* on gill)			
				5.43 ± 0.08^{b} grew to 5.94 ± 0.16^{b} (*Edwardsiella* on skin)			
	1×10^{4}			7.36 ± 0.11^{b} down to 5.86 ± 0.06^{b} (*Edwardsiella* in gut)	Not given	Not given	
				6.44 ± 0.08^{b} down to 5.44 ± 0.14^{b} (*Edwardsiella* on gill)			

(continued)

Table 3 (continued)

BALOs strains	BALOs final concentration (PFU mL^{-1})	Test duration/ ways of BALOs application	Reared organisms	Bacterial counts		Water quality parameters	References
				TCBC (%, or log CFU g^{-1}/mL^{-1})	TVC/TAC (%, or log CFU g^{-1}/ mL^{-1})		
				5.27 ± 0.07 [b] down to 4.92 ± 0.05 [b] (*Edwardsiella* on skin)			
	0	3 days / Bdm4 in feed	Sea bream (*Sparus aurata*)	Not given	TVC: 0 (control was set as)	Not given	
	1×10^7			Not given	TVC: decreased by 87.7[a]	Not given	
BDH12 and BDHSH06	0	7 days / BDH12 and BDHSH06 added to the test ponds at 1:1 ratio	Oyster (*Ostrea rivularis*)	Not given	TVC: 8.0 grew to 9.0 (in waters).	Not given	Li et al. (2011)
					TVC: 5.82 to 10.0 (in intestine)		
	1×10^5			Not given	TVC: 8.09 ± 0.05 down to 2.39 ± 0.01[b] (in water)	Not given	
					TVpC: 8.02 ± 0.04 down to 2.33 ± 0.01[b] (in water)		
					TVC: 5.72 ± 0.02 down to 2.28 ± 0.01[b] (in intestine)		
					TVpC: 5.69±0.01 down to 2.24±0.04[b] (in intestine)		
BDW03	0	60 days / every 7 days, water was partially exchange with fresh seawater. BDW03 added to the test ponds again	Turbot (*Sc. maximus*)	TCBC: 3.9 ± 0.16[b] (in water)	TVC: 2.6 ± 0.23[b] (in water)	Initial data: pH 8.1 ± 0.097, NH_4-N 0.061 ±0.006 mg L^{-1}, NO_2-N 0.04 ± 0.008 mg L^{-1}, NO_3-N 2.03 ± 0.280 mg L^{-1}, DO 7.70 ± 0.280 mg L^{-1}	Guo et al. (2016)
				TCBC: 4.1 ± 0.09[c] (in intestine)	TVC: 3.2 ± 0.17[c] (in intestine)		
	1×10^5				TVC: 1.8 ± 0.27[b] (in water)		

				TCBC: 2.5 ± 0.13[b] (in water)		End data: pH 8.1 ± 0.120, NH_4-N0.058 ±0.002mg L^{-1}, NO_2-N 0.037±0.007 mg L^{-1}, NO_3-N 1.99 ± 0.530 mg L^{-1}, DO 7.65 ± 0.310 mg L^{-1}	
				TCBC: 3.0 ± 0.15[c] (in intestine)	TVC: 1.9 ± 0.10[c] (in intestine)		
BDH12	0	90 days / every 7 days, water was partially exchanged with fresh seawater. BDH12 added to the test ponds again	Abalone *(Ha. discus hannai)*	TCBC: 3.52 ± 0.03 grew to 6.14 ± 0.16[b] (in water)	TVC: 1.64 ± 0.14 grew to 3.22 ± 0.24[b] (in water)	Initial data: pH 8.2 ± 0.07, NH_4-N 0.02 ± 0.076 mg L^{-1}, NO_2-N 0.04 ± 0.002 mg L^{-1}, NO_3-N 2.16 ± 0.307 mg L^{-1}, DO 7.6 ± 0.31 mg L^{-1}	Guo et al. (2017)
				TCBC: 4.75 ± 0.03 grew to 7.09 ± 0.14[c] (in gut)	TVC: 3.84 ± 0.07 grew to 5.29 ± 0.12[c] (in gut)		
	1×10^5			TCBC: 3.50 ± 0.08 down to 2.07 ± 0.19[b] (in water)	TVC: 1.62 ± 0.13 down to 0.83 ± 0.09[b] (in water)	End data: pH 8.2 ± 0.12, NH_4-N 0.02 ± 0.94mg L^{-1}, NO_2-N 0.04 ± 0.001 mg L^{-1}, NO_3-N 2.15 ± 0.142 mg L^{-1}, DO 7.6 ± 0.31 mg L^{-1}	
				TCBC: 4.75 ±0.04 down to 2.98 ± 0.13[c] (in gut)	TVC: 3.82 ± 0.02 down to 1.75 ± 0.18[c] (in gut)		

(continued)

Table 3 (continued)

BALOs strains	BALOs final concentration (PFU mL^{-1})	Test duration/ ways of BALOs application	Reared organisms	Bacterial counts		Water quality parameters	References
				TCBC (%, or log CFU g^{-1}/mL^{-1})	TVC/TAC (%, or log CFU g^{-1}/ mL^{-1})		
BDH12	0	63 days / every 9 days entire pond of water was replaced with fresh seawater. BDH12 added to the test ponds again	Abalone (*Ha. diversicolor aquatilis*)	TCBC: 3.11^{c} grew to 7.22^{c} (in intestine)	TVC: 1.36^{c} grew to 5.42^{c} (in intestine)	Not given	Li and Cai (2014)
				TCBC: 3.05^{b} grew to 4.28^{b} (in water)	TVC: 1.25^{b} grew to 2.55^{b} (in water)		
	3.3×10^{5}			TCBC: 3.10^{c} grew to 5.96^{c} (in intestine)	TVC: 1.45^{c} grew to 3.39^{c} (in intestine)	Not given	
				TCBC: 3.16^{b} grew to 3.57^{b} (in water)	TVC: 1.16^{b} grew to 1.9^{b} (in water)		
DA5	0	9 days /DA5 added to the larval shrimp tanks in test groups	Larval shrimp (*Lit. vannamei*) (nauplius to mysis)	See Table 4 for the details	See Table 4 for the details	No significant difference on pH. COD and NH_3-N increased by 4.52 ± 0.22 / 0.65 ± 0.02 (Control), 4.54 ± 0.14 / 0.65 ± 0.03 (Low DA5), 5.02 ± 0.06 /0.77 ± 0.04 (High DA5), respectively.	Wen et al. (2010)
	1.15×10^{3}			See Table 4 for the details	See Table 4 for the details		
	1.15×10^{4}			See Table 4 for the details	See Table 4 for the details		

BDHSH06	0	85 days (every 7 days, water was partially exchanged with fresh seawater. BDHSH06 added to the test tanks)	Black tiger shrimp *(Penaeus monodon)*	TCBC:7.43 ± 0.12[b] (in water, BPERW/4HA)	TVC: 5.32 ± 0.07[b] (in water, BPERW/4HA)	Not given	Li et al. (2014)
				TCBC: 10.52 ± 0.25[c] (in intestine, BPERW/4HA)	TVC: 6.51 ± 0.04[b] (in intestine, BPERW 4HA)		
	1×10^5			TCBC: 5.20 ± 0.09[b] (in water, BPERW/4HA)	TVC: 3.55 ± 0.13[b] (in water, BPERW/4HA)	Not given	
				TCBC: 6.04 ± 0.13[c] (in intestine, BPERW/4HA)	TVC: 5.18 ± 0.19[c] (in intestine, BPERW/4HA)		

BPERW/4HA denotes before the partial exchange of rearing water/4 h after BDHSH06 addition; TCBC denotes total cultivable bacterial counts; TVC denotes total vibrio counts, TVpC denotes total *V. parahaemolyticus* counts, TAC denotes total aeromonad counts: [a]%.; [b]log CFU mL^{-1}, [c]log CFU g^{-1}

Table 4 Effect of *Bacteriovorax* sp. DA5 on the heterogenic bacteria and vibrio numbers in rearing waters of white shrimp (*Lit. vannamei*) (adapted and modified from Wen et al. 2010)

	Heterotrophic bacteria ($\times 10^5$ CFU mL^{-1})			Vibrios ($\times 10^3$ CFU mL^{-1})		
Test days∗ (Larval stage)	Control	Low DA5	High DA5	Control	Low DA5	High DA5
0 (N_5-N_6)	6.67 ± 1.74^a	4.90 ± 1.41^a	6.48 ± 1.31^a	13.60 ± 0.57^a	11.38 ± 3.64^a	14.47 ± 1.08^a
0.5 (N_6-Z_1)	9.41 ± 1.90^a	9.00 ± 0.38^a	6.38 ± 0.26^a	18.23 ± 1.38^a	20.13 ± 5.69^a	13.38 ± 0.25^a
1 (N_6-Z_1)	174.33 ± 1.41^a	174.00 ± 8.49^a	144.50 ± 6.84^b	94.25 ± 10.96^a	109.50 ± 2.83^a	92.75 ± 9.55^a
2 (Z_1-Z_2)	22.33 ± 0.94^a	16.00 ± 0.47^a	11.67 ± 3.77^a	15.50 ± 2.83^a	16.00 ± 1.41^a	13.25 ± 1.77^a
3 (Z_1-Z_2-Z_3)	11.55 ± 2.57^a	9.60 ± 1.23^a	2.92 ± 0.87^b	5.35 ± 0.14^a	4.88 ± 0.25^a	3.35 ± 0.57^b
5 (Z_2-Z_3)	3.28 ± 0.49^b	4.58 ± 0.97^b	7.92 ± 1.06^a	0.83 ± 0.09^a	0.85 ± 0.21^a	2.02 ± 0.64^a
7 (Z_3-M_1)	32.00 ± 15.56^a	25.25 ± 1.06^a	15.50 ± 7.07^a	6.80∗∗	8.15∗∗	3.05 ± 1.27
Total increment (%)	864.71	651.71	336.56			
Total reduction (%)				52.01	45.74	72.22–88.55

Different superscript letters (^{a}, ^{b}) in the same line of data showed significant difference ($P < 0.05$) (Wen et al. 2010); ∗Test Day 0 meant samplings were done 30 min before adding DA5; ∗∗represented only one in two replicate samples could be counted effectively; No data were available on Test day 9 because of inappropriate dilutions on 2216E and TCBS plates

Table 5 BALOs applications in aquaculture practices and their effects on growth and survival of reared organisms

BALOs strains	BALOs final concentrations (PFU mL^{-1})	Test duration (BALOs added to the ponds directly)	Reared organisms	Survival rates (%)	Length gain (%)[a]	Weight gain (%)[b]	References
BDH12	0	90 days (every 7 days, water was partially exchanged with fresh seawater. BDH12 added to the test ponds)	Abalone juvenile (*Ha. discus hannai*)	41.8 ± 3.36	216 ± 17	4168 ± 47	Guo et al. (2017)
	1×10^5			63.3 ± 1.87	272 ± 15	6834 ± 39	
BDW03	0	60 days (every 7 days, water was partially exchange with fresh seawater. BDW03 added to the test ponds)	Turbot (*Sc. maximus*)	81 ± 3.2	56.7 ± 2.1	248.2 ± 5.3	Guo et al. (2016)
	1×10^5			92 ± 2.8	78.6 ± 1.5	387.1 ± 4.6	
BDHSH06	0	85 days (every 7 days, water was partially exchanged with fresh seawater. BDHSH06 added to the test tanks)	Black tiger shrimp (*Penaeus monodon*)	31.0 ± 2.1	86.0 ± 11.1	4.21 ± 1.56	Li et al. (2014)
	1×10^5			48.1 ± 1.2	99.8 ± 10.0	6.36 ± 1.50	
BDH12	0	63 days (every 9 days, entire pond of water was exchanged with fresh seawater. BDH12 added to the test ponds)	Abalone (*Ha. diversicolor aquatilis*)	27 ± 2.8	13.49 ± 0.1	47.33 ± 4.25	Li and Cai (2014)
	3.3×10^5			57 ± 6.8	15.43 ± 0.1	55.21 ± 4.59	
BDFM05	0	42 days (every 7 days, entire pond of water was exchanged with fresh seawater. BDFM05 added to the test ponds)	Abalone spat (*Ha. discus hannai*)	45.8	0 (average shell length: 4.332 mm)	Not given	Xiao and Cai (2011)
	1×10^3			75.8	31.7 (average shell length: 5.707 mm)	Not given	
	1×10^4			80.9	46.4 (average shell length: 6.343 mm)	Not given	

(continued)

Table 5 (continued)

BALOs strains	BALOs final concentrations (PFU mL^{-1})	Test duration (BALOs added to the ponds directly)	Reared organisms	Survival rates (%)	Length gain (%)[a]	Weight gain (%)[b]	References
DA5	0	9 days (DA5 added to the test groups)	Larval shrimp (*Lit. vannamei*) (from nauplius to mysis stage)	10.42 (metamorphosis rate: ca. 10%)	Not given	Not given	Wen et al. (2010)
	1.15×10^3			9.09 (metamorphosis rate: ca. 9.5%)	Not given	Not given	
	1.15×10^4			20.83 (metamorphosis rate: ca. 25%)	Not given	Not given	

[a]A percentage of the length gain (%) was performed by the shell length difference between the test group and control divided by the shell length of control. Set the shell length gain (%) in control as zero

[b]A percentage of the weight gain (%) was performed by the body weight difference between the test group and control divided by body weight of control. Set the weight gain (%) in control as zero

concentration of 1.15×10^3 PFU mL^{-1}) or significantly ($p < 0.05$) reduced (a high DA5 concentration of 1.15×10^5 PFU mL^{-1}) in the first 3 days of the test (Table 4); that is, the heterogenic bacterial numbers, based on 2216E agar plate counts, increased from $6.67 \pm 1.74 \times 10^5$ CFU mL^{-1} and $4.90 \pm 1.41 \times 10^5$ CFU mL^{-1} on Day 0 to $11.55 \pm 2.57 \times 10^5$ CFU mL^{-1} and $9.60 \pm 1.23 \times 10^5$ CFU mL^{-1} on Day 3 in the control and low DA5 groups, respectively, while their number was reduced from $6.48 \pm 1.31 \times 10^5$ CFU mL^{-1} to $2.92 \pm 0.87 \times 10^5$ CFU mL^{-1} in high DA5 group during the same period of time (Table 4). Heterogenic bacterial numbers then gradually rose in the high DA5 group, or went further down on day 5 and then rose again on Day 7 in the control and low DA5 groups (no data was available on Day 9 due to an over dilution of that days samples, as the authors explained). Overall, the increments of heterogenic bacteria in the control, low DA5 and high DA5 groups over the 7-day test period were 864.71%, 651.71% and 336.56%, respectively (Table 4). These data clearly indicated that DA5 was effective in the control of heterogenic bacteria numbers in postlarval rearing tanks, with higher efficiencies at relatively higher concentrations.

A similar trend was also noted in the total vibrio counts (Table 4), with reductions over the 7-day period in the control, low DA5 and high DA5 groups at 52.01%, 45.74% and 72.22–88.55%, correspondingly. Once more, these data fully demonstrate the effectiveness of *Bacteriovorax* sp. strain DA5 in the control of vibrios in postlarval rearing tanks.

With respect to water quality, there were no significant differences throughout the test period in pH, COD, and ammonia-N (NH_3-N) contents in waters among control, low DA5 and high DA5 groups, with the exception that the NH_3-N content in high DA5 group at mysis I-II stage (M_{1-2}, near the end of the test) increased significantly (Table 3). This difference could be due to the higher amount of feed given to high DA5 group as it had more postlarvae, rather than the effects directly exerted by BALOs (Wen et al. 2010).

On further reviewing existing documentation discussing the effects of BALOs on water quality, only two pieces of work showed the improvements after BALOs applications. The first one was done by Li et al. (2008), who showed that after a 7-day application of *Bd. bacteriovorus* at a dose of 0.75 mL per square meter of 1.0×10^8 PFU mL^{-1} stock, the NH_3-N, NO_2-N contents were significantly decreased ($p < 0.05$), and DO values were significantly increased ($p < 0.05$), but pH was not significantly changed ($p > 0.05$) (Table 3). The second one was done by Zhang et al. (2009a), who also demonstrated the increase of DO, and the decrease of NH_3-N and sulfide contents (Table 3). These two studies both pointed to the improvement of water quality by BALOs in aquaculture, although to various extents. On the other hand, Gou et al. (2016, 2017) also examined the effects of BALOs on water quality and showed no significant differences (Table 4).

As PCR-DGGE is a relatively powerful tool to provide information into a microbial community structure qualitatively and quantitatively, Chen et al. (2019) employed it to study the effects of *Bacteriovorax* sp. N1 on the bacterial community structures in aquaculture of both seawater sea cucumber (*Ap. japonicus*) and freshwater red carp. Bacterial community structures from the rearing waters were

analyzed using PCR-DGGE analysis over the 48 h-test period. They showed that in freshwater red carp rearing waters, the dominant vibrio and δ-*Proteobacteria* decreased significantly after 12 h of *Bacteriovorax* sp. N1 application, but *Ps. fluorescens* and *Thalassobius aestuarii* increased. In seawater *Ap. japonicus* rearing waters, the dominant δ-*proteobacteria* bacterium became a non-dominant one at 12 h while *Albirhodobacter* became the new dominant bacterium. Based on these results, the authors concluded that *Bacteriovorax* sp. N1 could not only lyse vibrios, δ-*proteobacteria* and many other Gram-negative bacteria, but also increase the number of some other bacteria in both seawater and freshwater aquaculture environments. Nevertheless, they also noted that *Bacteriovorax* sp. N1 concentrations decreased to its lowest level within 24 h and, therefore, it should be replenished per 24 h if it were used to control vibrios continuously.

The decrease of *Bacteriovorax* sp. N1 concentrations with time could well explain a phenomenon we noted in the study by Wen et al. (2010), that bacterial numbers, both heterotrophs and vibrios, went down first in the midst of the test period, and then rose up near the end of the test. The rise of both heterotrophs and vibrio numbers may well mean the decrease of DA5 numbers in the rearing waters. Unfortunately, the authors did not enumerate BALOs/DA5 numbers during the test period. This makes this association remain theoretical.

3.6 BALOs Applications in Aquaculture Practices

Various BALOs application studies have been performed in shrimp, turbot and abalone aquaculture practices with a view to control the overgrowth of various bacteria (including pathogens or potential pathogens) (Tables 3 and 4) and to enhance the growth and survival of reared organisms (Table 5).

In larviculture, Wen et al. (2010) applied *Bacteriovorax* sp. strain DA5 to white shrimp (*Lit. vannamei*), from nauplius stage ($N_{5–6}$) to mysis stage ($M_{1–2}$). They found that at the end of the 9-day test, shrimp survival and metamorphic rates were much higher in high DA5 group (20.83% and 25%, respectively) than those in control and low DA5 group (10.42%, 9.09% and 10%, 9.5%, correspondingly) (Table 5). A similar finding was also demonstrated by Xiao and Cai (2011) in abalone larviculture. They revealed that in comparison to controls with a 45.8% survival rate, BALOs BDFM05 application led to higher rates of survival (65.50% and 76.64% higher) in low and high BDFM05 groups, respectively (Table 5). Their shell length gain was 31.74% and 46.42% higher as compared to control (Table 5).

In grown out aquaculture, Li et al. (2014), Li and Cai (2014), and Guo et al. (2016, 2017) all demonstrated that BALOs applications brought about higher growth and survival rates of reared organisms as compared to controls (Table 5). That is, Li et al. (2014) performed an 85-day rearing test on black tiger shrimp (*Penaeus monodon*) and showed that the survival rate, body length and weight gains of black tiger shrimp were 70.59%, 46.60% and 196.60% higher respectively, in BDHSH06 group compared to control. On abalone tests, Gou et al. (2017)

performed a 90-day rearing test on abalone (*Ha. discus hannai*) and showed that the survival rate, body length and weight gains of abalone were 69.54%, 44.22% and 66.78% higher respectively, in BDH12 group as compared to control, while Li and Cai (2014) ran a 63-day rearing test on abalone (*Ha. diversicolor aquatilis*) and showed that the survival rate, body length and weight gains of abalone were 163.64%, 15.98% and 38.81% higher in BDH12 group compared to control, correspondingly. Regarding fish tests, Gou et al. (2016) performed a 60-day test on turbot (*Sc. maximus*) and showed that the survival rate, body length and weight gains of abalone were 21.85%, 46.70% and 61.26% higher in BDW03 group as compared to control, respectively.

To explore possible links among bacterial numbers with survival and growth rates of those reared organisms, we have performed statistical analyses (Tables 6 and 7). Statistical analyses were carried out using IBM SPSS Statistics (V23, New York, USA). Correlations among various parameters, including various bacterial numbers, survival rates, shell (body) length and body weight gains, as well as added BALOs concentrations, were assessed using Pearson's correlation coefficient, r. In terms of the strength of relationships, the value of the correlation coefficient varies between +1 and − 1. The meanings are as follows:

(i) A correlation coefficient of 1 means that for every positive increase in one variable, there is a positive increase of a fixed proportion in the other.
(ii) A correlation coefficient of −1 means that for every positive increase in one variable, there is a negative decrease of a fixed proportion in the other.
(iii) Zero means that for every increase, there isn't a positive or negative increase. The two just aren't related.

We first analyzed those relevant end-of-a-test data (viz., data at the end point of a test, instead of a series of data covering the beginning and the end as done in some original references) as shown in Table 5 and gave out the statistical results in Table 6.

Although analyses on the end-point data may not be as robust as we would like due to the limitation of available published data in the references, they at least show the trends of developments.

Pearson analysis on TCBC (total culturable bacterial counts), TVC (total vibrio counts), survival/metamorphosis rates, body length and weight gains revealed that in shrimp larviculture (Wen et al. 2010), TCBC had no significant correlations with the rates of larval survival ($r = -0.901$) or metamorphosis ($r = -0.927$). While TVC had a significant negative correlation with survival rates ($r = -0.997$), it had no significant negative link with metamorphosis rates ($r = -0.991$). Unfortunately, we were not able to perform such analyses on the study done by Xiao and Cai (2011) as they did not present data on TCBC and/or TVC. In the grown out aquaculture (Li et al. 2014; Li and Cai 2014; Guo et al. 2016, 2017), it is quite clear that the end-point data of the tests, viz., TCBC and TCVC, both in waters and intestines, all have very strong negative impacts ($r = -1.000$) on the survivals, length gains and weight gains of the reared organisms (Table 6).

Table 6 Pearson's correlations between relevant bacterial numbers and survival or length (gain) or weight (gain) of reared organisms[a]

BALOs strains	Sampling sites	Treatment	TCBC log CFU g^{-1}or mL^{-1}	TVC log CFU g^{-1} or mL^{-1}	S%	L%	W%	M%	Correlations								References
									TCBC × S	TCBC × L	TCBC × W	TVC × S	TVC × L	TVC × W	TCBC × M	TVC × M	
BDW03	Intestine	Control	4.1	3.2	81	56.7	248.2		**(r = −1.0)	**(r = −1.0)	**(r = −1.0)	**(r = −1.0)	**(r = −1.0)	**(r = −1.0)			Guo et al. (2016)
		Test	3.0	1.9	92	78.6	387.1										
	Water	Control	3.9	2.6	81	56.7	248.2		**(r = −1.0)	**(r = −1.0)	**(r = −1.0)	**(r = −1.0)	**(r = −1.0)	**(r = −1.0)			
		Test	2.5	1.8	92	78.6	387.1										
BDH12	Gut	Control	7.09	5.29	41.8	216	4168		**(r = −1.0)	**(r = −1.0)	**(r = −1.0)	**(r = −1.0)	**(r = −1.0)	**(r = −1.0)			Guo et al. (2017)
		Test	2.98	1.75	63.3	272	6834										
	Water	Control	6.14	3.22	41.8	216	4168		**(r = −1.0)	**(r = −1.0)	**(r = −1.0)	**(r = −1.0)	**(r = −1.0)	**(r = −1.0)			
		Test	2.07	0.83	63.3	272	6834										
BDH12	Intestine	Control	7.22	5.42	57	13.49	47.33		**(r = −1.0)	**(r = −1.0)	**(r = −1.0)	**(r = −1.0)	**(r = −1.0)	**(r = −1.0)			Li and Cai (2014)
		Test	5.96	3.39	27	15.43	55.21										
	Water	Control	4.28	2.55	57	13.49	47.33		**(r = −1.0)	**(r = −1.0)	**(r = −1.0)	**(r = −1.0)	**(r = −1.0)	**(r = −1.0)			
		Test	3.57	1.9	27	15.43	55.21										
BDHSH06	Intestine	Control	10.52	6.51	31.0	86.0	4.21		**(r = −1.0)	**(r = −1.0)	**(r = −1.0)	**(r = −1.0)	**(r = −1.0)	**(r = −1.0)			Li et al. (2014)
		Test	6.04	5.18	48.1	99.8	6.36										
	Water	Control	7.43	5.32	31.0	86.0	4.21		**(r = −1.0)	**(r = −1.0)	**(r = −1.0)	**(r = −1.0)	**(r = −1.0)	**(r = −1.0)			
		Test	5.20	3.55	48.1	99.8	6.36										
DA5	Water	Control	6.51	3.83	10.42			10	NS (r = −0.901)	/	/	*(r = −0.997)	/	/	NS (r = −0.927)	NS (r = −0.991)	Wen et al. (2010)
		Low DA5	6.4	3.91	9.09			9.5									
		High DA5	6.19	3.48	20.83			20.5									

[a]Due to the limitations of available data in most of the references, most of the Pearson analyses done here were on the data from the end points of the tests, instead of a series of data covering the whole test period. The *TCBC/TVC* units used for intestine samples were log CFU g^{-1}; The *TCBC/TVC* units used for water samples were log CFU mL^{-1}; *S* denotes survival rates, *L* denotes body (or shell) length or length gains, *W* denotes body weight or weight gains, *M* denotes metamorphosis rates; *denotes significant correlation ($p < 0.05$); ** denotes extremely significant correlation ($p < 0.01$); *NS* not significant.

Table 7 Pearson's correlations between BALOs additions and relevant bacterial numbers, survival or (shell) length (gain) or body weight (gain) of reared organisms[a]

BALOs strains	BALOs added concentration PFU mL^{-1}	Sampling sites	TCBC log CFU g^{-1} or mL^{-1}	TVC log CFU g^{-1} or mL^{-1}	S%	L%	W%	M%	Correlations						References
									BALOs × TCBC	BALOs × TVC	BALOs × S	BALOs × L	BALOs ×W	BALOs × M	
BDW03	Control: 0	Water	3.9	2.6	81	56.87	248.2		**(r = −1.0)	**(r = −1.0)	**(r = 1.0)	**(r = 1.0)	**(r = 1.0)		Guo et al. (2016)
	Test: 1×10^5		2.5	1.9	92	78.6	387.1								
	Control: 0	Intestine	4.1	3.2	81	56.87	248.2		**(r = −1.0)	**(r = −1.0)	**(r = 1.0)	**(r = 1.0)	**(r = 1.0)		
	Test: 1×10^5		3.0	1.8	92	78.6	387.1								
BDH12	Control: 0	Water	6.14	3.22	41.8	216	4168		**(r = −1.0)	**(r = −1.0)	**(r = 1.0)	**(r = 1.0)	**(r = 1.0)		Guo et al. (2017)
	Test: 1×10^5		2.07	0.83	63.3	272	6834								
	Control: 0	Gut	7.09	5.29	41.8	216	4168		**(r = −1.0)	**(r = −1.0)	**(r = 1.0)	**(r = 1.0)	**(r = 1.0)		
	Test: 1×10^5		2.98	1.75	63.3	272	6834								
BDH12	Control: 0	Water	4.28	2.55	27	13.49	47.33		**(r = −1.0)	**(r = −1.0)	**(r = 1.0)	**(r = 1.0)	**(r = 1.0)		Li and Cai (2014)
	Test: 3.3×10^5		3.57	1.9	57	15.43	55.21								
	Control: 0	Intestine	7.22	5.42	27	13.49	47.33		**(r = −1.0)	**(r = −1.0)	**(r = 1.0)	**(r = 1.0)	**(r = 1.0)		
	Test: 3.3×10^5		5.96	3.39	57	15.43	55.21								
BDHSH06	Control: 0	Water	7.43	5.32	31.0	86.0	4.21		**(r = −1.0)	**(r = −1.0)	**(r = 1.0)	**(r = 1.0)	**(r = 1.0)		Li et al. (2014)
	Test: 1×10^5		5.20	3.55	48.1	99.8	6.36								
	Control: 0	Intestine	10.52	6.51	31.0	86.0	4.21		**(r = −1.0)	**(r = −1.0)	**(r = 1.0)	**(r = 1.0)	**(r = 1.0)		
	Test: 1×10^5		6.04	5.18	48.1	99.8	6.36								
DA5	Control: 0	Water	6.51	3.83	10.42			10	NS (r= −0.968)	NS (r=−0.965)	NS (r = 0.981)			NS (r = 0.991)	Wen et al. (2010)
	Low DA5: 1.15×10^3		6.4	3.91	9.09			9.5							
	High DA5:														
	1.15×10^4		6.19	3.48	20.83			20							
								5							
BDFM05	Control: 0				45.8	0					NS (r = 0.681)	NS (r = 0.801)			Xiao and Cai (2011)
	Test: 1×10^3				75.8	31.7									
	Test: 1×10^4				80.9	46.4									

[a]Due to the limitations of available data in most of the references, most of the Pearson analyses done here were on the data from the end points of the tests, instead of a series of data covering the whole test period. The *TCBC/TVC* units used for intestine samples were log CFU g^{-1}; The *TCBC/TVC* units used for water samples were log CFU mL^{-1}; *S* denotes survival rates, *L* denotes length or length gains, *W* denotes weight or weight gains, *M* denotes metamorphosis rates; ∗denotes significant correlation ($p < 0.05$); ∗∗denotes extremely significant correlation ($p < 0.01$); *NS* not significant.

We then went on to analyze effects of BALOs additions on the test-end-point TCBC and TCVC, both in waters and intestines, and survivals, as well as body (shell) length gains and weight gains of the reared organisms (Table 7).

It is surprising to note that in both shrimp (Wen et al. 2010) and abalone (Xiao and Cai 2011) larviculture, BALOs added concentrations display no significant correlations with TCBC, TVC, survival or metamorphosis rates (Table 7). In abalone and turbot grow-out aquaculture, BALOs added concentrations did have significant negative links with the test-end-point TCBC and TVC ($r = -1.000$), in waters or guts, and positive correlations with survival, body (shell) length gains and weight gains ($r = 1.000$).The finding that showed no statistically significant links between BALOs added concentrations and the test-end-point TCBC, TVC, survival or metamorphosis rates indicate the complexities of larviculture, and more work need to be done before their potential interrelationships could be established.

Strong positive correlations between BALOs added concentrations and growth parameters (survival, body length and weight gains) were supported by the studies of Yang et al. (2016) and Xiong et al. (2017) who revealed a beneficial link between BALOs abundance in guts and shrimp health or growth. This is also supported by Iebba et al. (2013), who revealed a higher prevalence and abundance of *Bdellovibrio bacteriovorus* in the human gut of healthy subjects, implying that BALOs do contribute to the health, and by Shatzkes et al. (2017), who evaluated the effect of predatory bacteria on the gut bacterial microbiota in rats and predicted the changes in bacterial populations due to exposure to *Bd. bacteriovorus* would contribute to health.

4 BALOs Applications in the Infection Treatments in Aquaculture

Much rare work has been done, so far, regarding the use of BALOs to treat infections of reared organisms in aquaculture practice. Only Chen and Cai (2011) had conducted such a study.

Recognizing that hemorrhagic symptoms in the mouths of farmed turbot (*Sc. maximus*) was caused by *V. splendidus* (Angulo et al. 1994), Chen and Cai (2011) collected juvenile turbot (55 ± 2.5 g body weight) with some signs of red mouth symptom. They divided these fish into several groups, including groups of control, low BDM01 (10^3 PFU mL^{-1}), medium BDM01 (10^5 PFU mL^{-1}) and high BDM01 (10^7 PFU mL^{-1}). During the test, appropriate amounts of BDM01 were added every 2–3 days to the rearing waters to bath fish and to maintain BDM01 concentrations. No water flow was allowed during the test period so as to avoid BDM01 being diluted and the possible coming-in of new pathogens. Tests were run for 7 days. In comparison with a 47% survival rate in the control, the three different test groups achieved 98.67%, 99.33%, and 100% survival rates. Red mouth signs became fainter or disappeared in most of the fish in the test groups.

Though the use of BDM01 to treat red mouth symptoms in juvenile turbot proved to be successful, it does not mean it will be feasible in other occasions. There are four reasons to this. Firstly, the red mouth infections were at their very early stages as most fish with very faint reddish lips were selected. Secondly, the rearing temperature was relatively appropriate for the BDM01 to act (21–22 °C). Thirdly, the traditional flow-through water exchange was stopped. This should avoid the coming-in of any potential new pathogens and help maintain BDM01 concentrations. Fourthly, BDM01 was a relatively powerful lytic strain with higher efficiencies (unpublished data). This made it work faster in the elimination of vibrios.

5 Conclusions

Through the above comprehensive review on the relevant high quality documented studies, we can conclude that BALOs are naturally ubiquitous in aquaculture environments and even in the guts of reared organisms. They do show strong antibacterial activities against various Gram-negative bacteria and even some Gram-positives, including pathogens or potential pathogens in aquaculture. It is also quite clear that BALOs definitely have a role to play in aquaculture, in terms of controlling the number of bacteria, be it pathogenic or potentially pathogenic, and promoting growth and survival of the cultured organisms. Whether or not BALOs could improve water qualities, directly or indirectly, requires more rigorous work to be performed before definite answers could be given.

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Secondary Metabolism of Predatory Bacteria

Angela Sester, Juliane Korp, and Markus Nett

Contents

1 Introduction

Secondary metabolites are molecules exclusively produced by certain groups of microbes, plants or marine organisms. Unlike primary metabolites, these compounds are not essential for the growth or survival of an organism. Instead they can confer the producer specific advantages in its natural environment, *e.g.*, as volatile attractants towards the same or other species or as feeding deterrents. In the vast majority of cases, however, the biological function of secondary metabolites is still unclear (Dewick 2002). Some time ago, it was recognized that the production of secondary metabolites is widely distributed in predatory bacteria (Nett and Konig 2007). In particular, those taxonomic groups, which form predatory swarms and possess large genomes, stand out in their potential for the biosynthesis of such compounds (Korp et al. 2016). For example, more than 600 chemically distinct secondary metabolites have been isolated from myxobacteria (Findlay 2016), which have long been

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suspected to utilize antibiotics for the killing and degradation of prey organisms (Rosenberg and Varon 1984). Moreover, bacteria of the genus *Lysobacter*, which practice group predation (Seccareccia et al. 2015), have been identified as prolific producers of peptidic secondary metabolites (Panthee et al. 2016). In this chapter, we will give a brief overview on the type of molecules that can be expected from predatory bacteria and we will also discuss possible reasons for the accumulation of secondary metabolic pathways in the genomes of these organisms. For this purpose, we will take a detailed look on the secondary metabolomes of two model bacteria, *i.e.*, the facultative predator *Myxococcus xanthus* and the obligate predator *Bdellovibrio bacteriovorus*.

2 Classes of Secondary Metabolites

According to their biosynthetic origin, natural products can be categorized into various classes. Most bacterial secondary metabolites fall into one of four biosynthetic classes, namely ribosomally synthesized and post-translationally modified peptides (RiPPs), non-ribosomal peptides (NRPs), polyketides (PKs), or terpenes.

RiPPs are made from proteinogenic amino acids, but due to extensive post-translational modifications these molecules exhibit unusual structural features that are not generally associated with ribosomal peptides. By definition, RiPPs are smaller than 10 kDa to distinguish them from post-translationally modified proteins (Arnison et al. 2013). The preliminary primary structure of RiPPs is encoded by a structural gene and the transcribed mRNA is initially translated into a precursor peptide. In most cases this precursor peptide consists of an N-terminal leader peptide, followed by the core region and occasionally a C-terminal recognition sequence, which is relevant for dissection and cyclization. The processing of the precursor peptide is initiated upon recognition of the leader peptide by biosynthetic enzymes, which carry out various structural modifications in the core region. Such modifications can include the formation of α-β-unsaturated amino acids through dehydration of serine or threonine residues, intramolecular cyclizations to thiazol(in)-es or oxazol(in)es, *S*-adenosylmethionine (SAM)-dependent methylations, as well as head-to-tail (N-to-C) macrocyclization. Finally, proteolysis cleaves off the leader peptide (and the C-terminal recognition sequence) and, hence, releases the matured RiPP for consecutive export (Arnison et al. 2013). An illustrative example for RiPP biosynthesis in a predatory bacterium is provided by cittilin A, which is produced by several *M. xanthus* strains (Krug et al. 2008). For the biosynthesis of this tetrapeptide (Fig. 1), a leader and core peptide (encoded by one gene), a cytochrome P450 monooxygenase (P450) and a methyltransferase are sufficient. The core peptide, with its sequence Tyr-Ile-Tyr-Tyr undergoes two P450 catalyzed phenol coupling reactions to form a C–C as well as a C–O–C bridge between the tyrosine residues. An *O*-methylation of a phenolic hydroxyl moiety completes the post-translational modifications (Revermann 2012). Cittilin

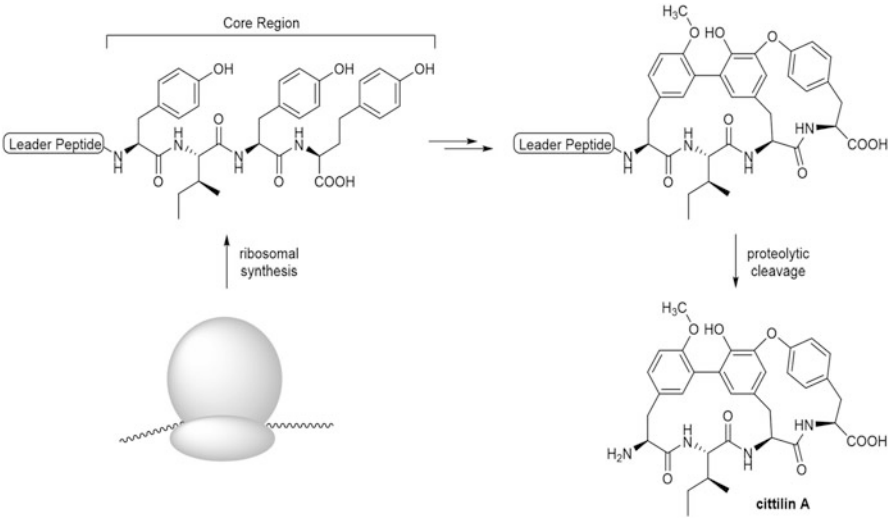

Fig. 1 Biosynthetic route to cittilin A. The two phenol coupling reactions are catalyzed by the P450 enzyme MXAN_0683, while the *O*-methylation in the N-terminal tyrosine moiety is due to the SAM-dependent methyltransferase MXAN_0682

biosynthesis exemplifies how a simple core structure of only four amino acids can be concisely transformed into a distinctive structural scaffold.

NRPs and also several PKs are produced by large enzyme complexes which operate in assembly line fashion (Nett 2014; Weissman 2015). For this, nonribosomal peptide synthetases (NRPSs) and polyketide synthases (PKSs) are organized into modules, each of which is responsible for the incorporation of a defined building block into the final product. The modules are composed of several domains with specific catalytic activities that are relevant for the assembly.

In NRPs a peptide chain forms the structural backbone. It can feature proteinogenic as well as non-proteinogenic amino acids including D-isomers and β-amino acids. The minimum NRPS module consists of three domains. The adenylation (A) domain is responsible for the recognition and ATP-driven activation of the amino acid substrate, which is thereby acyl-adenylated. Next, the A domain transfers the activated substrate to the phosphopantetheinyl side chain of the consecutive peptidyl carrier protein (PCP) domain where it is covalently attached through a thioester bond. This activated ester is targeted by the condensation (C) domain, which links the amino acid monomer with the peptidyl intermediate from the preceding module through an amide bond. Optional domains in NRPS modules can further perform reduction or oxidation steps, intramolecular cyclization (Cy), epimerization (E), methylation (MT) or dehydration and, thereby, increase the structural variety within the peptide products (Sussmuth and Mainz 2017). In the terminal NRPS module, a thioesterase (TE) domain releases the product from the enzyme machinery, either by simple hydrolysis or by intramolecular macrocyclization. A noteworthy NRP, due to its potent antibacterial properties, is

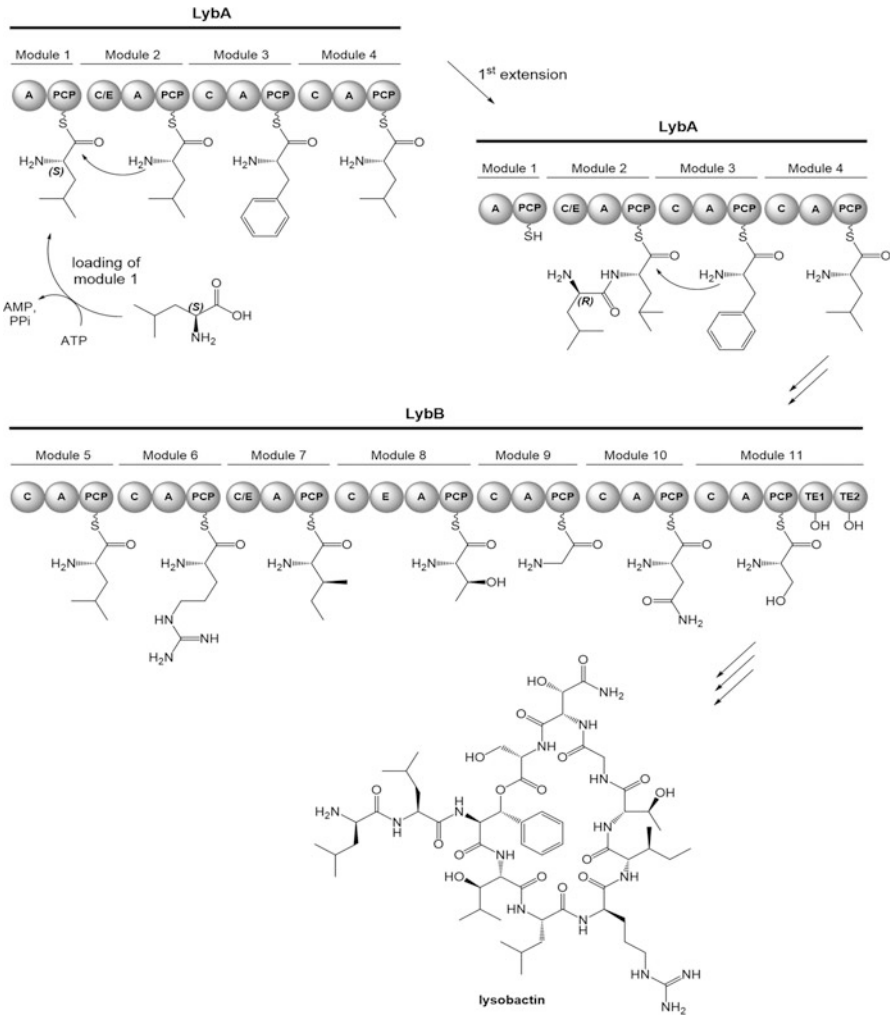

Fig. 2 NRPS assembly line of lysobactin with tethered amino acid monomers. The loading of module 1 and the first extension reaction including the stereochemical inversion of the initially primed leucine moiety are shown in detail

lysobactin which was discovered in *Lysobacter* sp. ATCC 53042 (Bonner et al. 1988; O'Sullivan et al. 1988).

The biosynthesis of this antibiotic (Fig. 2) follows the co-linearity paradigm by which an NRPS with 11 modules codes for 11 amino acids to form an undecapeptide (Hou et al. 2011). In accordance with the module number and the predicted number

of epimerizations, lysobactin is composed of nine L- and two D-amino acids. Interestingly, the epimerizations that give rise to the D-leucine and D-arginine residues in lysobactin are not catalyzed by distinct E domains. Instead, these reactions are mediated by dual function C/E domains (Balibar et al. 2005; Hou et al. 2011). The only dedicated E domain of the lysobactin assembly line, which is present in module 8, was proposed to be responsible for the side-chain epimerization from L-threonine to L-*allo*-threonine. A rather unusual feature in NRPS systems is the presence of a tandem TE domain. In the case of lysobactin, it was demonstrated that only the N-terminal TE domain is needed for macrocyclization and subsequent product release, whereas the C-terminal TE domain has a proofreading function through the deacylation of misprimed PCPs (Hou et al. 2011).

Similar to NRPSs, the majority of bacterial PKSs utilize thiotemplates for natural product biosynthesis, but their substrates are short-chain acyl-CoAs, such as malonyl-CoA or methylmalonyl-CoA (Hertweck 2009). These simple activated acyl units are selected by the acyltransferase domain (AT) of a PKS module and immediately tethered to an adjacent acyl carrier protein (ACP) domain, which previously underwent phosphopantetheinylation. A β-ketoacylsynthase (KS) domain then catalyzes the linkage of the monomer building block with the growing acyl chain via decarboxylative *thio*-Claisen condensation. The resulting β-keto group in the reaction product can be further processed by optional ketoreductase (KR), dehydratase (DH) and enoylreductase (ER) domains. At the end of the assembly-line biosynthesis, a TE domain cleaves off the acyl chain from the enzyme complex either by hydrolysis or by regioselective macrocyclization. The latter offloading mechanism was also observed in the biosynthesis of the gulmirecins, which are produced by the predatory myxobacterium *Pyxidicoccus fallax* (Schieferdecker et al. 2014). Many PKs, including the gulmirecins, are further subjected to post-assembly line modifications, such as acylations and glycosylations. Overall, the assembly of gulmirecins represents almost a textbook example of modular PK biosynthesis, except that the AT domain for the activation of the starter unit is located in the first extension module and not in the loading module (Fig. 3). Another peculiarity is the optional skipping of DH and ER domains, in the termination module, which was also described in the biosynthesis of the structurally related disciformycins (Surup et al. 2014).

In consideration of the similar construction mechanisms that are used by NRPSs and PKSs, it is no surprise that these two enzyme classes can also act in concert and, thereby, form mixed or hybrid assembly lines. All intermediates in NRP and modular PK biosynthesis are covalently bound to carrier proteins via thioester bonds, which guarantees a smooth transfer from a PKS to an NRPS module (or vice versa). Examples for secondary metabolites which derive from NRPS/PKS assembly lines are siphonazole and auriculamide (Fig. 4), both of which were reported from predatory bacteria of the genus *Herpetosiphon* (Nett et al. 2006; Schieferdecker et al. 2015a). Soon after the discovery of siphonazole, feeding studies with isotopically labeled precursors indicated its mixed biosynthetic origin

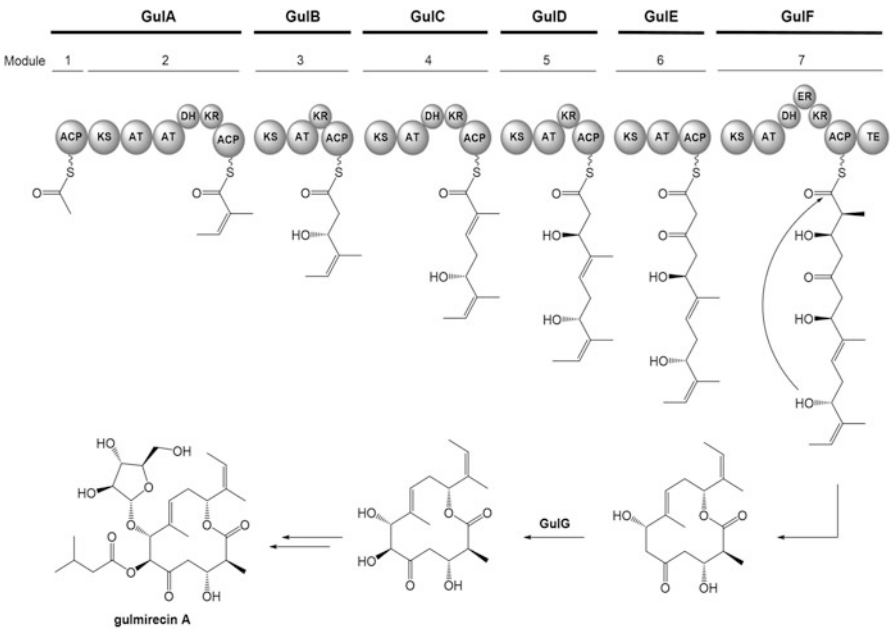

Fig. 3 PKS assembly line and biosynthesis of gulmirecin A. The ACP domains are depicted with the growing acyl chain

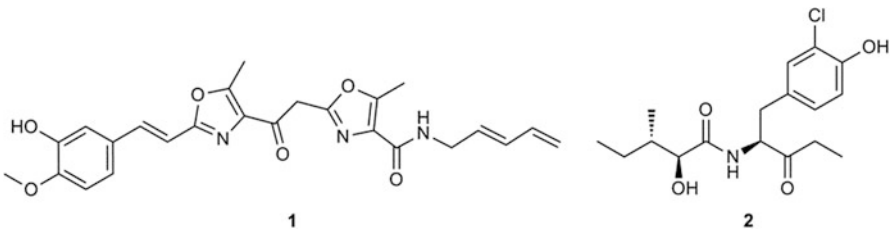

Fig. 4 Structures of siphonazole (**1**) and auriculamide (**2**)

(Nett et al. 2006). More recently, the biosynthetic gene cluster for the production of siphonazole was identified and the annotation of this locus revealed an assembly line consisting of 12 modules with a highly unusual domain architecture (Mohseni et al. 2016). In the case of auriculamide, a retrobiosynthetic analysis was conducted to trace candidate NRPS and PKS genes for its production in the genome of *H. aurantiacus* (Schieferdecker et al. 2015a). Subsequent biochemical analyses supported this assignment (Braga et al. 2016).

Terpenes derive from C_5 isoprene units, namely isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), which can be generated by two distinct pathways. The mevalonate (MEV) pathway is particularly prevalent in fungi and archaea, whereas the methylerythritol phosphate (MEP) pathway is commonly used by bacteria and the chloroplasts of higher plants for the synthesis of IPP and its isomer DMAPP.

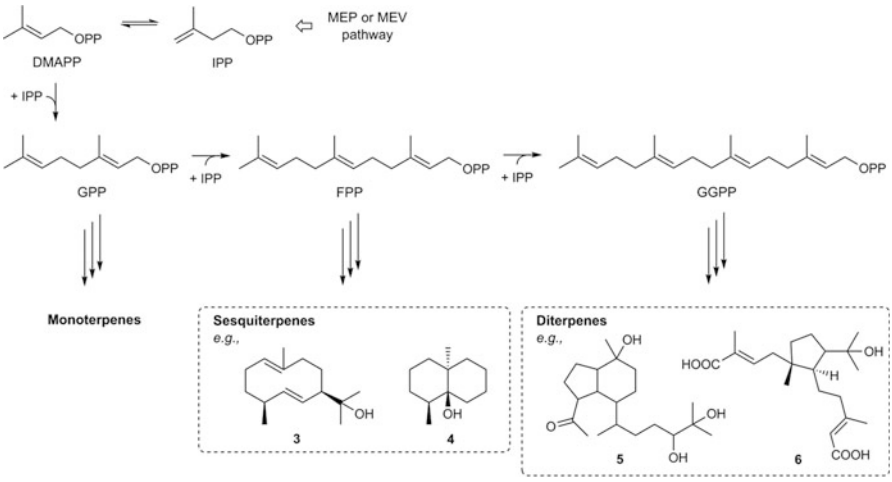

Fig. 5 Selected terpenes from predatory bacteria and their biosynthetic origin: (1(10)*E*,5*E*)-germacradien-11-ol (**3**), (−)-geosmin (**4**), herpetopanone (**5**), and cystodienoic acid (**6**). To date, no monoterpenes have been reported from predatory bacteria

Oligoprenyl diphosphate synthases catalyze the successive head-to-tail condensation of the C_5 monomers to geranyl (GPP), farnesyl (FPP) and geranylgeranyl diphosphate (GGPP). Subsequently, terpene cyclases convert the resulting polyprenyl chains into mono- (C10), sesqui- (C15), and diterpenes (C20). In addition to cyclizations, which can be accompanied by rearrangements and even elimination of carbon atoms, the structural modifications of a terpene precursor can further involve hydroxylations and glycosylations (Kuzuyama 2017). Examples of terpenes from predatory bacteria (Fig. 5) include volatile compounds, such as the sesquiterpene (1(10)*E*,5*E*)-germacradien-11-ol from *M. xanthus* (Dickschat et al. 2005b), as well as non-volatiles like the diterpenes herpetopanone from *H. aurantiacus* (Pan et al. 2017) or cystodienoic acid from *Cystobacter* sp. (Raju et al. 2015).

3 Myxobacteria

3.1 Fundamentals of Myxococcus xanthus *Predation*

Myxococcus xanthus is arguably the best characterized myxobacterium in terms of multicellular development, gliding motility, and predatory behavior (Munoz-Dorado et al. 2016; Nan and Zusman 2011; Velicer and Vos 2009). Taxonomically, this bacterium is ranked in the suborder Cystobacterineae of the Myxococcales (Dawid 2000; Garcia et al. 2010). Members of this division are easily distinguished by their facultative predatory lifestyle, which includes the consumption of living prey organisms as well as the saprophytic absorption of organic material (Casida 1988; Jurkevitch 2007). The predatory strategy of *M. xanthus* is in the literature often

referred to as wolfpack or group predation, in which the prey is killed through the concerted action of numerous predator cells secreting lytic enzymes and other harmful agents (Berleman and Kirby 2009; Martin 2002). Recently, the wolfpack model of cell density-dependent predation was challenged by the observation that *M. xanthus* dramatically changes its gene expression profiles upon contact with dead *E. coli* cells, whereas the presence of living prey results only in negligible transcriptional changes (Livingstone et al. 2018a). The authors of this study concluded that genes involved in the killing of prey must be expressed constitutively, whether or not suitable prey cells are nearby the predator. Once prey cells are affected by myxobacterial enzymes or antibiotics, they are assumed to release signals which can be sensed by the predator and induce the upregulation of genes necessary for the degradation and assimilation of prey-derived material. In this context, the predatory strategy of *M. xanthus* was compared with a lurking spider in its web, rather than a hunting pack of wolves (Livingstone et al. 2018a).

Like all myxobacteria, *M. xanthus* has a complex life cycle consisting of a vegetative part defined by the exponential growth of cells through binary fission, as well as a developmental cycle culminating in the formation of fruiting bodies and dormant myxospores in order to endure unfavorable environmental conditions. To guarantee both, successful predation as well as prolific fruiting body morphogenesis, myxobacterial cells are permanently forced to collaborate and to synchronize their actions. Therefore, myxobacteria developed a sophisticated social behavior and communication system (Dworkin 1996; Kaiser 2004, 2013, Shimkets 1990), which is at least partly based on chemical mediators (Kearns et al. 2001; Meiser et al. 2006; Plaga et al. 1998). For more details on the physiology and ecology of Myxococcales, see Chapter by Furness et al. "Predatory Interactions Between Myxobacteria and Their Prey."

3.2 *The Secondary Metabolome of* Myxococcus xanthus

Similar to other fruiting myxobacteria, *M. xanthus* possesses an exceptionally large genome (>9 Mbp) with a high number of genes encoding enzymes of secondary metabolism (Goldman et al. 2006; Korp et al. 2016). On the other hand, the genome of this bacterium lacks genes for the production of branched-chain amino acids, illustrating the dependency of *M. xanthus* on the consumption of protein-rich food sources (Goldman et al. 2006). Overall, 27 biosynthetic gene clusters (BGCs) were identified in the genome of the model strain DK1622 (Table 1). Roughly two-thirds of these loci are involved in the assembly of PKs, NRPs, or biosynthetic hybrids of these two compound classes. Genes for the production of RiPPs are also quite common and constitute together six loci in the DK1622 chromosome. In comparison, terpene biosynthesis is clearly underrepresented with only two loci, but the metabolic products of the respective pathways are easily recognized. While the terpene geosmin confers a characteristic earthy odor to the bacterium (Dickschat et al. 2004), the reddening of *M. xanthus* cultures upon light exposure is due to the formation of carotenoids (Burchard and Dworkin 1966; Moreno et al. 2001).

Table 1 Biosynthetic gene clusters in the genome of *M. xanthus* DK1622 and their predicted or known products

	Location of BGC on chromosome[a]	NP class	Predicted product[b]	Biological function or activity/Mode of action	References
1	MXAN_0682–0689	RiPP	**Cittilin**	Neurotensin receptor antagonist	Krug et al. (2008), Revermann (2012)
2	MXAN_0894–0904	Terpene	**Carotenoids**	Photoprotection	Moreno et al. (2001)
3	MXAN_1289–1292	NRP	Dipeptide		
4	MXAN_1527–1531	Other	**E signal**	Sporulation, fruiting body formation	Lorenzen et al. (2014), Bhat et al. (2014)
5	MXAN_1588–1608	NRP	Hexapeptide		
6	MXAN_2796–2798	NRP/PK	Unknown		
7	MXAN_2852–2857	RiPP	Type II lantibiotic		
8	MXAN_3459–3463	PK	Unknown		
9	MXAN_3554–3556	RiPP	Bacteriocin		
10	MXAN_3617–3625	NRP/PK	Unknown		
11	MXAN_3626–3638	NRP/PK	Lipopeptide		
12	MXAN_3639–3647	NRP	**Myxochelin**	Siderophore, lipoxygenase inhibitor	Kunze et al. (1989), Schieferdecker et al. (2015b)
13	MXAN_3779	NRP/PK	**Myxoprincomide**	Predation	Cortina et al. (2012), Muller et al. (2016)
14	MXAN_3928–3950	NRP/PK	**Antibiotic TA (myxovirescin)**	Predation, antibiotic/inhibition of type II signal peptidase	Rosenberg et al. (1973), Zafriri et al. (1981), Gerth et al. (1982), Xiao et al. (2011, 2012)
15	MXAN_4000–4003	NRP/PK	Lipopeptide		
16	MXAN_4077–4080	NRP/PK	**Myxochromide**	Unknown	Trowitzsch-Kienast et al. (1993), Wenzel et al. (2006), Burgard et al. (2017)

(continued)

Table 1 (continued)

	Location of BGC on chromosome[a]	NP class	Predicted product[b]	Biological function or activity/Mode of action	References
17	MXAN_4290–4305	NRP/PK	**DKxanthene**	Sporulation, fruiting body formation	Meiser et al. (2006, 2008)
18	MXAN_4402–4407	NRP/PK	Lipopeptide		
19	MXAN_4409–4415	NRP/PK	Lipopeptide		
20	MXAN_4525–4530	NRP/PK	**Myxalamid**	Antifungal/inhibition of NADH: Ubiquinone oxidoreductase	Gerth et al. (1983), Jansen et al. (1983), Bode et al. (2007)
21	MXAN_4589–4601	NRP	Lipopeptide		
22	MXAN_4602–4605	RiPP	Unknown		
23	MXAN_5829	RiPP	Bacteriocin		
24	MXAN_6247	Terpene	**Geosmin**	Unknown	Dickschat et al. (2004, 2005a), Lorenzen et al. (2009)
25	MXAN_6388–6389	RiPP	Type II lantibiotic		
26	MXAN_6392–6405	PK	Unknown		
27	MXAN_6635–6639	PK	**Alkylpyrones**	Inhibition of topoisomerase	Hayashi et al. (2011), Hug et al. (2019)

[a]The assignments were made on the basis of literature data or manual annotations. They differ from previously reported data, which had been obtained using automated genome mining tools (Korp et al. 2016)
[b]Products which were actually observed are highlighted in bold

The secondary metabolites of nine further BGCs could be identified by chemical analyses of the DK1622 strain (Table 1). The molecules that have been retrieved so far possess versatile biological functions, such as iron-acquisition, predation or development. The following passages will highlight the discovery, biosynthesis, and physiological role of selected secondary metabolites from *M. xanthus* DK1622.

Antibiotic TA First reports of antibiotic TA can be traced back to 1973 when the production of this bactericidal agent was observed during the late exponential growth of *M. xanthus* strain TA, which had been isolated from the bark of an olive tree in Tel Aviv (Rosenberg et al. 1973; Vaks et al. 1974). Although the compound was purified and its biological activities were thoroughly characterized at this time,

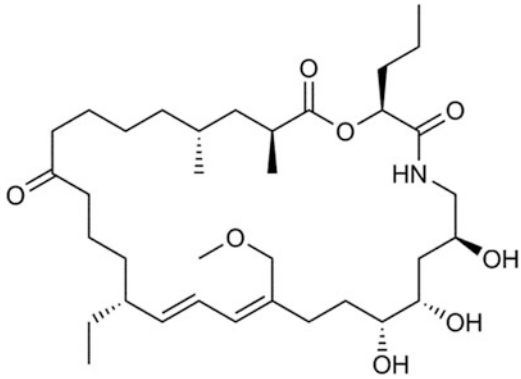

Fig. 6 Structure of myxovirescin A_1

the structure of antibiotic TA remained initially unclear (Rosenberg and Dworkin 1996). Almost 10 years later, several macrolide antibiotics were discovered in *M. virescens* Mx v48 and designated myxovirescins (Gerth et al. 1982). The subsequent comparison of spectroscopic data confirmed that myxovirescin A_1 (Fig. 6), the major metabolite from *M. virescens* Mx v48, and antibiotic TA have the same chemical constitution (Rosenberg et al. 1982; Trowitzsch et al. 1982). Even though the stereochemical identity of the two compounds is not certain according to the *Dictionary of Antibiotics and Related Substances* (Bycroft and Payne 2013), the terms antibiotic TA and myxovirescin A_1 have been used as synonyms by several researchers in the past years (Calderone et al. 2007; Simunovic et al. 2006; Xiao et al. 2011).

Genetic analyses of a *M. xanthus* TA-derived strain yielded first insights into the molecular basis of antibiotic TA biosynthesis (Paitan et al. 1999a, b, c, d, e, 2001, Varon et al. 1992). In 2006, the complete biosynthetic *ta* locus was identified in the chromosome of *M. xanthus* DK1622 (Simunovic et al. 2006). The gene cluster spans about 83 kb of DNA and includes 23 open reading frames (ORFs). Five *ta* genes code for type I PKSs and a hybrid PKS/NRPS enzyme complex, respectively. A closer inspection of the corresponding assembly line (Fig. 7) reveals several deviations from the prototypical domain organization of modular PKSs. First, all but one PKS module are lacking acyltransferase (AT) domains. These "AT-less" PKS modules depend on the discrete *trans*-acting tandem AT domain TaV for substrate selection and loading. Furthermore, the enoyl reductase (ER) domains show an unusual arrangement. They are either placed behind the respective acyl carrier proteins in modules 7 and 12 or outsourced, as proposed for modules 4, 8, 10, and 11 (Simunovic et al. 2006). Lastly, the domains of the two modules 7 and 11 are distributed over two proteins each. In addition to the uncommon assembly line architecture, myxovirescin biosynthesis exhibits further peculiarities, such as the presence of apparently redundant proteins involved in polyketide chain initiation (Simunovic et al. 2006) or two independent routes for substrate-specific β-branching (Calderone et al. 2007; Simunovic and Muller 2007a, b).

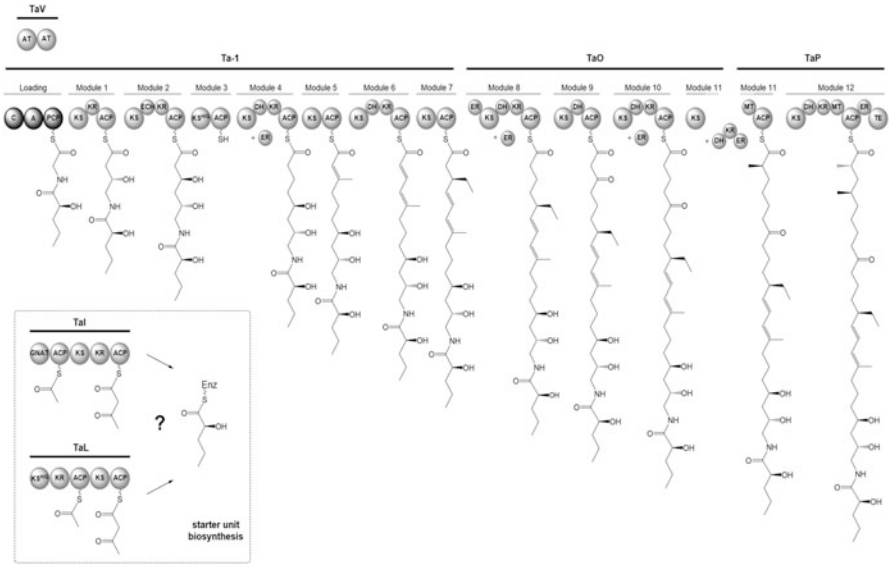

Fig. 7 NRPS/PKS assembly line and postulated biosynthesis of antibiotic TA (Piel 2010). The carrier protein domains are depicted with the growing acyl chain

It is long known that antibiotic TA and the myxovirescins interfere with peptidoglycan synthesis in bacteria (Gerth et al. 1982; Rosenberg et al. 1973). More recently, the molecular target of these antibiotics was identified as LspA, a type II signal peptidase required for maturation of lipoproteins during murein processing (Xiao et al. 2012). While most bacterial genomes harbor a single *lspA* gene (Sutcliffe et al. 2012), *M. xanthus* DK1622 possesses four copies of this gene (*lspA*1 – *lspA*4), of which two are located in the *ta* locus (Xiao et al. 2012). Subsequent studies showed that the *lspA* genes of *M. xanthus* are redundant and that each of these genes individually confers resistance against antibiotic TA, albeit at different degrees. Furthermore, it was proposed that the *lspA* genes in the antibiotic TA locus regulate the production of this secondary metabolite (Xiao and Wall 2014).

The true biological function of antibiotics is a matter of ongoing debate (Davies 2006). In case of predatory bacteria, it is very tempting to assume a link between antibiotic production and feeding strategy. Up to now, however, there is only limited evidence supporting this hypothesis. One of the noteworthy exceptions is a study on antibiotic TA (Xiao et al. 2011). After abolishing the production of antibiotic TA in *M. xanthus* DK1622 through deletion of a biosynthesis gene, the predator was severely affected in its ability to kill and consume the prey bacterium *E. coli*, which is often found together with myxobacteria on herbivore dung (Dawid 2000; Pan et al. 2013). This predation defect of the mutant could be compensated by addition of exogenous antibiotic TA. Moreover, it was demonstrated that *lspA* overexpressing *E. coli* strains are resistant against predation of the antibiotic TA-producing DK1622 wild-type strain. In sum, these analyses confirmed that antibiotic TA production is essential for the feeding of *M. xanthus* DK1622 on

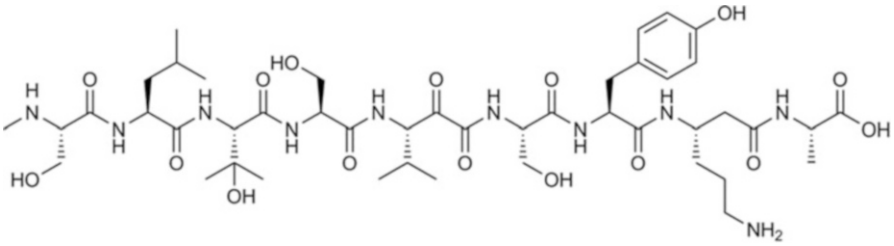

Fig. 8 Structure of myxoprincomide

E. coli. On the other hand, it also became clear that antibiotic TA had no effect on the killing of another prey bacterium, *i.e.*, *Micrococcus luteus* (Xiao et al. 2011). Apparently, the DK1622 strain does not rely exclusively on the production of antibiotic TA for predation. It is further noteworthy that the biosynthesis of antibiotic TA is not conserved in all *M. xanthus* strains. Out of 98 strains previously tested, only 39 were able to produce this compound (Krug et al. 2008). Nevertheless, all members of the species *M. xanthus* possess the ability to lyse and feed on other bacteria. It is hence likely that these bacteria utilize different sets of antibiotics in combination with lytic enzymes in order to attack a variety of prey bacteria.

Myxoprincomide Myxoprincomide is a linear peptide (Fig. 8), which was discovered in *M. xanthus* DK1622 after an extensive metabolome analysis involving statistical data evaluation (Cortina et al. 2012). The method used also provided the name for this secondary metabolite (*Myxococcus* compound found using principle component analysis). Biosynthetically, myxoprincomide is the product of a giant NRPS, which features a PKS module. The assembly involves several unusual transformations and is not fully understood (Cortina et al. 2012). An inspection of myxobacterial genome sequences suggests that the capacity for the biosynthesis of this natural product is restricted to bacteria of the genus *Myxococcus* (Nett, unpublished data).

Up to now, biological activities have not been reported for myxoprincomide (Herrmann et al. 2017). Although the peptide is apparently not an antibiotic, it seems to influence the ability of *M. xanthus* to feed on *Bacillus subtilis* NCIB3610. The latter bacterium can, at least to some extent, evade myxobacterial predation through the production of a polyketide and the formation of tree-like megastructures, which are filled with endospores (Muller et al. 2014, 2015). In case of myxoprincomide-deficient *M. xanthus* strains, the predatory resistance of *B. subtilis* NCIB3610 is even further increased. Moreover, the predators show reduced growth rates in the presence of their prey. These results demonstrate that the secondary metabolite contributes to the predatory success of *M. xanthus* (Muller et al. 2016), even though the precise function of myxoprincomide remains elusive.

DKxanthenes The DKxanthenes are yellow pigments, which are commonly produced by *M. xanthus* strains (Krug et al. 2008; Meiser et al. 2006) and can also be found in other myxobacteria, such as *Myxococcus stipitatus* DSM14675 (Hyun et al. 2018) and *Stigmatella aurantiaca* DW4/3–1 (Meiser et al. 2008). Their basic

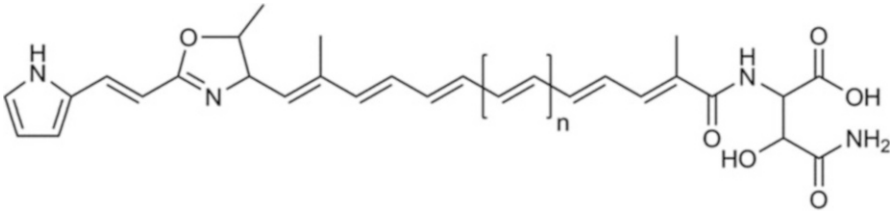

Fig. 9 Structures of DKxanthene-534 (n = 1) and DKxanthene-560 (n = 2), the two main pigments produced by *M. xanthus* DK1622

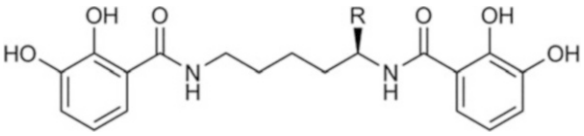

Fig. 10 Structures of bis-catecholate siderophores: azotochelin (R = COOH), myxochelin A (R = CH_2OH), and myxochelin B (R = CH_2NH_2)

structure includes a hydrophilic asparagine residue and a hydrophobic polyene chain, which confer an amphiphilic character to these molecules. Members of this pigment family differ in their polyene chain length, the extent of methylation and the hydroxylation pattern (Fig. 9) (Meiser et al. 2006, 2008).

To identify the genes involved in DKxanthene biosynthesis, the production of these pigments was abolished in *M. xanthus* by transposon mutagenesis and the insertion sites were subsequently tracked (Meiser et al. 2006). This approach led to the discovery of a gene locus encoding several PKS and NRPS enzymes (Meiser et al. 2008). An inspection of the corresponding assembly line quickly revealed a discrepancy between the number of PKS modules and the polyene chain length in DKxanthenes. Preliminary analyses indicated that one or more PKS modules act iteratively and that the variable rounds of chain extension catalyzed by these enzymes give rise to the structural diversity in this natural product family. According to the authors of the study, this variability might be due to non-ideal docking interactions involving a PKS module of the assembly line that was potentially acquired in the course of horizontal gene transfer (Meiser et al. 2008).

M. xanthus mutants, which are deficient in DKxanthene biosynthesis, display a significant delay in fruiting body formation and maturation of their myxospores compared to the wildtype (Meiser et al. 2006). Surprisingly, the loss of DKxanthene production in *M. stipitatus* was reported to have no effect on the developmental program of the corresponding bacterium (Hyun et al. 2018).

Myxochelins The myxochelins constitute a family of catecholate-type siderophores, which supply the producing bacterium with essential iron (Kunze et al. 1989; Silakowski et al. 2000). Structurally and biosynthetically these secondary metabolites are closely related to azotochelin (Fig. 10), a siderophore from the diazotrophic

bacterium *Azotobacter vinelandii* (Corbin and Bulen 1969), albeit their assembly involves a distinctive reduction of the central lysine motif (Li et al. 2008). This peculiarity was recently demonstrated to enable further biosynthetic transformations, giving rise to structurally more complex molecular scaffolds, such as pseudochelin A (Korp et al. 2018; Sonnenschein et al. 2017).

Myxochelin production is widely distributed in myxobacteria, but occurs occasionally also in bacteria belonging to different lineages (Korp et al. 2018). The biosynthetic assembly of these siderophores is well understood both on the genetic and on the biochemical level (Gaitatzis et al. 2005; Korp et al. 2015; Li et al. 2008; Silakowski et al. 2000) and was even reconstituted *in vitro* (Gaitatzis et al. 2001). Briefly, an NRPS catalyzes the condensation of two 2,3-dihydroxybenzoate building blocks with the amino acid L-lysine and releases the product following a reductive offload as an unstable aldehyde intermediate. The latter is either converted to myxochelin A via another reductive step or to myxochelin B through a transamination reaction.

Unlike other siderophores, the myxochelins possess only very weak antimicrobial properties (Kunze et al. 1989). Instead researchers observed noteworthy activities against human leukemic cells (Miyanaga et al. 2006; Schieferdecker et al. 2015b). This activity was traced to an inhibition of the enzyme 5-lipoxygenase (Schieferdecker et al. 2015b). In general, lipoxygenases (LOXs) form a class of non-heme iron containing enzymes widely distributed in eukaryotic organisms including mammals, plants, marine invertebrates, and fungi. In recent years, however, the number of reports on the identification of bacterial lipoxygenases is constantly increasing (Hansen et al. 2013). Most of these enzymes were found in bacteria with Gram-negative cell wall architecture (Hansen et al. 2013), including members of the ß-proteobacteria (*e.g.*, *Burkholderia thailandensis* (An et al. 2015)), γ-proteobacteria (*e.g.*, *Pseudomonas aeruginosa* (Vance et al. 2004)), δ-proteobacteria (*e.g.*, *Sorangium cellulosum* (Porta and Rocha-Sosa 2001)) and cyanobacteria (*e.g.*, *Nostoc punctiforme* (Koeduka et al. 2007)). In *M. xanthus* DK1622, two different LOXs have been discovered (An et al. 2018; Qian et al. 2017). The two proteins share low sequence identity with each other (An et al. 2018) but, interestingly, one enzyme shows structural similarities with eukaryotic 5-lipoxygenases (Qian et al. 2017). Plant and fungal LOXs predominantly metabolize linoleic acid and α-linolenic acid (Oliw 2002; Porta and Rocha-Sosa 2002), whereas in mammalian cells, arachidonic acid and linoleic acid serve as major substrates (Kuhn and Thiele 1999; Kuhn et al. 2015). Considering that 13-methylmyristic acid forms the main cellular fatty acid component in *M. xanthus*, it is surprising that both of its LOX enzymes possess a substrate preference for arachidonic acid and linoleic acid (An et al. 2018; Qian et al. 2017). Since there is no concrete evidence for the biological role of myxobacterial lipoxygenases, it is unclear whether these enzymes metabolize cellular substrates or whether they utilize exogenous fatty acid sources for catalysis, as observed in *P. aeruginosa* (Vance et al. 2004). Anyway, the presence of endogenous LOXs in

M. xanthus might indicate an additional biological function for the myxochelins beyond their role as siderophores (Sester et al. 2019).

4 *Bdellovibrio* and Like Organisms (BALOs)

4.1 Characteristics of BALOs

Bdellovibrio and like organisms (BALOs) are predators of Gram-negative bacteria. They are widely distributed in nature and grow usually host-dependently. Many BALOs invade their prey after attachment and replicate inside its periplasmic space, but some are also known to pursue an epibiotic feeding strategy (Jurkevitch 2007). Among predatory bacteria, the bacterium *Bdellovibrio bacteriovorus* has received particular attention from the scientific community due to its interesting life cycle, which involves the switch from an axenic to a periplasmic growth phase, and due to the morphological transformation of the host prey bacterium, which culminates in the formation of a structure called bdelloplast (Sockett 2009). For details on BALO's life cycle and ecology and for environmental effects on BALO predation see Chapters by Jurkevitch "The Ecology of *Bdellovibrio* and Like Organisms in Wastewater Treatment Plants" and by Im et al. "Environmental and Biotic Factors Impacting the Activities of *Bdellovibrio bacteriovorus*", respectively.

4.2 The Secondary Metabolome of BALOs

As opposed to myxobacteria, not much is known about secondary metabolites from *B. bacteriovorus* or from BALOs, in general. The importance of such molecules for the lifestyle of these bacteria is not clear, but this situation is about to change. Over the past years, several genome sequencing projects of BALO species have been completed (Hobley et al. 2012; Oyedara et al. 2018; Pasternak et al. 2013; Rendulic et al. 2004; Wurtzel et al. 2010) and the accumulated data now provides comprehensive insights into the secondary metabolism of this bacterial group. Before BALO pathways to secondary metabolites are introduced, it is appropriate to take a closer look at general features of their genomes.

All sequenced BALOs were found to possess a single circular chromosome. The length of the BALO replicons vary from 2.5 Mbp for the epibiotic *B. exovorus* up to 4 Mbp for the periplasmic predators (Pasternak et al. 2014). In comparison to myxobacteria, the BALO genome sizes are hence rather small. In several studies, a positive correlation between the capacity for secondary metabolite biosynthesis and genome size was observed (Baltz 2017; Donadio et al. 2007). Consistent with these findings, a bioinformatic analysis of the fully sequenced BALO chromosomes reveals only few (≤ 3) loci for the production of secondary metabolites (Table 2).

Table 2 Genomic and biosynthetic features of BALOs

BALO strain (GenBank accession no.)	Genome size [Mbp]	GC content [%]	Biosynthetic gene cluster (BGC): predicted secondary metabolite	Location of BGC on chromosome
B. bacteriovorus strain HD100 (NC_005363)	3.78	50.60	1: Aromatic polyketide 2: Siderophore (aerobactin) 3: Carotenoid	Bd0330–0332 Bd1572–1578 Bd1723–1730
B. bacteriovorus strain 109 J (NZ_CP007656)	3.83	50.70	1: Siderophore (aerobactin) 2: Carotenoid 3: Aromatic polyketide	EP01_RS04000–04030 EP01_RS04640–04675 EP01_RS13390–13,400
B. bacteriovorus strain SSB218315 (NZ_CP020946)	3.77	50.50	1: Carotenoid 2: Siderophore (aerobactin) 3: Aromatic polyketide	B9G79_10365–10,330 B9G79_11005–10,975 B9G79_16665–16,655
B. bacteriovorus strain Tiberius (NC_019567)	3.99	49.90	1: Aromatic polyketide 2: Siderophore (aerobactin)	Bdt_0327–0329 Bdt_1565–1571
B. bacteriovorus strain W (NZ_CP002190)	3.01	43.30	Not detected	–
B. exovorus strain JSS (NC_020813)	2.66	41.90	Not detected	–

The smaller BALO genomes of *B. bacteriovorus* strain W and *B. exovorus* JSS appear to be completely devoid of genes for the biosynthesis of specialized metabolites. This finding suggests that such molecules are not absolutely required for the predatory activity of BALOs. On the other hand, the biosynthetic loci, which were detected in the other strains, are highly syntenic and conserved. It is hence expected that they are responsible for the synthesis of structurally identical metabolites.

The first secondary metabolite gene cluster, which was noticed in *B. bacteriovorus* genomes, governs the biosynthesis of carotenoids (Hobley et al. 2012). Although the BALO carotenoids have not been chemically characterized, it is long known that *B. bacteriovorus* uses these compounds for photoprotection (Friedberg 1977). The carotenoid pathway is present in all BALO genomes with a size larger than 3.5 Mbp except that of the Tiberius strain. This circumstance was attributed to the aquatic origin of the latter and a different protection strategy against free radicals (Hobley et al. 2012). Genes that are needed to produce the isoprene building blocks for carotenoid biosynthesis were found to be induced when *B. bacteriovorus* switches to predatory growth (Lambert et al. 2010), which might indicate that protection from oxidative damage could be particularly relevant in the periplasm of the prey host. Interestingly, the isoprene units originate from the MEV pathway and not from the MEP pathway (see Sect. 2), even though the latter is more common in prokaryotes. This peculiarity seems to be a characteristic signature in the

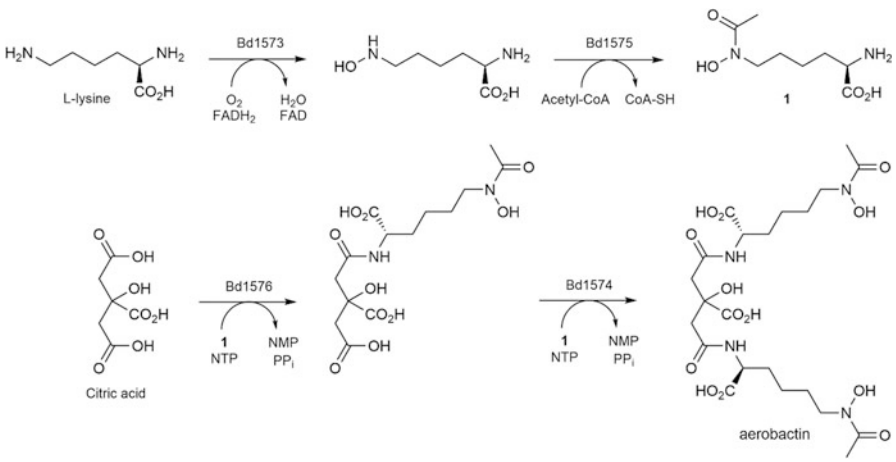

Fig. 11 Pathway for aerobactin biosynthesis in *B. bacteriovorus* HD100

genomes of predatory bacteria and was observed in BALOs and myxobacteria alike (Pasternak et al. 2013).

A pathway directing the biosynthesis of the siderophore aerobactin (Fig. 11) is generally conserved in periplasmic BALOs (Pasternak et al. 2014). According to a global transcriptome analysis of *B. bacteriovorus* HD100, aerobactin is produced during the growth phase of the bacterium (Karunker et al. 2013), which implies an iron limitation in the periplasm of the prey cell. It is thus possible that aerobactin promotes, at least to some degree, the predacious replication of *B. bacteriovorus*.

Another biosynthetic locus, which is widely distributed in *B. bacteriovorus* strains but has not been mentioned in the literature to our knowledge, is predicted to govern the production of an aromatic polyketide. This locus consists of a three-gene operon including open reading frames for an oxidoreductase, a type III PKS, and a hypothetical protein. Unlike the modularly organized enzymes introduced in Sect. 3.2, type III PKSs act iteratively, *i.e.*, a single enzyme carries out multiple chain elongations. Another mechanistic difference to other PKSs is the usage of free acyl-CoA thioesters as substrates. Indeed, type III PKSs lack ACP domains and are solely composed of KS domains, which form homodimers (Shimizu et al. 2017). These modest-sized condensing enzymes have long been known from plants as chalcone and stilbene synthases and were only discovered in microorganisms with the advent of genome sequencing (Moore et al. 2002). Despite the minimalist domain architecture of type III PKSs, it is possible to predict their metabolic products. This is usually achieved on the basis of a phylogenetic comparison with characterized representatives (Shimizu et al. 2017). A phylogenetic analysis of the *B. bacteriovorus* type III PKSs (Fig. 12) indicates that they are most closely related to ArsB and ArsC from *Azotobacter vinelandii* (Funa et al. 2006). These enzymes are clearly positioned in the subclade that is associated with phenolic lipid and alkylpyrone biosynthesis. In the case of *A. vinelandii* it is known that

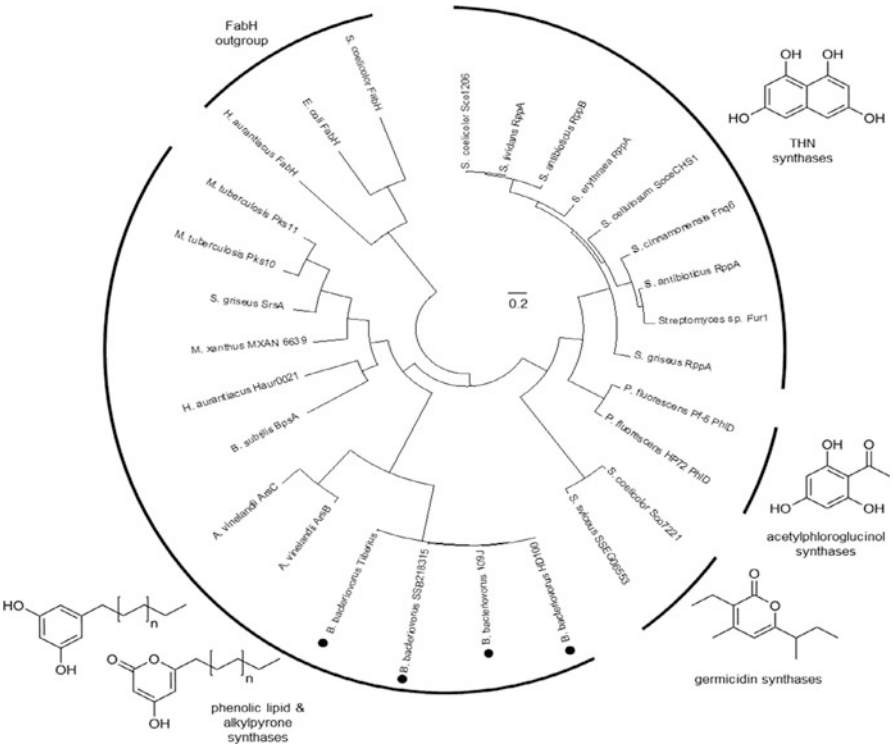

Fig. 12 Phylogenetic tree of bacterial type III PKSs. Multiple alignment was performed with Clustal Omega (Sievers et al. 2011) and the tree was constructed by using maximum-likelihood methods in MEGA7 (Kumar et al. 2016). Type III PKS sequences from *B. bacteriovorus* strains are marked with black dots

5-alkylresorcinols can be incorporated into the plasma membrane during encystment in order to enhance its resistance (Reusch and Sadoff 1983). Up to now, the production of phenolic lipids or alkylpyrones has not been reported for *B. bacteriovorus*, though it is tempting to speculate that such compounds might also be relevant in the switch between axenic and periplasmic growth phase.

5 Conclusions and Perspectives

In recent years, secondary metabolite gene clusters have been detected in the genomes of many predatory bacteria, which were not associated with natural product biosynthesis before (Kiss et al. 2011; Livingstone et al. 2018b). Evidence has now accumulated that the potential for the production of specialized metabolites is almost omnipresent in predatory bacteria, irrespective of taxonomic affiliation. Although the number of secondary metabolite gene clusters in a genome does not allow direct

conclusions on the biological importance of the encoded molecules, it can still be used as an indicator for the biosynthetic versatility of an organism. In this regard, significant differences exist among predatory bacteria. The extent of secondary metabolism, as reflected by the number of biosynthetic loci, is obviously correlated with the genome size and, in the case of predatory bacteria, possibly with the respective feeding strategy. Obligate solitary predators, such as BALOs, possess comparatively small genomes and, consequently, exhibit less biosynthetic loci than facultative predators. Those predators, which show extensive collaborative and synchronized behavior, rely on chemical mediators for the coordination of swarm movement and fruiting body formation (Bhat et al. 2014; Kearns et al. 2001; Lorenzen et al. 2014; Meiser et al. 2006). The required pathways are encoded in their genomes together with many other biosynthetic loci. It is thus not farfetched to speculate about a role for secondary metabolites in the coordinated process of group predation.

The strategic use of secondary metabolites for predation purposes was first shown for antibiotic TA (see Sect. 3.2) and could indeed be a widely distributed trait in predatory bacteria. Additional support for this assumption comes from *Corallococcus coralloides*, which feeds on *E. coli*. Once the prey bacterium develops resistance against the *C. coralloides*-derived antibiotic corallopyronin, it also becomes resistant toward predation by this myxobacterium (Xiao et al. 2011). Another example is given by the gulmirecins. The activity spectrum of these macrolide antibiotics was found to precisely match the prey range of the producing *P. fallax* strain (Schieferdecker et al. 2014), though it still remains to be demonstrated that gulmirecin tolerance confers predation resistance as well. Correlations between antibiotic biosynthesis and predatory lifestyle were also postulated for bacteria outside the Myxococcales, such as *Aristabacter necator* (Cain et al. 2003) or *Lysobacter enzymogene*s which was recently demonstrated to deliver antibiotics to fungal prey using outer membrane vesicles (Meers et al. 2018). The recent finding that myxoprincomide is required for efficient predation (see Sect. 3.2) further suggests that some secondary metabolites affect the predatory success in a more subtle way than direct antibiosis. In this context, aerobactin production by *B. bacteriovorus* should also be mentioned, as this siderophore might be relevant for the survival in the periplasm of the prey cell (see Sect. 4.2).

Despite the many insights from genomic analyses and an increasing number of studies addressing the biological function of small molecules, it is evident that our current understanding of the secondary metabolism in predatory bacteria as well as its contribution to the specific lifestyle of these microorganisms is still limited. Aside from myxobacteria, only a minor fraction of predatory bacteria have been analyzed in this context. Furthermore, there is a deplorable lack of studies focusing on the biological role of antibiotics beyond their killing effect. It is thus necessary to further unlock the biosynthetic potential predicted by genomics. This is likewise important for the BALOs, which were almost completely neglected in the field of natural product chemistry, as well as for established secondary metabolite producers, such as the model strain *M. xanthus* DK1622, for which only 11 out of 27 biosynthetic loci could be associated with known compounds. The generation of mutant strains

impaired in the biosynthesis of selected secondary metabolites and their subsequent testing in appropriate (predation) assays will certainly foster the development of new hypotheses on the biological function of small molecules. The authors would be glad, if this brief review gave some incentive for such efforts.

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Environmental and Biotic Factors Impacting the Activities of *Bdellovibrio bacteriovorus*

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1 *Bdellovibrio bacteriovorus*-and-Like Organisms, Collectively: Bacterial Predators with Much Potential

Bdellovibrio bacteriovorus-and-like organisms, collectively referred to as BALOs, are bacterial predators that attack and consume other Gram-negative bacterial species. BALOs have been isolated from habitats all over the world in various abundances and with different adaptations, most notably their differing tolerance to salt concentrations (Amat and Torrella 1989; Chauhan et al. 2009; Fry and Staples 1976; Jurkevitch et al. 2000; Schoeffield and Williams 1990) (Fig. 1). Isolates from samples taken thousands of kilometres away from each other maybe similar enough to potentially be the same BALO species, whereas BALO isolates within a single sample may differ tremendously from one another (Snyder et al. 2002), with some strains possessing a much broader predation spectrum than others. For instance, the type strain is *B. bacteriovorus* HD100, an intraperiplasmic predator that is capable of attacking over 100 different human pathogens, including strains of *Acinetobacter*, *Klebsiella* and *Salmonella* (Dashiff et al. 2011a; Im et al. 2017b; Sun et al. 2017). Similarly, the epibiotic predator *Micavibrio aeruginosavorus* has broad spectrum activity, albeit much more restricted than *B. bacteriovorus*, against a number of pathogenic strains (Dashiff et al. 2011a). However, the prey range for *Peredibacter starrii*, another intraperiplasmic predatory strain, is restricted to only Pseudomonads (Stolp and Starr 1963).

Although differences exist between these strains and their activities, some characteristics are true for all three. Most prominently and long known is their dependency on magnesium and calcium. The presence of these ions has been linked to diverse functions necessary for predation to occur successfully. In their absence, for instance, predator-prey attachment rates are much lower (Starr and Seidler 1971) and

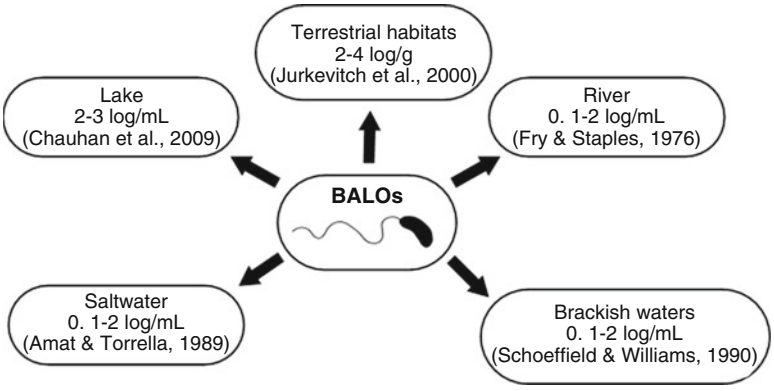

Fig. 1 Examples of the different environmental habitats occupied by BALO species and their measured populations within each. It should be noted that these values were based on plaque-forming units using top agar plates, which is inherently biased as the prey was pre-selected and does not represent the complete predatory complement. Quantitative PCR analyses imply these values may be orders of magnitude higher. (Van Essche et al. 2009; Zheng et al. 2008)

the bdelloplasts appear to be significantly less stable (Seidler and Starr 1969). Moreover, the activity of their extracellular lytic enzymes is greatly reduced if these ions are not provided (Huang and Starr 1973). These requirements are not as stringent for all strains as *B. bacterivorous* 109 J seems to be able to recycle Ca^{2+} from prey cells (Huang and Starr 1973). Moreover, marine BALOs additionally require potassium for high motility and attachment rates (Marbach and Shilo 1978).

Given their propensity to attack Gram-negative pathogens, several groups have considered applying BALOs as a therapeutic to reduce or remove these harmful bacteria, as reviewed in several articles (Choi et al. 2017; Dwidar et al. 2012b). BALOs also mitigate plant (Barel et al. 2005; McNeely et al. 2017) and animal pathogens (Cao et al. 2014, 2018; Li et al. 2014), with all of these studies hinting at the potential application of predators as biocontrol agents within the agricultural and aquacultural sectors to reduce spoilage and loss in productivity.

However, recent research has found BALOs face many hurdles, impediments that may be biotic or abiotic in nature. From the presence of sugars or salts within the media to the production of secondary metabolites by prey strains, researchers are currently defining the limitations of predation while also seeking ways to overcome these hurdles or, as in the case of *Vampirovibrio chlorellavorus*, to employ them a means of controlling undesired predatory activities (Bagwell et al. 2016; Ganuza et al. 2016).

2 Abiotic Factors Impacting the Predatory Activities of *Bdellovibrio bacteriovorus* and Other BALOs

2.1 Oxygen

As strict aerobes, it comes as no surprise that BALOs and their predatory activities strongly correlate with the availability of oxygen (Kadouri and Tran 2013; Schoeffield et al. 1996; Varon and Shilo 1968). In one of the first studies on this topic, Varon and Shilo (1968) measured the predator-prey attachment rate when either agitated or stationary. In the agitated cultures, the attachment rates at 20 min were greater than 70% but hovered only near 20% in the stationary tubes. This was evaluated further by Kadouri and Tran (2013) where the activities of three BALO strains, *i.e.*, *B. bacteriovorus* HD100, *B. bacteriovorus* 109 J and *M. aeruginosavorus*, were measured under different oxygen concentrations (0 to 100%). Similar with Varon and Shilo (1968), the BALO strains did not effectively attack planktonic bacteria under microaerobic or anaerobic conditions, but prey biofilms were reduced by as much as 60% in the former environment. These results suggest predation of surface attached prey is still possible when oxygen levels are low, but not when the environment is anaerobic.

One predatory strain, *B. bacteriovorus* W, however, can attack planktonic prey even when the oxygen partial pressure is very low (3–5 mm Hg) (Burger et al. 1968),

a level where many anaerobes are also capable of growing. As several obligate (aerotolerant) anaerobes, including *Prevotella intermedia* and some strains of *Fusobacterium nucleatum*, may be used as prey by BALOs (Dashiff and Kadouri 2011; Van Essche et al. 2011), *B. bacteriovorus* W represents a class of predators that may be better adapted for survival within low-oxygen environments and biocontrol of these anaerobic pathogens.

Aside from controlling predation, oxygen also impacts the long-term survival of predatory strains, as illustrated in the study by Schoeffield et al. (1996) where the viabilities of several different BALO species, representing both halotolerant and non-halotolerant species, were measured over several days when under either aerobic or anaerobic conditions. Their study found anaerobic conditions led to significantly faster losses with both classes of predators. However, these results conflict with those of Williams and Falkler (1984), where predatory bacteria were isolated from the anaerobic region (13 m depth) within the Miles River. In fact, they found the oxygen concentration had no impact on the predatory numbers, with similar numbers of isolates at each of the depths tested (*i.e.*, 0.5–13 m). Whether these differences are due to the bacterial strain, their overall concentration (which was several log higher in the lab) or some unidentified environmental factors that contribute to stabilizing the predator under anaerobic conditions remains to be elucidated.

2.2 Temperature

Predatory strains that have been studied to date generally have mesophilic preferences with optimal temperatures between 28 °C and 35 °C, although limited predatory activities have been seen in the range of 10–45 °C (Atterbury et al. 2011; Fratamico and Whiting 1995; Varon and Shilo 1968). As with oxygen, Varon and Shilo (1968) studied predator-prey attachment at different temperatures, spanning from 4 °C to 45 °C. As the temperature rose from 4 °C to 25 °C, the percentage of *B. bacteriovorus* 109 cells that were attached to prey increased in a fairly linear manner from 1% to 64%, and remained somewhat steady thereafter until 35 °C, which was the maximum permissible temperature. Increasing the temperature further reduced the number of attachment events significantly, with only 7% of predators attached to prey at 45 °C. These results were corroborated by Fratamico and Whiting (1995), who measured prey viabilities at set times over 24 h at temperatures between 4 °C and 37 °C. In both studies, predation and attachment was optimal at or near 37 °C and decreased as the temperature was lowered, with 4 °C showing no predation (Fratamico and Whiting 1995) and only 1% of predators attached to prey cells (Varon and Shilo 1968).

Although the majority of BALO studies use mesophilic predatory strains, this does not preclude their presence in hotter or colder environs. As potential proof that thermophilic BALOs exist, predatory strains were reportedly found in significant numbers (~ 1% of total counts) in two hot spring microbial mats where the surface

temperatures were 57 ± 2 °C and 91 ± 3 °C (Sangwan et al. 2015). A draft genome of this BALO strain was constructed and homologues for *Bd0108 and Bd0105*, two genes required for intraperiplasmic stages of predation, were not found, hinting this strain has a highly specialized genome to cope with the atypical conditions within this environment. Although the authors were unsuccessful in culturing the predators, they did manage to capture images of them attacking *E. coli* and found an unusual predation mechanism; the predator was epibiotic but was attached side-on with the prey cell, as opposed to the polar attachment seen with *M. aeruginosavorus*. Along with other culture-independent studies that revealed a significantly higher BALO-diversity in soil (Davidov et al. 2006), fresh water (Li et al. 2015) and saltwater habitats (Li et al. 2015; Pineiro et al. 2007b) than originally expected, the above study highlights a potentially untapped diversity of BALOs that grow at extreme temperatures, and should encourage BALO researchers to consider other environments, such as glacial pools or the deep ocean, when seeking novel predatory strains.

2.3 pH

Attachment of the predator to its prey is most effective when the pH is between 6 and 9.3 based on Varon and Shilo (1968), with a maximum at the slightly basic pH of 8. At lower pH levels, attachment rates and predator motility were heavily impaired (Fratamico and Whiting 1995; Varon and Shilo 1968) but was still possible at a pH of 5.6. Dashiff et al. (2011b) evaluated this further by measuring the predatory viabilities at different pH values. They reported that incubating either *B. bacteriovorus* 109 J or *M. aeruginosavorus* at a pH of 4 or lower for 24 h completely kills (<1 PFU/ml) both predators while, for *M. aeruginosavorus*, no loss was seen when incubated in DNB media at a pH of 5. Host-independent (HI) variants of *B. bacteriovorus* 109 J, which grow axenically, were much more sensitive than the wild-type (host-dependent) strain; 1 h at pH 4 completely eradicated the HI population (> 7-log loss) while the wild-type population dropped by only 3-log.

The sensitivity of predatory strains to acidic pHs was used by one group to control *Vampirovibrio chlorellavorus*, a predatory non-photosynthetic cyanobacterium, and its predation of *Chlorella* HS26, an algae used to produce lipids for biodiesel (Ganuza et al. 2016). Shifting the pH to 3.5 for only 15 min with the small addition of acetate (0.5 g/L) reduced the *V. chlorellavorus* viability by 2-log without significantly affecting that of *Chlorella* HS26. Using this cost-effective protocol, they were able to protect open ponds of *Chlorella* HS26 from predation, extending their longevity and overall productivity.

2.4 Salinity and Osmolality

Given their pervasive presence throughout nature, it is not surprising researchers have found some BALO strains prefer low levels of salt while others are more suited for growth in seawater, where the osmolality is around 1000 mOsm/kg. For instance, the best studied strain, *B. bacteriovorus* HD100, was isolated from soil (Stolp and Starr 1963), prefers freshwater and is generally unable to predate when the osmolality is greater than 250 mOsm/kg, or approximately 0.82% NaCl (Im et al. 2017b). If the salinity was reduced slightly to 0.65% (200 mOsm/kg), predation was as effective as in HEPES buffer as based on the 24-h prey viabilities. The loss of activity seen with osmolalities of between 250 and 350 mOsm/kg was not due a reduced *B. bacteriovorus* HD100 viability; the 24-h values were not significantly different from those within HEPES, where the osmolality was typically around 40 mOsm/kg.

On the opposite side of the BALO spectrum one finds *Halobacteriovorax* spp., including *B. litoralis* and *B. marinus*, which are ubiquitous in saltwater environments and require NaCl concentrations of 0.5% or greater for optimal predation rates (Koval et al. 2015). Predatory bacteria have also been isolated from estuaries, where midline salinities are found (Pineiro et al. 2007a, 2013; Williams and Falkler 1984) and, in fact, certain clades of *Bacteriovorax* are only found in less saline waters and disappear as the river mixes with and enters ocean waters (Pineiro et al. 2013).

In a separate but related study, Kandel et al. (2014) identified predatory strains within fresh and saltwater zero discharge systems (ZDS) over a seven-month period. These ZDS mesocosms, where the water is continuously recycled, were developed to rear fish and use microbial activities to remove nitrogen, sulphate and organic materials. An analysis of both freshwater and saltwater ZDS found relatively equal numbers of *Bacteriovorax*/*Bacteriolyticum* within each (between 10^4 and 10^5) and a similar concentration of *Bdellovibrio* spp. within the freshwater ZDS. Interestingly, within the saltwater ZDS, where the salt concentration was 20 ppt (approximately 600 mOsm/kg), *Bdellovibrio* were still found at an average concentration of around 10^3 PFU/ml. Phylogenetic analysis identified a relatively large number of sequences that were somewhat related to *B. bacteriovorus* HD100 but, since the maximum likelihood tree had low bootstrap values, reliable annotation is difficult and further analysis needs to be done to confirm the heritage of these strains. However, their results strongly imply halo-tolerant *Bdellovibrio* species do exist within nature and, as of yet, remain an uncharacterized group of BALOs.

2.5 Environmental Factors and Niche Partitioning

In a recent study, the distribution and abundance of three BALO families (*Peredibacteraceae*, *Bdellovibrionaceae* and *Bacteriovoracaceae*) was investigated in perialpine lakes (Paix et al. 2019). The spatially separated, seasonally changing coexistence of these families suggests that each have different strategies for their

respective environmental niches, with depth and temperature reportedly as the main factors. Similar observations could be made for soil and rhizosphere isolates, which display a locally separated coexistence of various BALO strains, each with a different prey spectra, in relatively close proximity to one another (Jurkevitch et al. 2000). Both studies show a clear adaptation or selection of BALOs that is driven by environmental factors, leading to niche partitioning of the different predatory species and strains within a local environment. However, the causes are still under investigation. For instance, it cannot be ruled out that a predator may "follow" a prey organism into a given niche that is beneficial to the prey and slowly adapt to that environment over time.

2.6 Susceptibility of BALOs to Some Environmental Factors May Be Mitigated When in a Bdelloplast

When present intraperiplasmically, *i.e.*, within the periplasm of a prey, predators may be protected from some environmental conditions and survive significantly longer than free attack-phase BALOs. This was proven to be true for anoxic conditions and/or at elevated temperatures (Schoeffield et al. 1996). Similarly, during winter, when the overall temperatures drop, some BALOs "hibernate" in estuarine sediments within bdelloplasts, and these sediments later on serve as a reservoir to recolonize the above waters during the warmer seasons (Williams 1988). Prey biofilms also offer protection against environmental stresses, as illustrated by different studies showing *Bacteriovorax* spp. survival rates under naturally occurring, unfavourable habitat conditions (temperature/salinity) improved significantly when associated with biofilms (Kelley et al. 1997; Williams et al. 1995, 2009). In each case, *Bacteriovorax* was less susceptible when associated with a biofilm rather than as attack-phase planktonic cells. All of these studies illustrate a potential survival mechanism used by BALOs to reduce the impacts of salinity and temperature. As discussed in the following sections, however, being within a prey does not provide blanket protection against all conditions, though.

3 Biotic Factors that Impact the Predatory Activities of *Bdellovibrio bacteriovorus* and Other BALOs

In addition to the abiotic factors listed above, research over the past decade has identified a range of biotic elements from bacteria and eukaryotes that impact predation. The former is showing us that predation may not be as straight-forward as previously thought, with prey secreting factors that inhibit predation and non-prey bacteria offering some benefits, while the latter (eukaryotic factors) is important if application of BALOs as living antibiotic is to be realized.

3.1 Bacterial Factors

Prey Metabolic Activities The prey metabolic activities may also influence the local environment in a way that is less suitable for the predator, such as through acidification of the medium. As discussed above, the pH can have a tremendous impact on predation. Within their study, Dashiff et al. (2011b) found the addition of either glycerol or glucose to co-cultures of *E. coli* and *B. bacteriovorus* 109 J blocked predation. They initially evaluated if these carbohydrates alone killed the predator and found this was not the case. Intriguingly, neither was able to block predation when the prey cells were heat killed, suggesting the metabolic activity of *E. coli* was responsible for the inhibitions seen. Further evaluation found the cause was the media pH, which dropped from pH 6.5 to less than pH 4 within the first 5 h as the carbohydrates were consumed by the prey. As was reported by Varon and Shilo (1968), this pH was both inhibitory and lethal, leading to a significant and rapid killing of *B. bacteriovorus* 109 J (Dashiff et al. 2011b). Importantly, the same experiments performed in buffered media did not give the same results. This suggests there may be microenvironments within nature that inhibit bacterial predation due to the activities of the prey cells within them.

Another example of prey activity that actively hinders predation was recently reported by Duncan et al. (2018) in their article discussing *B. bacteriovorus* HD100's ability to predate on *Vibrio cholera*. *V. cholerae* is highly motile which puts stress (literally) on the predator when it is attempting to attack it; the predator is dragged along while attached to the prey as *V. cholerae* continues to swim. Although predation was still relatively successful (99.4% killing with a wild-type, motile V. cholerae over 14 h with an initial MOI of 0.1), the non-motile V. cholerae ΔmotY mutant was more susceptible to predation by B. bacteriovorus 109J.

Prey Secondary Metabolites Substances produced by potential prey bacteria can also significantly impact BALO activities. Indole is a secondary metabolite produced by various bacteria and reported to be involved in quorum sensing (Lee and Lee 2010). This molecule, which was not toxic towards *B. bacteriovorus* HD100, slowed predation when present at a concentration of 0.25–1 mM and completely blocked it when added to a final concentration of 2 mM (Dwidar et al. 2015). Through activity assays and transcriptomics, the authors found that indole represses expression of many flagellar genes, compromising the predator's motility during the attack-phase, *i.e.*, they stop swimming. Moreover, indole interfered with the growth of *B. bacteriovorus* HD100 within bdelloplast, bringing it to a halt and preventing further development. As such, the bdelloplast offers no apparent protection against indole.

Another secondary metabolite that impacts predation is cyanide. Strains of *Pseudomonas* and *Chromobacterium* are cyanogenic and produce significant quantities of cyanide when amino acids are available (Askeland and Morrison 1983; Freeman et al. 1975; Gallagher and Manoil 2001; Michaels et al. 1965; Mun et al. 2017). When this occurs and the cyanide concentration was below 100 μM,

predation with *B. bacteriovorus* HD100 slowed down but it's viability was stable (Mun et al. 2017), much like what is seen with indole. Increasing the cyanide concentration to 200 μM or higher completely blocked predation and led to a slight but statistical drop (~50%) in the *B. bacteriovorus* HD100 viabilities. This concentration (202 μM) was achieved with *C. piscinae* when incubated in dilute nutrient broth (1:10 diluted NB) while much higher concentrations (600–800 μM) were obtained when NB was used, illustrating the small amount of amino acids needed to achieve resistance in this strain. Another similarity between cyanide and indole is the bdelloplast-associated predatory strains were also susceptible, *i.e.*, being within the prey did not protect the intraperiplasmic predator. As with indole, they were just as sensitive to cyanide as attack-phase cells (Dwidar et al. 2015; Mun et al. 2017).

Antibiotics Strains of *Streptomyces* are known for their ability to produce a wide-range of antibiotics (Procopio et al. 2012) but, as Gram-positive bacteria, they are not prey for known BALOs. In the study by Varon and Shilo (1968), it was reported three protein synthesis inhibitors, *i.e.*, streptomycin, chloramphenicol and puromycin, produced by strains of *Streptomyces* all blocked predation with *B. bacteriovorus* 109. A deeper analysis found the predator still attached to the prey when exposed to these antibiotics but invasion did not occur, suggesting *de novo* protein synthesis is needed after attachment to the prey. In contrast, attachment and invasion both occurred when ampicillin, a β-lactam antibiotic that inhibits cell wall synthesis, was tested. A subsequent study reported treatment of *B. bacteriovorus* with penicillin, a different β-lactam antibiotic, leads to the stable formation of spheroplasts (Thomashow and Rittenberg 1978). Both lysozyme and D-cycloserine, another antibiotic produced by *Streptomyces* that inhibits cell wall synthesis (Kuehl et al. 1955), had similar effects on this predator (Thomashow and Rittenberg 1978), implying *B. bacteriovorus* is tolerant to cell wall-targeting antibiotics. Another important finding was penicillin did not have the same effect when used in combination with chloramphenicol, *i.e.*, there was no spheroplast formation. As with the protein inhibitors mentioned above, this illustrates *de novo* protein and peptidoglycan synthesis both occur during the attack-phase.

A more recent study evaluated the use of *B. bacteriovorus* HD100 in the presence of violacein (Im et al. 2017a). Violacein, a bisindole compound formed through a condensation reaction involving two tryptophan molecules (Hoshino et al. 1987), is produced by a wide-range of Gram-negative bacteria (Choi et al. 2015a, b). As an antibiotic, the spectrum of violacein primarily encompasses Gram-positive strains where it appears to attack the cellular membrane, causing loss in integrity and leakage of the cellular components (ATP, protein, etc.) (Aruldass et al. 2018). As BALOs are Gram-negative bacteria, the limited spectrum of violacein these two antibacterials were combined and used together (Im et al. 2017a). They demonstrated the specificity of both, *i.e.*, violacein against only Gram-positive and *B. bacteriovorus* HD100 against only Gram-negative, and that they did not interfere with the activity of the other. Moreover, when used together, their combined activities reduced the viability of mixed cultures (*i.e.*, four different pathogens) by 4-log, and was much more effective than the combined use of gentamycin and

chloramphenicol. Consequently, that study showed predatory bacteria can successfully be used alongside antibiotics that specifically target Gram-positive bacterial strains.

Bacterial Proteins In contrast with the above inhibitors, *Serratia marcescens* employs a different class of biological defence to protect it from the epibiotic predator, *M. aeruginosavorus*. Garcia et al. (2018) reported *S. marcescens* expresses and secretes PrtS, a serralysin family metalloprotease, which protects it from predation. Not only did PrtS reduce predation of *S. marcescens* by more than 95%, it similarly protected *E. coli*, reducing *M. aeruginosavorus* predation by as much as 98%. However, experiments with the intraperiplasmic *B. bacteriovorus* 109 J found this protease affords no protection, implying its inhibitory activity may be specific for epibiotic predators. Another important characteristic of PtrS is, rather than working against the predator like many of the factors discussed here, this enzyme hydrolyzes some yet unidentified surface protein(s) in the outer membrane of the prey (*E. coli* and *S. marcescens*) but does not impact their ability to grow. This study also suggests the recognition mechanisms used by *B. bacteriovorus* and *M. aeruginosavorus* are likely distinct, with the latter recognizing a specific outer membrane protein within its prey that is susceptible to proteolytic hydrolysis.

On the other hand, extracellular proteins may also be beneficial to *B. bacteriovorus*, particularly its own, as shown recently in work done with *S. aureus* biofilms (Im et al. 2018). As a Gram-positive bacterium, *S. aureus* is not a prey for *B. bacteriovorus* (Im et al. 2017a; Monnappa et al. 2014), although Iebba et al. (2014), using unconventional methods, allegedly claims otherwise. Monnappa et al. (2014) found proteases secreted by a host-independent variant of *B. bacteriovorus* (HIB) extensively hydrolysed the surface proteins of planktonic *S. aureus* cells. In two subsequent studies, Dwidar et al. (2017) and Im et al. (2018) demonstrated wild-type attack-phase *B. bacteriovorus* HD100 also respond to extracellular amino acids and secrete the same proteases in response. In the first study, *B. bacteriovorus* HD100 was found to secrete proteases in both a time- and dose-dependent manner when incubated alone in different nutrient media preparations (HEPES, 0.2x NB, 1x NB and 5x NB). Moreover, the *B. bacteriovorus* HD100 gene expression patterns in 1x NB mimicked those seen during intraperiplasmic phase, as reported by Karunker et al. (2013). In Im et al. (2018), this was expanded to studies with *S. aureus* biofilms. Although this bacterium is not a prey for *B. bacteriovorus* HD100, results which were confirmed once more in that study, the authors found the predator benefitted from interacting with *S. aureus* biofilms, specifically by hydrolysing proteins present within the extracellular polymeric substances (EPS). The extracellular proteases responsible were produced *de novo* by attack-phase *B. bacteriovorus* HD100 when they encountered the *S. aureus* biofilms, while the supply of amino acids translated into significantly higher ATP pools within the predators and improved killing rates. Together, these three studies (Dwidar et al. 2017; Im et al. 2018; Monnappa et al. 2014) prove predatory bacteria gain a clear benefit from extracellular amino acids, even if they are from non-prey biofilms and their EPS layers.

3.2 Eukaryotic Factors

Predators Are not Harmful Towards Eukaryotes In 1996, Lederberg coined the term 'living antibiotic' in an article where he mentioned bacteriophage and BALOs may be developed as new therapeutic agents (Lederberg 1996). Since that time, work by different groups reports BALOs actively predate a large number of human pathogens, including drug-resistant strains (Dashiff et al. 2011a; Im et al. 2017a; Sun et al. 2017), and their biofilms (Dwidar et al. 2012a, 2013; Kadouri et al. 2007). Later work also demonstrated BALOs are not harmful towards human cells (Monnappa et al. 2016), neither inducing strong cytokine responses nor leading to any observed increase in cell death. One reason for their mild nature is their unique lipid A, which contains α-D-mannopyranose residues instead of phosphate, making it the first example of a lipid A that lacks negatively charged groups (Schwudke et al. 2003). Due to this change in structure, the *B. bacteriovorus* HD100 lipid A did not induce strong immunogenic responses, *i.e.*, cytokines, from human macrophage cells.

More than not being harmful towards eukaryotic cells, several studies demonstrated BALOs actually protected animal cells from pathogens. Boileau et al. (2011) reported *B. bacteriovorus* 109 J lowered *Moraxella bovis* attachment to Madin-Darby bovine kidney cells by sixfold. Similarly, the study by Dwidar et al. (2013) showed *B. bacterivorous* HD100 protected mammalian cells from a strain of *Pseudomonas*. In that study, when the predator was not present, a biofilm of *Pseudomonas* sp. DSM 50906 killed human cells located beneath it, leading to an "footprint" zone of clearing. With the addition of *B. bacterivorous* HD100, though, the human cells within this zone were healthy. Regarding non-prey pathogens, as mentioned above, BALO proteases hydrolysed *S. aureus* surface proteins, which reduced the ability of this pathogen to invade human epithelial cells by 80% (Monnappa et al. 2014).

Although the above studies illustrate the gentle, and potentially helpful, nature of BALOs towards host cells, the same cannot be said for the host impact on BALOs and their activities. This is discussed further in the following section.

Serum Albumin, a Proteinaceous Inhibitor Within in Blood Sera As noted in Sect. 2.4, predation is inhibited by the osmolality. As the value for blood sera in most higher organisms hovers around 300 mOsm/kg (Hall et al. 2012), this would limit the activity of BALOs. However, this is not the only inhibiting factor associated with blood sera. As reported by Im et al. (2017b), human serum albumin also coats *B. bacteriovorus* HD100 and prevents it from attacking its prey. Blood sera contains several different proteins but albumin is the most common one, present at a concentration of approximately 35–53 mg/ml (Choi et al. 2004). Using both an immunoassay, *i.e.*, dot blot analyses with antibodies specific for albumin, and FITC-labelled bovine serum albumin, they unequivocally demonstrated that albumin is binding to

and coating the predator, not the prey, and that a subsequent treatment with proteinase K restored their ability to attack. All of these suggest mammalian serum albumin proteins bind a component present on the surface of *B. bacteriovorus* HD100 and block its ability to bind and/or recognize its prey. Although albumin also binds other bacterial strains, all previously reported strains were Gram-positive (de Chateau et al. 1996, Johansson et al. 2002; Willcox et al. 1993), making *B. bacteriovorus* HD100 the first clear example of a Gram-negative bacterium to be bound by this class of proteins.

Their study also showed the impact of albumin varied somewhat based on the prey. Whereas predation of both *E. coli* and *Salmonella enterica* were inhibited by human serum albumin, a clinical isolate of *K. pneumoniae* was still attacked slightly, but significantly (4.5-fold reduction), even when albumin was present (Im et al. 2017b). Similarly, in the study by Baker et al. (2017), long-term experiments using a different strain of *K. pneumoniae* saw a temporary, approximately 4-log reduction 32–78 h after initiating predation. The results with *K. pneumoniae* in both studies indicate that predation, though heavily impaired, may still occur with some select pathogenic strains.

4 Conclusions – A Move Towards Using Native BALOs?

Predatory microorganisms are a remarkable group of bacteria that possess a very distinctive lifestyle. The unique properties possessed by BALOs make them a potential alternative to chemical antibiotics against diverse human and animal pathogens, including multi-drug resistant strains. It should come as no surprise, therefore, that as research into their activities has progressed, there continues to be a clear move towards their use as living antibiotics within higher organisms, such as cows, rabbits, rats and zebrafish (Atterbury et al. 2011; Boileau et al. 2016; Shatzkes et al. 2017; Willis et al. 2016). However, discrepancies exist between the observed *in vitro* and *in vivo* predatory activities with most of the *in vivo* results being underwhelming, *i.e.*, only mild reductions in the pathogen viability or only a slight benefit. The only *in vivo* applications so far where BALOs have consistently been effective are within aquaculture (Cao et al. 2014, 2015; Guo et al. 2017).

As presented in this chapter, BALOs and their activities are negatively impacted by different environmental and biotic factors, many of which may contribute to the less than ideal results seen in the *in vivo* studies. As such, effort should be given (1) to identify the limitations of and hurdles to be overcome for these bacteria, as they pertain to their use within animal hosts, and (2) to seek out other predatory strains that are inherently resistant to the offending factors currently holding back breakthroughs in *in vivo* experiments.

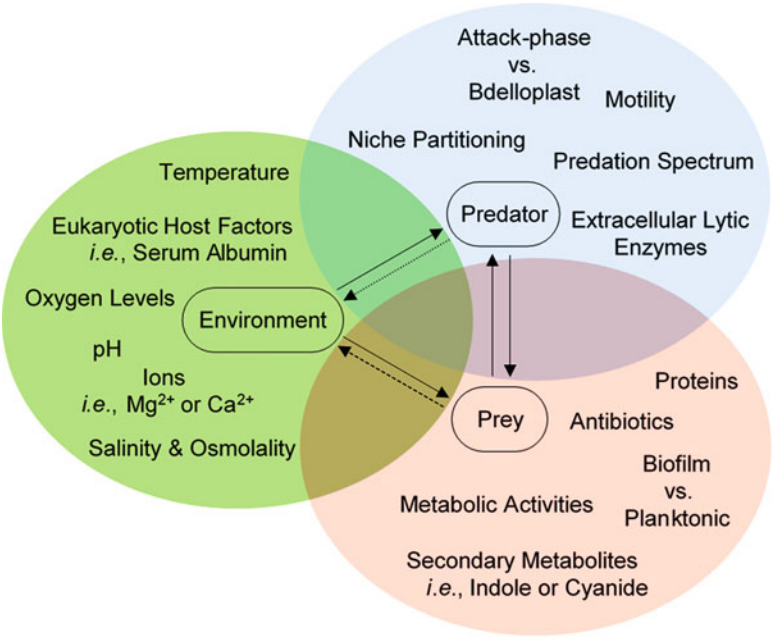

Fig. 2 Simplified relationship showing the three-way connection between predator and prey populations and their environment

Much effort has been given to the first, as shown by the many studies referenced here, but the second is an area that has not been extensively tapped, yet holds much promise. A case in point is the natural preference of *Halobacteriovorax* spp. for higher osmolalities, which may make them a better choice for use within the blood sera if one with the proper prey spectrum can be isolated. As these strains have structurally different lipid A molecules than *B. bacteriovorus* (Beck et al. 2010; Jayasimhulu et al. 2007), though, host cell responses would need to be evaluated to determine if they, like *B. bacteriovorus* HD100 and other isolates (Monnappa et al. 2016), do not elicit strong cytokine responses. In addition, as discussed briefly in the beginning of this chapter, BALOs are fairly ubiquitous throughout nature, yet most studies have limited their characterization to three main strains, *B. bacteriovorus* HD100, *B. bacteriovorus* 109 J and *M. aeruginosavorus*. With a plethora of different predators in nature, and possibly extreme environments, an untapped resource still exists and should be explored, particularly within an environmental setting that befits their application. As illustrated in Fig. 2, the predator and prey are not the only condition that governs their respective activities; the environmental setting needs to be considered as well. Consequently, finding an active predator within a certain locale dictates that the predator is likely adapted to the conditions within that location. This is exemplified in the successful aquaculture studies mentioned above – the predators were not the three powerhouse strains but, rather, were isolated from the environments in question, an aspect of those studies that helped to ensure

their success. Extending this perspective to other concerns, for example, gut dysbiosis within humans, rather than using soil organisms, *i.e.*, *B. bacteriovorus* HD100 and *B. bacteriovorus* 109 J, researchers should perhaps identify and characterize BALOs found within the guts of mammals (Schwudke et al. 2001).

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Emerging Horizons for Industrial Applications of Predatory Bacteria

C. Herencias, S. Salgado-Briegas, and M. A. Prieto

Contents

1 Introduction

Industrial microbiology and metabolic engineering are becoming key strategies for the biotechnological industries due to the increasing interest in circular economy strategies (Ortiz-Marquez et al. 2013). Environmental protection and sustainability are the central promises. However, successful microbial processes have to be economically efficient in order to compete with traditional manufacturing routes. The economic success of a microbial strategy in a bioprocess is led by three main points: the renewable carbon source, the specific process (i.e. bioconversion), and the downstream process (i.e. purification of the product) (Du et al. 2011). The

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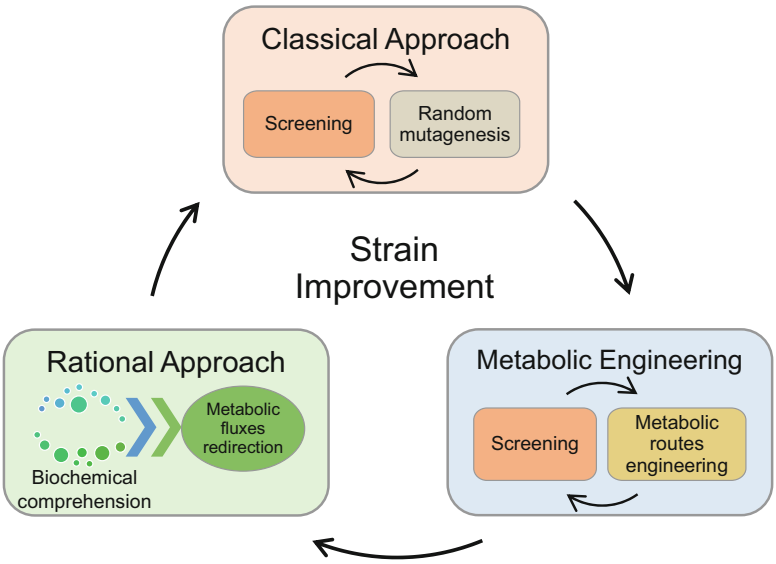

Fig. 1 Workflow for the improvement of a strain for potential use in industry as a cyclic process

"microbial factory" has to be adapted to the specific process, which can be achieved using three different approaches (Fig. 1): (i) classical strain improvement, that involves the screening of the desired phenotype, random mutagenesis, and re-screening, (ii) development of cell factories using metabolic engineering by a cyclic process of analysis and engineering of the desired strains, iii) rational strain engineering, where the strategy was initially based on the comprehension of the biochemical stoichiometry and the expected metabolic pathways (Goel et al. 2012).

In the last few decades, *Bdellovibrio* and like organisms (BALOs) have attracted the attention of the scientific community due to their particular lifestyle, their physiological and metabolic versatility to colonize different niches and their ability to diminish bacterial populations (Sockett 2009). The extraordinary repertoire of species susceptible to predation by BALOs (see below) enables a wide range of potential applications based on their predatory capabilities, such as biocontrol agents in medicine, in agriculture, aquaculture and water treatment (Fig. 2) (Atterbury et al. 2011; Lin et al. 2007; Loozen et al. 2015; Scherff 1973). Apart from their well-documented application as clinical biocontrol agents, predatory bacteria have been proposed as an excellent source of valuable biotechnological enzymes (Bratanis et al. 2017; Lambert and Sockett 2013; Martinez et al. 2012; Rendulic et al. 2004) and as a biological lytic tool for intracellular product release, due to their hydrolytic arsenal (Martinez et al. 2013, 2016). In view of their unique lifestyle, they represent a sound model for evolution studies. Penetration into other cells, as observed with periplasmic BALOs, constitutes a new adaptation that could be subject to studies focusing on the origin of the eukaryotic cells (Davidov and Jurkevitch 2009; Margulis 1996).

BALOs are the group of predatory bacteria best characterized. This group is composed by small vibrioid to rod-shaped gram-negative aerobic and mesophilic

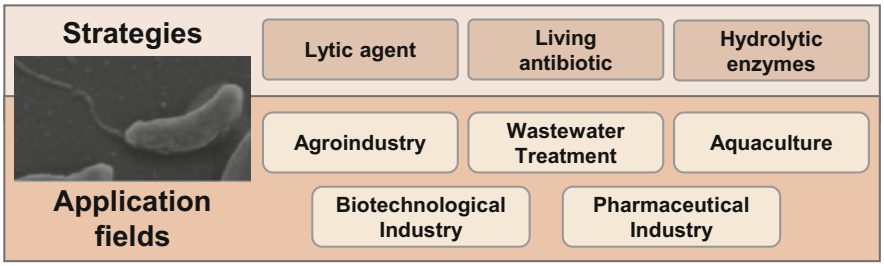

Fig. 2 Summary of the applications of *B. bacteriovorus* and the more relevant fields where the predator can be applied

bacteria (0.2–0.5 μm wide, 0.5–2.5 μm long) propelled by a single sheathed flagellum, that confers them high motility, reaching velocities of 160 μm s^{-1} (Thomashow and Rittenberg 1978). Although they were first isolated in soil, they are ubiquitous in nature and can be found in aquatic and terrestrial environments, including hypersaline systems (Piñeiro et al. 2008), biofilms (Kadouri and O'Toole 2005), mammalian guts (Hobley et al. 2012; Schwudke et al. 2001) and cystic fibrosis lung microbiota (de Dios Caballero et al. 2017).

Although predatory bacteria have been proposed as promising microorganisms to be applied in different fields, there is still poor knowledge available to control and use them efficiently. Thus, a deeper understanding of their lifestyle, genetics, and metabolism becomes necessary for BALOs to be developed as microbial cell factories.

In this chapter, we will address the state of the art of the potential use of *Bdellovibrio* strains in industrial applications. We will expose the applications that have been proposed so far, as well as discuss the drawbacks of the use of BALOs considering the cultivability, the prey range and the possible genetic manipulations to improve the predatory bacteria to be used as a biotechnological tool.

2 BALOs from an Industrial Perspective

Different applications in agriculture, food industry or aquaculture have been recently reported in which *B. bacteriovorus* is used (Fig. 2). Most of these applications are focussed on the direct application of the wild type predator cells. Until the work by Martínez et al. (2016), it had not been proposed the engineering and optimization of the predator as a biotechnological catalyst.

The first attempt to use predatory bacteria as biocontrol agents was in 1973 when Sherff described the effectiveness of *B. bacteriovorus* preying on *Pseudomonas syringae* to avoid the development of bacterial blight of soybean (Scherff 1973). In 2011 this predatory bacterium was used *in vivo*, highlighting its successful use as living antibiotic in chicken guts with *Salmonella* infection (Atterbury et al. 2011).

B. bacteriovorus was later applied to treat and prevent the spoilage in post-harvest steps for mushrooms (*Agaricus bisporus*) infected with *Pseudomonas tolaasi,* which causes blotches on their surface decreasing the quality of the product resulting in economic losses (Saxon et al. 2014).

Bdellovibrio spp. have been also found in several bacterial communities in bioreactors for wastewater treatment. During this process, the contaminants or pathogenic microorganisms potentially present in the industrial or domestic wastewater are removed. In the biological-based steps of the processes, the predator cells could be involved in the process of auto-purification of water by shaping the microbial community and favouring the proliferation of some beneficial bacteria (anaerobic in most of the cases) that remove the more persistent contaminants during the treatment (Guelin et al. 1967; Paoletti et al. 1967). Moreover, BALOs can even be employed to kill pathogenic bacteria from water avoiding the use of hazardous chemicals (Chen et al. 2014).

Apart from the use of the predators directly to decontaminate equipments or the soil from pathogenic bacteria, the interest on biological remediation of land contaminated with hazardous chemicals, such as aromatics compounds, is increasing in the last decades due to the adverse effects on human health and the environment. To this aim, several microorganisms are being used due to their naturally or synthetically ability to degrade those compounds. However, the effectiveness of the treatment is determined by the dispersion of the degrader microorganism (Banitz et al. 2012; Furuno et al. 2010). A very peculiar application of *B. bacteriovorus* based on its ability to reduce prey strains from the predatory zone has recently been described. In this study of the potential of *B. bacteriovorus* as an adjuvant for the bioremediation of phenanthrene, it was found that under certain conditions, the predator increased phenanthrene degradation by promoting prey dispersion (Otto et al. 2017).

The susceptibility of biofilms to the attack of *B. bacteriovorus* has been described (Kadouri and O'Toole 2005). The hydrolytic arsenal encoded in its genome allows the dispersion on the surface of the biofilm releasing the potential prey bacteria to the medium. Also, biofilms degradation products can be used by *B. bacteriovorus* for protein synthesis and as a source of energy generating ATP (Im et al. 2018). Although there are no examples reported in the literature yet, this capability could be important for use in different bioprocess, where the formation of these scaffolds supposes a bottleneck in the process, because, besides the contamination issue, it could affect the functionality of the equipments (Chmielewski and Frank 2015; Kumar and Anand 1998).

Aquaculture, beyond doubt, is the fastest growing food-producing sector in the world. Its important role is to provide aquatic animal protein to balance out the deficit in the wild fisheries. Likewise, its socio-economic role in providing livelihood opportunities and economic security, particularly for the less-developed regions in the world, is being recognized (Naylor et al. 2000). The threat of diseases has now become a primary constraint and risk to the growth of this sector. The importance of prevention and control of disease risks as a measure to reduce production losses in commercial and small-scale aquaculture systems has thus received increased attention. In particular, outbreaks caused by fish pathogens such as *Aeromonas*

hydrophyla or *Yersinia ruckeri* among others are considered to be a major problem to fish farming and quality, leading to severe losses on the production (Cao et al. 2012). These infections are now partially controlled by fish farmers with direct application of antibiotics such as terramycin and florfenicol. However, antibiotic treatment is cost-prohibitive to farmers in many undeveloped and developing countries, and antibiotic use may be detrimental to the environment and human health (Harikrishnan et al. 2010). The use of predatory bacteria constitutes an attractive alternative and several reports using them have been published (Cao et al. 2012; Lu and Cai 2010).

The most considered application of BALOs has been as potential antimicrobial agent against animal and human pathogens. Over last decades there has been a decrease in the discovery/development of new antibiotics alongside with an increment in resistance to current antibiotics. Therefore, the need to develop new therapies to treat bacterial infections points at predatory bacteria as a good alternative and they have been proposed as "living antibiotic". In this sense, there has been increasing research assessing predatory bacteria both *in vitro* and *in vivo* for being able to eradicate the population of a wide range of gram-negative bacteria from diverse genera, including multi-drug resistant clinical isolates (Dashiff et al. 2011a; Im et al. 2017).

Taking into account the interesting lifecycle of BALOs (for details, see Chapter "The Ecology of *Bdellovibrio* and Like Organisms in Wastewater Treatment Plants", by Jurkevitch) and the crucial role played by their hydrolytic arsenal, it is unsurprising that they are considered to constitute a rich source of hydrolytic enzymes of great interest for industry. Lipases, nucleases, glucanases or hydrolases are some of the potential candidates contained within their genomes (Rendulic et al. 2004). The use of enzymes in industry provides high and superior performances of catalytic processes and can be used on different fields: pharmaceutical and analytical industry, food and feed industry, paper and pulp industry, leather and textile industry and polymer industry among others (Singh et al. 2016). Interestingly, *B. bacteriovorus* possesses two depolymerases of polyhydroxyalkanoate (PHA) as part of its hydrolytic repertoire. These enzymes are able to specifically degrade short- or medium-chain-length PHA, respectively, in an efficient manner (Martinez et al. 2012). PHA are biodegradable polyesters composed by *R*-3-hydroxyalkanoate monomers. They are produced by a wide variety of bacteria and have similar physicochemical properties than the conventional polymers, being attractive alternatives to petroleum-based plastics (Prieto et al. 2016). Apart from its use as promising biomaterial, several biotechnological applications have been described for the PHAs involving their synthesis and degradation mechanisms. For instance, as all the 3-hydroxyalkanoates (HAs) incorporated to the pathway are pure enantiomers (*R* form), they are an important source of quiral syntons in medicine (Philip et al. 2007). Hence, the development of sustainable bioprocesses for producing these quiral intermediates are interesting in industry (Sudesh et al. 2000). One of the more commonly used methods for obtaining HAs is the *in vivo* and *in vitro* depolymerization of the PHA, which is based on PHA depolymerase enzymes (de Eugenio et al. 2007). In relation with PHA and taking into account the lytic

ability of *B. bacteriovorus*, this predator has been used as a biological lytic tool for extracting PHA as a value-added intracellular bio-product. This would entail employing a PHA-producing bacterium, such as *Pseudomonas putida*, as prey (Martinez et al. 2016). This application is explained in detail in the next sections.

3 *B. bacteriovorus* as an Industrial Lytic System

B. bacteriovorus is the model microorganism among BALOs. It exhibits a biphasic growth cycle, including a free-swimming attack phase (AP) in which *B. bacteriovorus* search for its prey, and an intraperiplasmic growth phase (GP) inside the prey's periplasm, forming the so-called bdelloplast structure, where it will digest the prey cellular components to synthesize its own. It is worthwhile to note that, within its large genome (~3.8 Mb), this predator contains a wide-ranging hydrolytic arsenal (150 genes coding for proteases, 10 glycanases, 20 DNases, 9 RNases and 15 lipases) which is crucial during the penetration to the prey cell and also for the lysis of the ghost prey cells, when the progeny is released (Rendulic et al. 2004). From an industrial perspective, *B. bacteriovorus* is attractive not only for its predation ability but also for its enormous hydrolytic arsenal.

To implement *B. bacteriovorus* as a biotechnological cell catalyst it should be possible to be controlled rationally (Fig. 3a). This requires a deep knowledge of its physiology and metabolism that allows the construction of metabolic models. Specifically, for predatory bacteria, the understanding of the growth cycle is crucial as well as the prey range in which the predator is efficient. All these along with a set of genetic tools would allow for predator domestication. However, the particular requirements of *B. bacteriovorus*, such as the prey and high concentrations of oxygen (please see Chapter "Environmental and Biotic Factors Impacting the Activities of *Bdellovibrio bacteriovorus*", by Im et al. for more information), will be crucial for the bioprocess design. An optimal inoculum of predator needs to be determined according to the prey concentration reached during the fermentation as well as the moment in which predation will be maximal. Taking everything into account specific parameters for scaling-up processes needs to be calculated. The requirements for *B. bacteriovorus* to be used as a cell catalyst will be explained in detail in this section.

3.1 Domestication of B. bacteriovorus

One of the principal requirements of *B. bacteriovorus* to be used as a biotechnological tool is for it to be domesticated, i.e. to have a repertoire of genetic tools that allows its manipulation at a genomic level. Most genetic tools that have been developed to date are addressed for model organisms, which divide by binary fission or gemmation. In contrast, *B. bacteriovorus* elongates to form an intracellular

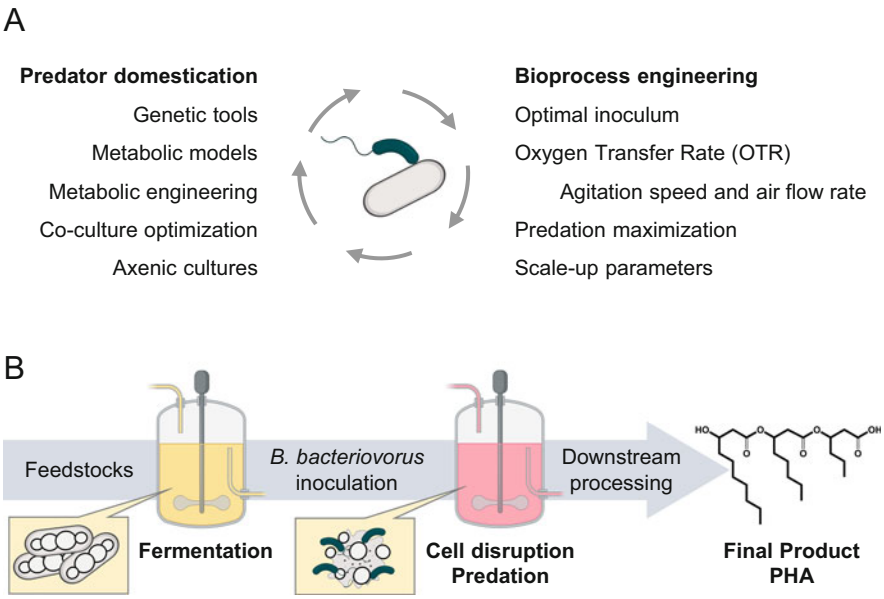

Fig. 3 *B. bacteriovorus* in industrial bioprocesses as a biological catalyst. (**a**) Requirements for *B. bacteriovorus* to be used as a biotechnological tool. In an industrial bioprocess the microorganism employed needs to be domesticated. That means to have a rational control over it with a battery of genetic tools. In the case of *B. bacteriovorus*, the bioprocess needs to be adapted to the prey range, i.e. it has to be susceptible to be preyed by *B. bacteriovorus*. The last step in the design of a bioprocess involving *B. bacteriovorus* is the scale-up: culture parameters, such as agitation rate, flow gas rate or inoculum size, must be calculated to achieve the highest yields. (**b**) Schematic representation of a bioprocess. In this integrated bioprocess, *P. putida* KT2440 produces PHA granules intracellularly from a pool of feedstock. *P. putida* cells are subjected to a biological disruption using *B. bacteriovorus*, which will facilitate downstream processing to recover the final product (purified PHA). *Figure partially made with biorender* (https://www.biorender.com)

filament inside the bdelloplast and septates into daughter cells afterwards, promoting an unequal partition of plasmids and making it difficult to develop fully controlled expression systems.

B. bacteriovorus was genetically modified in 1992 for the first time. *B. bacteriovorus* 109J and its host-independent (HI) derivative *B. bacteriovorus* BB5, which is able to grow in a rich medium in the absence of prey, were transformed to elucidate the mechanism which drives the axenic growth of HI strains. In that report, two plasmid incompatibility groups were tested, IncQ and IncP, to confer antibiotic resistance to *B. bacteriovorus.* Constructed plasmids were transferred by conjugation to *B. bacteriovorus* strains from *E. coli* SM10 derivatives, which has RK2 transfer functions integrated into its genome (Simon et al. 1983). The RSF1010 (IncQ) derivative plasmids (pSUP204, pSUP304.1 and pMMB33) yielded antibiotic resistance to *B. bacteriovorus* whereas the RK2 (IncP) derivative plasmids (pRK290, pVK100 and pTC3) did not. Nevertheless, when the latter included a

B. bacteriovorus chromosomal region, they conferred antibiotic resistance. Therefore, they concluded that it was possible to perform conjugal transformation of *B. bacteriovorus* employing RK2 machinery resulting in either autonomous replication with RSF1010 derivative plasmids or chromosomal homologous recombination if the plasmid replicon is an RK2 derivative (Cotter and Thomashow 1992a). In later experiments, Cotter and Thomashow, demonstrated that the cosmid pVK100 including chromosomal sequences of *B. bacteriovorus* led to merodiploid formation via homologous recombination. They used pVK100 derivative cosmids to identify the *hit* locus and to restore plaque-forming ability of HI *Bdellovibrio* isolates (Cotter and Thomashow 1992b). Overall, the works of Cotter and Thomashow described for the first time the possibility to genetically modify *B. bacteriovorus* as well as described some of the genetic features of the HI phenotype.

The capability of *B. bacteriovorus* to incorporate exogenous DNA to its chromosome via homologous recombination was exploited to carry out directed mutagenesis experiments. In 2003, a methyl-accepting chemotaxis protein (MCP), *mcp2*, and a homologous gene (*mviN*) were disrupted with a kanamycin cassette. Suicide plasmids derived from the pSET151 plasmid (IncP) with disrupted versions of those genes were transferred by conjugation to *B. bacteriovorus* 109J, resulting in merodiploid strains (Lambert et al. 2003). Following this strategy, several genes of *B. bacteriovorus* HD100 have been disrupted to better understand predation mechanism: flagellar genes (Lambert et al. 2006), type IV pili (Evans et al. 2007), cytoskeletal elements (Fenton et al. 2010a), shape related proteins (Fenton et al. 2010b), flagellar genes (Morehouse et al. 2011), transporters (Chang et al. 2011) and sigma factors genes (Lambert et al. 2012). To identify more predation related genes, random mutagenesis using a Tn5 transposon was exploited (Medina et al. 2008; Roschanski et al. 2011; Tudor et al. 2008).

The next step forward in the genetic modification of *B. bacteriovorus* was the development of a system to generate markerless mutants. This system included an stringent suicide vector (pSSK10) with an R6K origin of replication, that only replicates in *pir*$^{+}$ strains (Rakowski and Filutowicz 2013). To counterselect recombinant strains, the pSSK10 vector included the *sacB* gene, a toxic gene when 5% sucrose is present in the culture media. Employing this system, they eliminated the gene that confers streptomycin resistance, *strB*, from *B. bacteriovorus* HD100. Mutant strains were complemented with the expression of this gene in a pMMB206 derivative plasmid, demonstrating that this plasmid can be autonomously replicative in *B. bacteriovorus* HD100 (Steyert and Pineiro 2007). They used the same technique to delete a dGTPase from *B. bacteriovorus* HD100 (Steyert et al. 2008).

The widely used pK18mob*sacB* vector (Schafer et al. 1994), with the same counter-selection gene as pSSK10, was used for the first time in *B. bacteriovorus* HD100 to fluorescently tag proteins fusing the gene of interest to a green fluorescent protein (GFP) and conjugating the plasmid to obtain recombinant strains (Fenton et al. 2010b). This vector can be also used to generate markerless deletion mutants.

As it is shown in Table 1, all replicative plasmids that have been used in *B. bacteriovorus* are RSF1010 derivatives. Although these plasmids were employed to complement mutant strains in general, few experiments to express heterologous

Table 1 Plasmids used in *B. bacteriovorus* strains

Name	Replicon	Selection marker	Replicative or integrative	References
pSUP204	RSF1010 (IncQ)	Ap^R, Cm^R, Tc^R	Replicative	Cotter and Thomashow (1992a)
pSUP304.1	RSF1010 (IncQ)	Ap^R, Km^R	Replicative	Cotter and Thomashow (1992a)
pMMB33	RSF1010 (IncQ)	Km^R	Replicative	Cotter and Thomashow (1992a)
pRK290	RK2 (IncP)	Tc^R	Integrative	Cotter and Thomashow (1992a)
pVK100	RK2 (IncP)	Tc^R, Km^R	Integrative	Cotter and Thomashow (1992a)
pSET151	pUC	Km^R Th^R Ap^R *xylE* *lacZα*	Integrative	Lambert et al. (2003, 2006, 2012), Evans et al. (2007), Fenton et al. (2010) and Morehouse et al. (2011)
pUI800	pMB1	Tc^R, Cm^R, Km^R	Integrative[a]	Lambert et al. (2003)
pMMB206	RSF1010 (IncQ)	Cm^R *lacZα*	Replicative	Flannagan et al. (2004) and Steyert and Pineiro (2007)
pSSK10	R6K	Km^R Cm^R *sacB*	Integrative	Steyert and Pineiro, (2007), Dori-Bachash et al. (2009), Chanyi and Koval (2014), Rotem et al. (2015) and Avidan et al. (2017)
pBT20	R6K	Ap^R	Integrative[a]	Medina et al. (2008)
pRL27	R6K	Km^R	Integrative[a]	Tudor et al. (2008)
pSUP202	pMB1	Ap^R Tc^R Cm^R	Integrative	Roschanski and Strauch (2010)
pSUP404.2	RSF1010 (IncQ) and p15A	Cm^R Km^R	Replicative	Roschanski and Strauch (2010)
pMiniCm	R6K	Cm^R	Integrative[a]	Roschanski et al. (2011)
pK18mob*sacB*	pMB1	Km^R *sacB*	Integrative	Schäfer et al. (1994), Chang et al. (2011), Roschanski et al. (2011), Hobley et al. (2012), Lambert and Sockett (2013), Milner et al. (2014) and Martínez et al. (2016)
pMQ414	RSF1010 (IncQ) and p15A	Gm^R URA3	Replicative	Mukherjee et al. (2016)
pK18mob	pMB1	Km^R	Integrative	Martínez et al. (2016)

[a]Random chromosomal integration via Tn5 transposon
Ap ampicillin, *Cm* Chloramphenicol, *Gm* Gentamycin, *Km* Kanamycin, *Nm* Neomycin, *Tc* Tetracycline, *Th* Thiostrepton

proteins have been also carried out. Plasmids carrying green or red fluorescent proteins were also transferred by conjugation into *B. bacteriovorus* resulting in fluorescent strains (Flannagan et al. 2004; Mukherjee et al. 2016; Roschanski and Strauch 2011). These experiments demonstrated the viability to use *Bdellovibrio* strains as a cell catalyst suitable for producing heterologous proteins. However, there is still a remarkable lack of genetic tools to domesticate *B. bacteriovorus*. For instance, there is not any inducible nor repressible promoter reported so far. To overcome this problem, a recent work has been lately published where synthetic theophylline–responsive riboswitches are employed to control GFP expression (Dwidar and Yokobayashi 2017). This system was used also to control predation by regulating the flagellar sigma factor FliA which may control up to 66% of attack phase genes. In terms of biotechnological tools, it would be interesting to develop suitable genetic tools allowing not only multiple genes deletions or under-expression, but also the expression of heterologous genes in order to recreate metabolic routes or to produce heterologous proteins.

3.2 Prey Range

Predatory bacteria attack and digest other bacteria and may therefore play a role in shaping microbial populations. This ability might be very useful and challenging in biotechnological processes driven by microbial communities. The prey range will determine the efficiency or feasibility to use predators in specific processes, such as the recovery of interesting intracellular bioproducts. To develop predatory bacteria as a biotechnological tool, it is important to characterize the variation in predation characteristics, such as prey range, and to examine the evolution of predatory bacteria lineages at different scales.

The manner in which BALOs shape microbial communities depends in part on which bacterial species are susceptible to predation and how efficient it is. Traditionally, the most common prey used to isolate and characterize BALOs were almost exclusively from the phylum Proteobacteria: *Escherichia coli*, *Pseudomonas* spp. and *Erwinia* spp. for terrestrial habitats and *Vibrio parahaemolyticus* for marine ecosystems (Jurkevitch and Davidov 2006).

Despite the wide range of susceptible prey for BALOs, predatory efficiency is strain-dependent. Indeed, *Bdellovibrio* spp. has been reported to be able to distinguish between different prey species in heterogenic co-cultures (Rogosky et al. 2006). Moreover, several reported cases describe *B. bacteriovorus* as unable to prey upon specific gram-negative bacteria. One example involves the presence of an extracellular proteinaceous layer (S-layer) that can block attachment between predator cells and the lipopolysaccharide (LPS) layer in *Caulobacter* sp. (Koval and Hynes 1991). Another example refers to predation by *B. bacteriovorus* on α-proteobacteria, such as *Rhodobacter*, which possess a lipopolysaccharide in its envelope that differs significantly from that of other gram-negative bacteria (Strittmatter et al. 1983), and predation on these strains is therefore generally slower.

Table 2 Industrially relevant prey bacterial strains

Bacterial prey strain	Industrial product	References
P. fluorescens	Vanillin	Di Gioia et al. (2011)
E. coli	Amino acids, organic acids, hydrogen and alkanes, fatty acids, sugar alcohols, isoprenoids, polymers, Coumarin, valinomycin, proteins	Theisen and Liao (2016)
P. putida	Polymers, lipopolysaccharides, glycogen, cell wall constituents, lipids, amino acids, nucleotides, tetrapyrrols	Nikel et al. (2016) and Nikel and de Lorenzo (2018)
P. aeruginosa	Biosurfactants, rhamnolipids, antioxidants	Maier and Soberón-Chávez (2000), Allouche et al. (2004), Sinumvayo (2015) and Bagheri Lotfabad et al. (2017)
Alcaligenes faecalis	Acidic polysaccharide, succinoglucan	Harada et al. (1965)
Fusobacterium nucleatum	Tannase (tannin acylhydrolase)	Tomás-Cortázar et al. (2018)
Serratia marcescens	Pigments (prodigiosin), β-carotene	Abdelhafez et al. (2016) and Elkenawy et al. (2017)
Klebsiella pneumoniae	2-Butanone	Chen et al. (2015)
Acinetobacter spp.	Polymers (emulsan)	Gutnick et al. (1991)
Enterobacter aerogenes	2,3-Butanediol	Perego et al. (2000)
Morganella moganii	Histamine	Kim et al. (2002)

Table 2 compiles the list of susceptible preys of the BALOs commonly studied and relevant in industry due to the production of some high-value products. It is important to highlight the value that *E. coli* and *P. putida* entail for the biotechnology industry, since they are involved in a multitude of bioprocesses. Hence *B. bacteriovorus* emerge as an important downstream tool for intracellular bioproducts such as the above-mentioned biopolymer PHA (Martinez et al. 2016) or as lytic agent of gram negative cell catalysts whenever required for the bioprocess.

3.3 Cultivation: The Major Drawback

Designing microbes as successful biotechnological catalysts requires some considerations, such as the complexity of the particular industrial process, the nature or toxicity of the products or by-products in the process, and the physiological and

metabolic requirements of the selected bacteria. Then, during the selection and evaluation of a cell catalyst for a specific process, the potential bottlenecks must be identified. In the case of predatory bacteria, which have never been applied in industrial processes, several obstacles derived from their own physiology emerge, for example the co-culture requirement and predation inhibition.

Routinely, *Bdellovibrio* strains are propagated by growing them in a co-culture on gram-negative prey cells such as *E. coli* or *Pseudomonas* strains by the double-layer technique or in liquid co-cultures (Herencias et al. 2017; Lambert and Sockett 2008). This particularity makes the bioprocess especially challenging. Remarkably, it is well reported that part of the population cells of *Bdellovibrio* culture mutates to being able to grow axenically in the absence of prey in rich medium. These cells are the so-called host-independent (HI) derivatives (Seidler and Starr 1969). Since the isolation of *B. bacteriovorus* in 1962, it has been noted that it can also form saprophytic colonies on hard agar plates in the presence of heat-treated prey bacteria. The successful isolation of HI variants requires a much higher number of predatory cells compared to that needed for plating on prey lawns (Stolp and Starr 1963). This is due to the low frequency of development of these saprophytic predators (one in 10^6–10^7 cells) in rich medium (Dwidar et al. 2017). This rate is similar to the mutational rate of bacteria (Schaaper 1993). It was not until the 1990s that the HI phenotype was attributed to mutations in the predator's genome. The region containing these mutations is called the "*hit*" locus (host-interaction locus) and no metabolic function is assigned so far. This region has heretofore been associated to the Type IVa pili (Capeness et al. 2013). In addition, some HI isolates lack mutations at the *hit* locus, and other genes may therefore be involved in the switching pathway from host dependent to HI phenotype (Capeness et al. 2013; Wurtzel et al. 2010). The genomic alteration of the *hit* locus was analyzed by means of next-generation sequencing (NGS) and the gene *bd0108* was identified as being related to the HI phenotype. This gene encodes a 101 amino acid protein and has no homologs outside the *Bdellovibrionaceae* family. The gene *bd0108*, those in the surroundings (*bd0109-bd0113*, *bd0118*, *bd0119*) and other ones associated with the HI phenotype (*bd3461*, *bd3464* or *bd3852*) are related to the formation of the Type IV pili, which is involved in the prey invasion process (Chanyi and Koval 2014). Mutant strains in some of these genes are unable to recognize and to attach to the prey cell in liquid co-cultures. In the context of industrial bioprocesses, the rational development of axenic predator cultures for generating predator cells suitable of preying under controlled conditions remains as a challenge. Meanwhile, to produce *B. bacteriovorus* at a large scale, or to use it as a lytic tool, it is necessary to establish a liquid predator-prey co-culture. The axenic growth of *Bdellovibrio* HI strains would be applicable as well in processes focused to purifying hydrolytic enzymes with industrial interest from the *Bdellovibrio's* arsenal.

Bdellovibrio strains, high oxygen-demanding microorganisms, are unable to grow under anoxic conditions but capable of surviving for a limited period of time (Schoeffield et al. 1996). Under microaerobic conditions, the predator cells are able to prey, albeit more slowly than in the optimal oxygen conditions (Kadouri and Tran 2013). Hence, oxygen concentration is a crucial variable that needs to be considered

in industrial bioprocesses involving *B. bacteriovorus*. Fermenter agitation, gas flow rate, and oxygen uptake are parameters to be controlled for ensuring an adequate oxygen concentration during the predation events in the bioreactor (Garcia-Ochoa and Gomez 2009). This is particularly relevant in high cell density cultivations.

Finally, predation and survival of *B. bacteriovorus* could be affected by the presence of certain compounds. Although, this is discussed more thoroughly in the Chapter "Environmental and Biotic Factors Impacting the Activities of *Bdellovibrio bacteriovorus*" by Im et al., it is possible to take advantage of them to control predation adapting it to the requirements of an industrial bioprocess. Several compounds have been reported to enhance or inhibit predation. For example, carbohydrates play an important role in predation inhibition provoking a medium acidification (pH ~4.0) due to the release of by-products (Dashiff et al. 2011b). This pH predation dependence might be exploited to precisely control the predation along the process. On the opposite side, certain ions enhance predation such as copper sulphate, a widely used algicidal in aquaculture. In concentrations ranging from 0.1 to 1.0 mg $\cdot$ L^{-1} it stimulates *Bdellovibrio* sp. strain BDF-H16 predation as calcium chloride or magnesium sulphate do (Huang and Starr 1973), suggesting that copper ions may act synergistically with other cations improving the bacteriolytic activity of the predator (Cao et al. 2018).

In conclusion, *B. bacteriovorus* has a tremendous potential as a biotechnological tool, but there are many issues that need to be addressed before it can be considered as a scalable industrial microorganism.

4 The Case of Polyhydroxyalkanoates

Given its ability to lyse other bacteria, *B. bacteriovorus* has been proposed as a novel downstream living lytic agent for the production of valuable intracellular bio-products (Figs. 3b and 4). One of the most challenging downstream processes is the isolation of bacterial polyesters or polyhydroxyalkanoates (PHAs) at industrial scale. The PHA is accumulated as intracellular granules in the bacterial cytoplasm and can reach up to 90% of cell dry weight.

Depending on the length of the lateral chain, these polymers have different mechanical and physicochemical properties. Several short-chain-length-PHAs (scl-PHA) such as poly-3-hydroxybutyrate (PHB), are currently produced at large scale by several companies (Chanprateep 2010) and have extensive applications in packaging, moulding, fibre production and other commodities. Medium-chain-length-PHAs (mcl-PHA, with carbon numbers ranging from 6 to 14) are also promising candidates as bioplastics given their longer-side-chain-derived properties of reduced crystallinity, elasticity, hydrophobicity, low oxygen permeability and biodegradability. Moreover, mcl-PHA are being used as resorbable materials for medical applications, and as food coatings, pressure-sensitive adhesives, paint binders and biodegradable rubbers (Sudesh et al. 2011). However, their condition

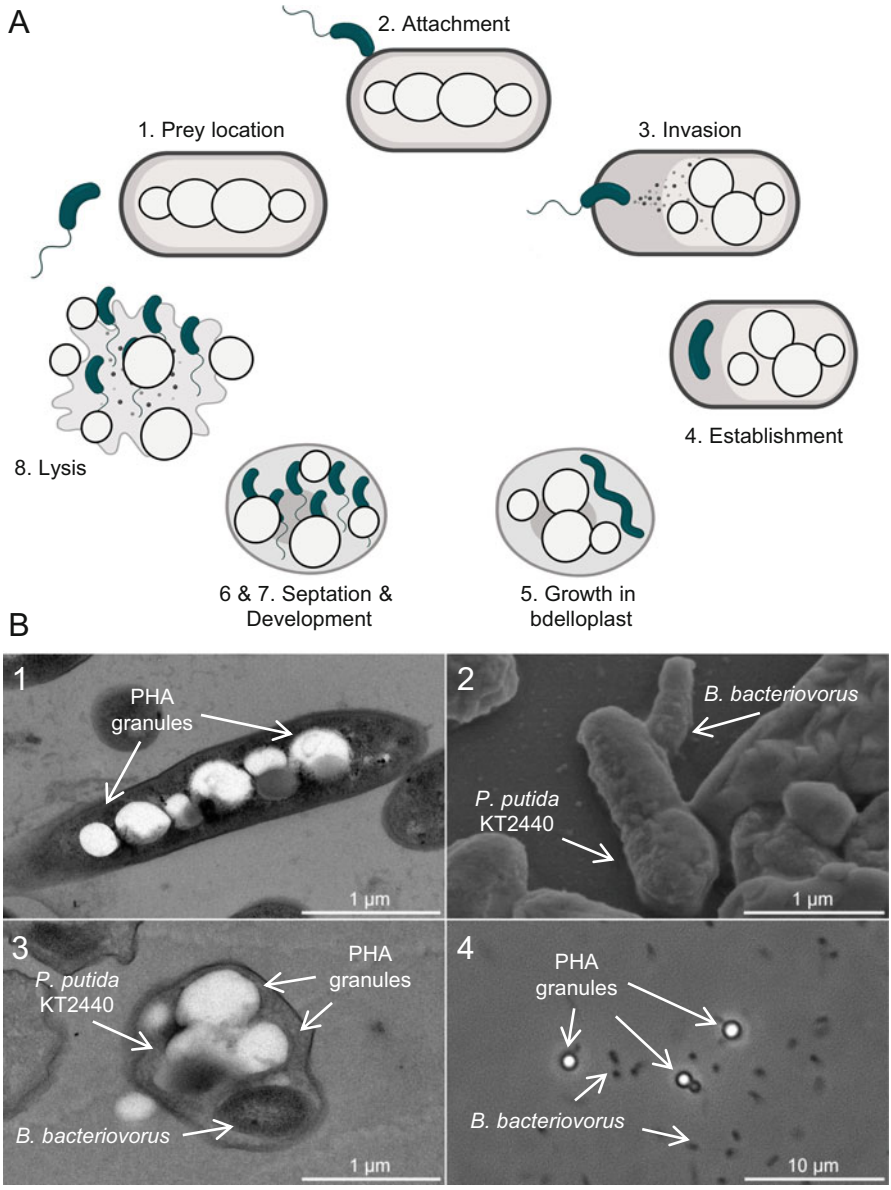

Fig. 4 (**a**) The predatory cycle of *B. bacteriovorus* preying on PHA accumulating *P. putida* KT2440. (1) Attack phase: *Bdellovibrio* cells move towards prey-rich regions. (2) Attachment: predator anchors to the host cell, which leads the infection. 3) Penetration: it enters the periplasm of the prey cell. (4 and 5) Growth in bdelloplast: the prey turns rounded due to cell wall modification and the predator grows in the periplasm and replicates its DNA. (6 and 7) Septation and development: *B. bacteriovorus* uses the prey as a source of nutrients. When resources become limited the predator septates and matures into individual attack phase cells. (8) Lysis: mature attack-phase cells lyse the cell-wall of the bdelloplast, beginning the search of fresh prey. PHA granules are therefore released to medium. The complete cycle takes about 4 h. *Figure partially made with biorender* (https://www.biorender.com) (**b**) Microphotographies show the different steps of *P. putida*

as intracellular bio-products makes their recovery difficult and costly (Jacquel et al. 2008; Madkour et al. 2013).

In the last years, a great effort has been made for isolating these biopolymers, which is one of the key step for process profitability in the fermentation system (Fig. 3b) (Madkour et al. 2013). Different methods such as mechanical cell disruption, separation processes (filtration, froth flotation, continuous centrifugation), enzymatic digestion or use of detergents and solvents have been investigated (Jacquel et al. 2008). However, the high costs of the traditional downstream processing or the reduced quality of the recovered polymer suppose a handicap for high-scale biopolymers production. It has been shown that *B. bacteriovorus* can prey upon PHA-producers such as *P. putida* KT2440 while the latter accumulates large amounts of mcl-PHA within its cells (Martinez et al. 2013). After lysing the prey, the predator hydrolyzes and consumes part of the PHA released into the extracellular environment; indeed, significant quantities of PHA granules and free hydroxyalkanoic acid (HAs) oligomers (54% and 25%, respectively, of PHA accumulated by the prey bacteria) can be recovered. This is due to the activity of an extracellular-like mcl-PHA depolymerase ($PhaZ_{Bd}$, encoded by the gene *bd3709*), which forms part of the hydrolytic arsenal of *B. bacteriovorus* (Martinez et al. 2012, 2013; Rendulic et al. 2004). In order to optimize polymer recovery, *B. bacteriovorus* was engineered to avoid the degradation of prey-produced PHA by mutating *bd2637* and *bd3709* genes (which encoded for two different PHA depolymerases). The use of these mutant strains in the PHA depolymerase enzymes led to the recovery of larger amounts of the polymer (more than 80% of the PHA accumulated in the prey cells). Moreover, the use of these predator mutant strains provided a high-quality polymer, due to the lack of hydrolyzation by the PHA depolymerases. Besides, it was shown that *B. bacteriovorus* has the ability to attack high cell density prey cultures, allowing the release of the polymer (Martinez et al. 2016). Thus, although the system needs to be tested at larger scales in an industrially relevant environment, the results suggest that the industrial-scale upgrade is possible. To further demonstrate the feasibility of the system, engineered *B. bacteriovorus* strains were tested against different gram-negative bacteria that accumulate PHA (including scl-PHA).

Regarding the metabolism of the predator and the impact that the PHA has into its physiology, mcl-PHA degradation provided ecological advantages in terms of motility and predation efficiency, associated to an increment of the ATP intracellular levels. In contrast, preying on scl-PHA rewards the predator fitness in terms of the

Fig. 4 (continued) KT2440/*B. bacteriovorus* predation event when the prey is producing PHA. (1) Transmission electronic microphotography of *P. putida* KT2440 cell of containing PHA granules inside the cytoplasm. (2) Scanning electronic microphotography of the attachment of the predator to the surface membrane of the prey cell. (3) Transmission electronic microphotography of the bdelloplast structure containing the predator and the PHA granules inside. (4) Phase-contrast images of PHA granules released by *B. bacteriovorus* after 24 h of predation upon *P. putida* KT2440

number of progenies. Overall, the results obtained in that report provide a proof-of-principle that this system could be used for intracellular bio-products recovery.

Taking into account the successful development of the lytic system by using predatory bacteria, other compounds with industrial interest could be considered for extraction: polyphosphates, hormones or pigments (Table 2).

5 Future Perspectives

With the renewed excitement and the successive promising findings opening for BALOs application, the possibility to use predators designed "à la carte" to treat bacterial infections and to exploit their possibilities seems endless. However, the future use of BALOs needs a deeper understanding of the predatory lifestyle and metabolism in order to control them rationally and to develop predators as cell factories. For that, some points should be addressed: (i) control the growth conditions taking into account that the group of BALOs have a biphasic growth cycle, (ii) control the predatory ability in terms of killing efficiency, (iii) control the metabolic state and be able to switch between the different growth phases by identifying the responsible factor/s and (iv) predator storage in suitable formulations preserving their viability over the time.

There is a need to develop genetics tools that allow the use of predatory bacteria as a lytic tool. To this aim, computational modelling and simulation are becoming crucial strategies for metabolic engineering of microorganisms. Computational models are focused on characterizing and engineering the cell at the systems level. Genome-scale metabolic modelling aims to predict gene targets to be engineered taking into account the different components of the biological system and their connections at the same time.

Currently, the availability of high-throughput experimental tools and quantitative analytical techniques allows for the design of more robust metabolic engineering strategies aimed at providing a better understanding of the behaviour of predatory bacteria. Furthermore, integration of the information and omics data at a system level constitutes a useful platform in order for BALOs to be developed as a biotechnological chassis for different purposes.

The abundance and the ubiquitous presence of BALOs in the environment highlights their potential use for control pathogenic bacteria in human, animal, plants and food as well as to be use as co-adjuvant in different processes such as wastewater treatment.

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