Emerging Horizons for Industrial Applications of Predatory Bacteria

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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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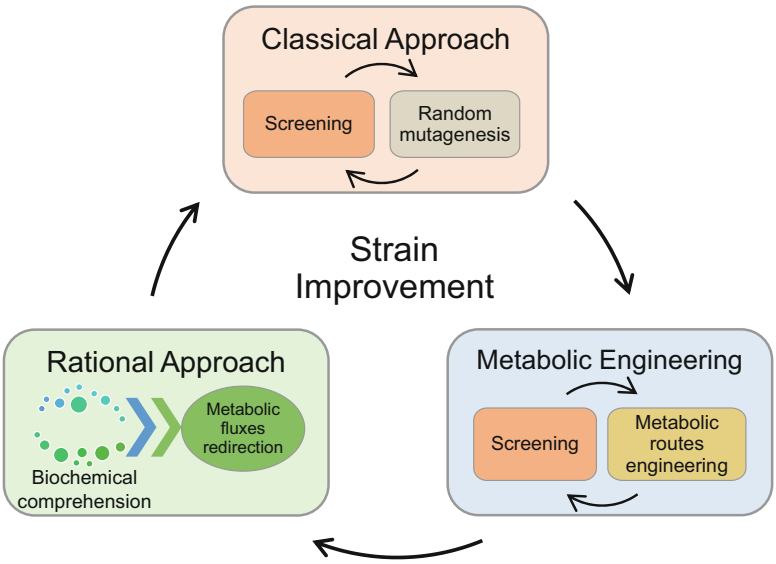

Fig. 1 Workflow for the improvement of a strain for potential use in industry as a cyclic process

"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

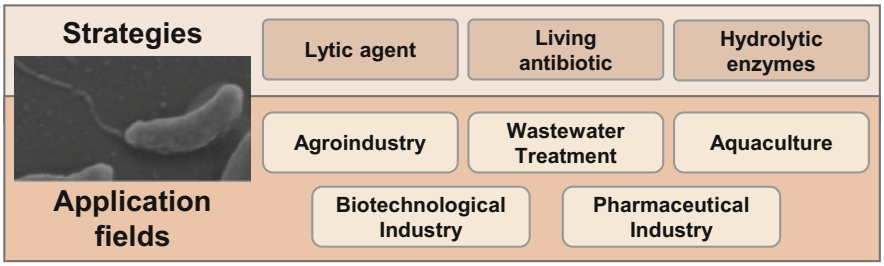

Fig. 2 Summary of the applications of *B. bacteriovorus* and the more relevant fields where the predator can be applied

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

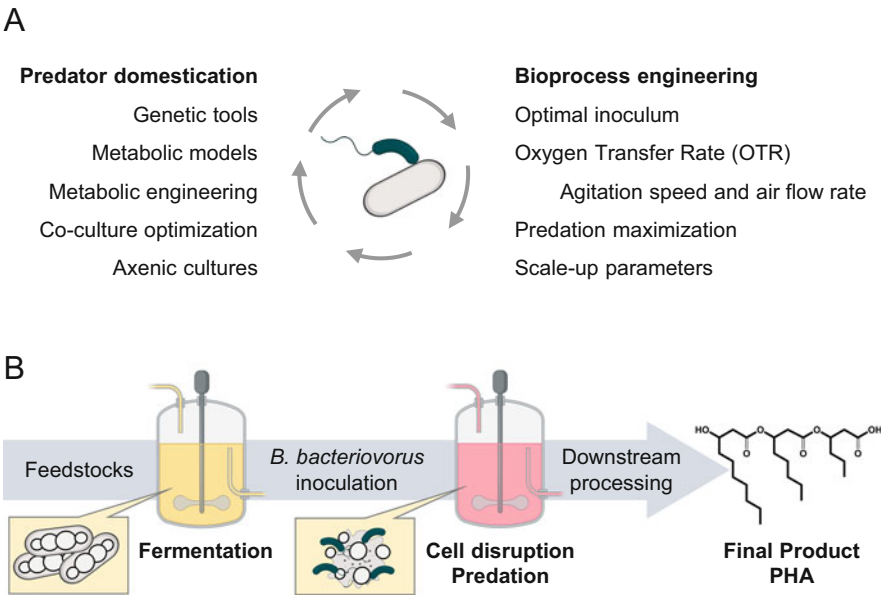

Fig. 3 *B. bacteriovorus* in industrial bioprocesses as a biological catalyst. (**a**) Requirements for *B. bacteriovorus* to be used as a biotechnological tool. In an industrial bioprocess the microorganism employed needs to be domesticated. That means to have a rational control over it with a battery 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

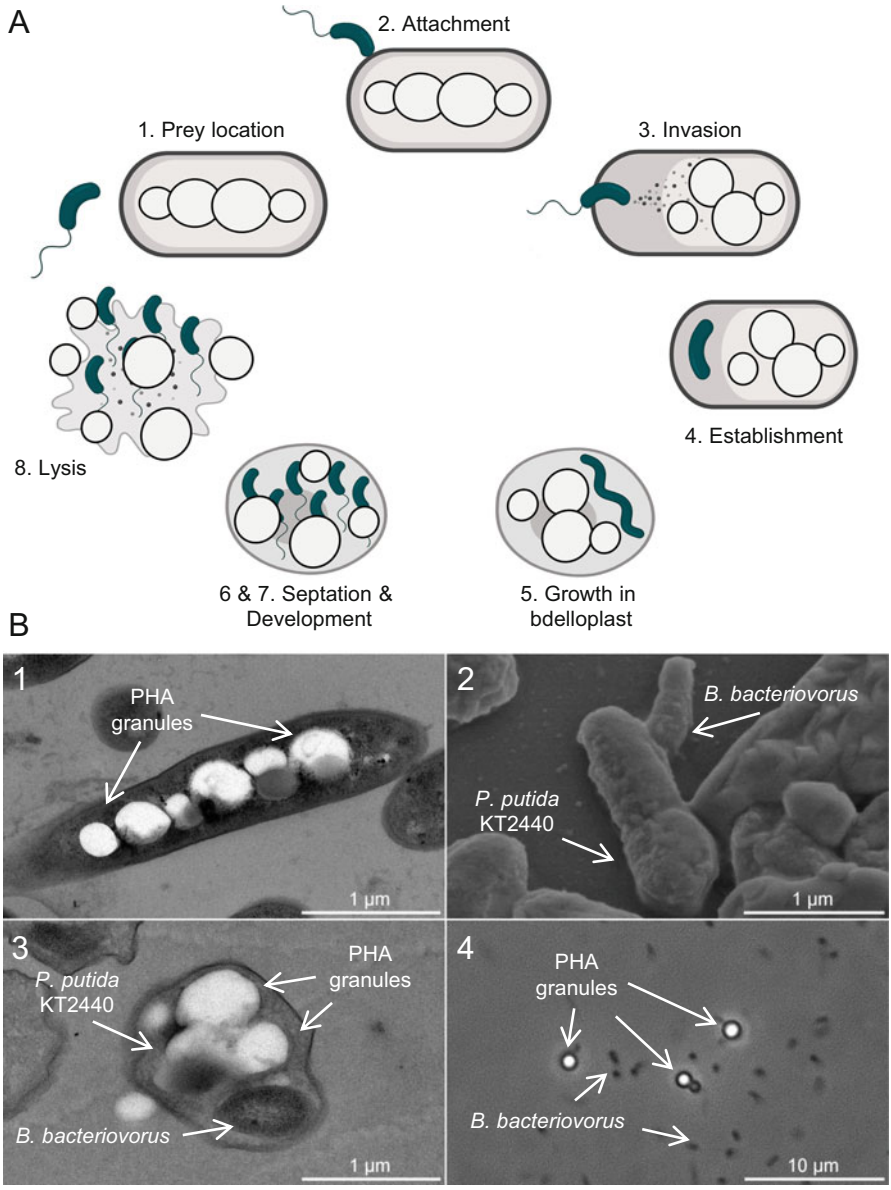

Fig. 4 (**a**) The predatory cycle of *B. bacteriovorus* preying on PHA accumulating *P. putida* KT2440. (1) Attack phase: *Bdellovibrio* cells move towards prey-rich regions. (2) Attachment: predator anchors to the host cell, which leads the infection. 3) Penetration: it enters the periplasm of the 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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