Reward for *Bdellovibrio bacteriovorus* for preying on a polyhydroxyalkanoate producer

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Summary

*Bdellovibrio bacteriovorus* HD100 is an obligate predator that invades and grows within the periplasm of Gram-negative bacteria, including mcl-polyhydroxyalkanoate (PHA) producers such as *Pseudomonas putida*. We investigated the impact of prey PHA content on the predator fitness and the potential advantages for preying on a PHA producer. Using a new procedure to control *P. putida* KT2442 cell size we demonstrated that the number of *Bdellovibrio* progeny depends on the prey biomass and not on the viable prey cell number or PHA content. The presence of mcl-PHA hydrolysed products in the culture supernatant after predation on *P. putida* KT42Z, a PHA producing strain lacking PhaZ depolymerase, confirmed the ability of *Bdellovibrio* to degrade the prey’s PHA. Predator motility was higher when growing on PHA accumulating prey. External addition of PHA polymer (latex suspension) to *Bdellovibrio* preying on the PHA minus mutant *P. putida* KT42C1 restored predator movement, suggesting that PHA is a key prey component to sustain predator swimming speed. High velocities observed in *Bdellovibrio* preying on the PHA producing strain were correlated to high intracellular ATP levels of the predator. These effects brought *Bdellovibrio* fitness benefits as predation on PHA producers was more efficient than predation on non-producing bacteria.

Introduction

*Bdellovibrio bacteriovorus* HD100 is a small, predatory, soil deltaproteobacterium that preys upon a wide range of other Gram-negative bacteria, including human pathogens (Jurkevitch and Davidov, 2007; Sockett, 2009). The life cycle of *B. bacteriovorus* is unique in being biphasic, alternating a free non-replicative attack phase and a prey-dependent intraperiplasmic growth phase (Stolp and Starr, 1963). During the growth phase, which takes place inside the periplasm of the prey cell, *Bdellovibrio* replicates its DNA and grows using the prey as a source of nutrients, forming a bdelloplast (a predator-containing prey cell rounded by cell-wall modifications). When prey resources are exhausted, *Bdellovibrio* septates into individual attack phase cells and escapes the prey ghost to invade fresh prey (Lambert et al., 2006a; Sockett, 2009). *Bdellovibrio* bacteria are famously fast, swimming at high speeds (an average of 60 μm s⁻¹) (Lambert et al., 2012) by rotating a single polar flagellum, which is a crucial feature for attack phase *Bdellovibrio* (Lambert et al., 2006b).

*Bdellovibrio bacteriovorus* HD100’s genome revealed a large set of proteases and other hydrolases, which are used throughout the predatory life cycle for prey entry, degradation of prey components and exit from the bdelloplast (Rendulic et al., 2004). Very recently, we have demonstrated the existence of an extracellular-like polyhydroxyalkanoate (PHA) depolymerase in *B. bacteriovorus* HD100 (Martínez et al., 2012) as part of the hydrolytic arsenal. PHAs are accumulated as inclusions in the cytoplasm of various bacteria in response to inorganic nutrient limitations, acting as sinks for carbon and reducing equivalents. According to the length of the side-chain of the polymer, these storage bacterial polyesters can be classified into two major classes, both formed by enantiopure R-(hydroxyalkanoic) acids as monomers: short-chain-length PHAs (scl-PHA), with C4–C5 monomers, and medium-chain-length PHAs (mcl-PHA), with C6–C14 monomers. Investigation of enzymatic hydrolysis of PHA by many microorganisms distinguishes between extracellular and intracellular processes, depending on polymer location (Jendrossek and Handrick, 2002; Prieto et al., 2007). Intracellular PHA can be catabolized by intracellular depolymerases of PHA producers, which are permanently associated to the PHA granule (de Eugenio et al.,...
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2007). In extracellular degradation, exogenous PHA can be utilized as a carbon and energy source by many microorganisms. The granules that are released to the medium by producer microorganisms after death can be hydrolysed by PHA depolymerases secreted by surrounding microorganisms, generating water-soluble oligomers and monomers which can be catabolized by the bacterial community (Schirmer et al., 1993; Schirmer and Jendrossek, 1994; Behrends et al., 1996; Abe and Doi, 1999; Handrick et al., 2001; Jendrossek and Handrick, 2002; Braaz et al., 2003; Knoll et al., 2009; Garcia-Hidalgo et al., 2012; Santos et al., 2012). The physiological meaning of PHA degradation in bacterial predators remains unknown, although it could be presumed that the prey’s PHA may be used as carbon source during predation, leading to an increase in fitness, i.e. number of progeny. The biochemical characterization of a new depolymerase in a predatory bacterium such as B. bacteriovorus HD100 (Martínez et al., 2012), opens new possibilities for studying the role of these hydrolytic enzymes during predatory interactions.

In this study we describe the influence of the prey PHA content on B. bacteriovorus HD100 progeny and the ecological advantages for preying on a PHA accumulating strain. PHA metabolism provides an increase in the ATP intracellular levels in Bdellovibrio cells, generating an increase in predator motility that facilitates the encounter with the prey cells during the attack phase.

Results

B. bacteriovorus HD100 can prey on Pseudomonas putida KT2442 accumulating mcl-PHA granules: influence of prey PHA accumulation on the predatory capacity

The ability of Bdellovibrio to grow on PHA-accumulating prey bacteria was first investigated by using the mcl-PHA model producer Pseudomonas putida KT2442 strain (Table 1) as prey. Pseudomonas putida KT2442 was grown under PHA producing conditions, prepared in Hepes buffer [3·10⁸ colony-forming units (cfu) ml⁻¹] and inoculated with 3·10⁷ plaque-forming units (pfu) ml⁻¹ of Bdellovibrio (see Experimental procedures for details). Examination by phase-contrast microscopy at different times of incubation, revealed the presence of many bdelloplasts 8 h post inoculation, and free-swimming Bdellovibrio cells accompanied by extracellular PHA granules at 24 h (Fig. 1A). These results suggested that PHA accumulation did not protect the cells from invasion and lysis by the predator. Furthermore, lytic plaques of Bdellovibrio growing on a lawn of P. putida KT2442 accumulating PHA were observed after 48 h of incubation (Fig. 1B). Since PHA disturbs the culture’s turbidimetry (de Eugenio et al., 2010) optical density could not be used to determine the number of viable prey cells during the incubation with the predator. Therefore, cell counts of the co-culture were performed by plating appropriate dilutions. After 24 h of predation, a 3–4 log prey reduction was observed (Table 2), which confirmed the susceptibility of PHA accumulating P. putida KT2442 cells to predation. Transmission electron microscopy (TEM) studies further supported this result (Fig. 1C).

To investigate the ability of the predator to degrade the prey’s PHA during the life cycle, we first studied the expression of the PhaZas depolymerase-encoding gene (Martínez et al., 2012) by real-time RT-PCR throughout the predatory cycle of Bdellovibrio. In these experiments, synchronous co-cultures of Bdellovibrio with a P. putida KT2442 accumulating-PHA prey were developed by mixing 2·10⁷ cfu ml⁻¹ of the prey with 10⁶ pfu ml⁻¹ of the predator. The phaZas expression level did not vary, suggesting that this gene was constitutively expressed at all stages of Bdellovibrio’s life cycle (Fig. 2A). These results suggested that the enzyme could be active along the whole cycle and may therefore support degradation the prey’s mcl-PHA. We also investigated whether prey PHA content affected the number of Bdellovibrio progeny. For this purpose, the wild-type strain P. putida KT2442, and its PHA-deficient mutant P. putida KT42C1, which carries a disrupted phaC1 PHA synthase-encoding gene (de Eugenio et al., 2010) and are therefore PHA-

Table 1. Bacterial strains and primers used in this study.

<table>
<thead>
<tr>
<th>Strain or primer</th>
<th>Relevant genotype, description or sequence (5′ to 3′)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>B. bacteriovorus HD100</td>
<td>Type strain, genome sequenced</td>
<td>Stolp and Starr (1963); Rendulic et al. (2004)</td>
</tr>
<tr>
<td>P. putida KT2442</td>
<td>P. putida mt-2 without TOL plasmid, hsdR, Rf’</td>
<td>Franklin et al. (1981)</td>
</tr>
<tr>
<td>KT42Z</td>
<td>KT2442 derivative strain, phaZ disruptional mutant, Km’</td>
<td>de Eugenio et al. (2010)</td>
</tr>
<tr>
<td>KT42C1</td>
<td>KT2442 derivative strain, phaC1 insertional mutant, Km’</td>
<td>de Eugenio et al. (2010)</td>
</tr>
<tr>
<td>Primers</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PhaZBd-F</td>
<td>GGCCTAGCTATTCTGTATCA</td>
<td>This work</td>
</tr>
<tr>
<td>PhaZBd-R</td>
<td>GTCAATCATGCGCACACCTCAA</td>
<td>This work</td>
</tr>
<tr>
<td>Bd2400-8</td>
<td>GCGACTCCAGAACACGACATT</td>
<td>Dori-Bachash et al. (2009)</td>
</tr>
<tr>
<td>Bd2400-9</td>
<td>GAATCCGGACTGCAATTGA</td>
<td>Dori-Bachash et al. (2009)</td>
</tr>
</tbody>
</table>
Fig. 1. *Bdellovibrio bacteriovorus* HD100 preying on *P. putida* KT2442 accumulating PHA.
A. Phase-contrast microscopy of *B. bacteriovorus* HD100 growing on *P. putida* KT2442 for 24 h. Attack phase *Bdellovibrios* and extracellular mcl-PHA granules can be observed. White bar corresponds to 2 μm.
B. *Bdellovibrio bacteriovorus* HD100 growing on a lawn of *P. putida* KT2442 accumulating mcl-PHA after 48 h of incubation.
C. Transmission electron microscopy image showing *B. bacteriovorus* HD100 inside *P. putida* KT2442 prey cells forming the bdelloplast.

Table 2. Growth parameters and PHA content at the start of the experiment (time zero) and after 24 h of co-cultures of *B. bacteriovorus* HD100 preying on *P. putida* strains (KT2442, KT42C1 and KT42Z).

<table>
<thead>
<tr>
<th>Strain cultures</th>
<th>Prey biomass 0 h (g l⁻¹)</th>
<th>Prey PHA content 0 h (g l⁻¹)</th>
<th>PHA content in the sediment 24 h (g l⁻¹)</th>
<th>PHA hydrolysis products content in the supernatant 24 h (g l⁻¹)</th>
<th>Prey cell count 24 h (10⁸ cfu ml⁻¹)</th>
<th><em>Bdellovibrio</em> cell count 24 h (10⁸ pfu ml⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>KT2442 (Bd⁻)ʰ</td>
<td>1.33 ± 0.11</td>
<td>0.84 ± 0.1</td>
<td>0.75 ± 0.0</td>
<td>&lt; 0.05</td>
<td>3.6 ± 1.5</td>
<td>--</td>
</tr>
<tr>
<td>KT2442 + Bd</td>
<td>1.33 ± 0.11</td>
<td>0.84 ± 0.1</td>
<td>0.45 ± 0.1³</td>
<td>0.2 ± 0.03</td>
<td>0.001 ± 0.0</td>
<td>15.4 ± 3</td>
</tr>
<tr>
<td>KT42C1 (Bd⁻)ʰ</td>
<td>0.057 ± 0.0</td>
<td>&lt; 0.005</td>
<td>0</td>
<td>nd</td>
<td>1.9 ± 0.15</td>
<td>--</td>
</tr>
<tr>
<td>KT42C1 + Bd</td>
<td>0.057 ± 0.0</td>
<td>&lt; 0.005</td>
<td>0</td>
<td>nd</td>
<td>0.001 ± 0.0</td>
<td>2.6 ± 0.4</td>
</tr>
<tr>
<td>KT42Z (Bd⁻)ʰ</td>
<td>1.22 ± 0.2</td>
<td>0.76 ± 0.02</td>
<td>0.72 ± 0.04</td>
<td>nd</td>
<td>1.1 ± 0.2</td>
<td>--</td>
</tr>
<tr>
<td>KT42Z + Bd</td>
<td>1.22 ± 0.2</td>
<td>0.76 ± 0.02</td>
<td>0.4 ± 0.05³</td>
<td>0.16 ± 0.03</td>
<td>0.001 ± 0.0</td>
<td>16 ± 2.5</td>
</tr>
</tbody>
</table>

a. Viable prey cell numbers were adjusted to a mean value of 3·10⁸ cfu ml⁻¹ at time zero of predation.
b. Control cultures of the prey without predator.
c. Value has been corrected by discounting biomass of *Bdellovibrio*.
nd, not determined.
accumulating and non-accumulating strains, respectively, were used as prey in *Bdellovibrio* predation experiments. Prey cultures were adjusted to 3·10⁸ cfu ml⁻¹ and subsequently inoculated with 3·10⁷ pfu ml⁻¹ of *Bdellovibrio*. Remarkably, *Bdellovibrio* progeny after 24 h of growth was almost sixfold higher in the wild-type cell culture than in the PHA mutant cell culture (Table 2). This suggested a benefit for *Bdellovibrio* preying on cells containing an extra carbon source in the form of PHA. To investigate the effect of PHA degradation on this phenomenon, prey PHA content during the predatory cycle was quantified by gas chromatography (GC) (Table 2). After 24 h of co-culture on the parental strain, PHA content decreased from 0.84 g l⁻¹ at time zero to 0.45 g l⁻¹ (Table 2), i.e. 54% of the prey’s PHA had been hydrolysed. Control culture of the prey without the predator showed 90% of the previous PHA after 24 h of incubation (Table 2). However, 0.2 g l⁻¹ PHA remained in the supernatant of the co-culture suggesting that PHA was partially released to the culture medium. To check for the presence of PHA hydrolytic products in the culture supernatant due to polymer degradation, the composition of PHA was further examined by high-performance liquid chromatography-mass spectrometry (HPLC-MS). The chromatograms revealed the presence of five chromatographic peaks, corresponding to the molecular masses of the deprotonated HO monomer (m/z 159), the deprotonated HX-HO diester (m/z 273); the deprotonated HO diester (m/z 301); the deprotonated HO-HX-HO triester (m/z 415); and the deprotonated HO triester (m/z 443). Polymers of higher molecular weight were not found. After 24 h of predation, the mcl-PHA hydrolytic products present in the culture medium were composed of 20% monomers, 30% dimers and 50% trimers. These results confirmed that PHA was degraded during the predation cycle, but interestingly the released hydrolysed products accumulated in the supernatant, instead of being metabolized by *Bdellovibrio*. Remarkably, 0.19 ± 0.02 g l⁻¹ (about 25% of the prey’s PHA), i.e. the remaining part of the initially accumulated PHA was not detected in the pellet nor in the supernatant of the co-culture, suggesting that PHA was partially hydrolysed and metabolized by growing *Bdellovibrio*.

To exclude the implication of *P. putida* KT2442’s intracellular mcl-PHA depolymerase (de Eugenio et al., 2007) in PHA hydrolysis, we analysed *Bdellovibrio* predation on a *P. putida* KT42Z prey. *Pseudomonas putida* KT42Z is a mutant strain of *P. putida* KT2442 lacking intracellular PHA depolymerase (de Eugenio et al., 2010). After 24 h of co-culture, the level of *Bdellovibrio* progeny in the medium and the degradation level of the PHA (52% of the prey’s PHA) were similar to those of *Bdellovibrio* preying on the wild-type strain (Table 2). Moreover, 0.16 g l⁻¹ PHA

**Fig. 2.** PhaZBd depolymerase determination in *Bdellovibrio* cells.

A. Transcription profile of the phaZBd gene across the predatory cycle when growing synchronously on *P. putida* KT2442 accumulating PHA. Primers were designed against the phaZBd gene and no amplification was detected in the *P. putida* KT2442 RNA. The expression levels of phaZBd were normalized by comparison with *Bd2400* gene expression. Expression levels are shown for mRNA prepared after 15 min, 45 min, 2 h, 3 h and 4 h after infection.

B. Qualitative depolymerase activity measured in mcl-PHA agar plates of 20 μl of the sonicated co-culture of 24 h *Bdellovibrio* preying on PHA accumulating *P. putida* KT42Z cells.
remained in the culture supernatant, and HPLC-MS analysis showed a similar ratio of PHA hydrolysed products than that detected in the supernatant of *Bdellovibrio* preying on wild-type strain KT2442. This definitively demonstrates that PHA was hydrolysed by the predator’s own PHA depolymerase.

PhaZBd depolymerase activity in the soluble fraction of *Bdellovibrio* cells after 24 h growing within PHA accumulating *P. putida* KT42Z cells, was analysed by spot-test on mcl-PHA-agar plates (Fig. 2B). The formation of a clearing zone around the spot well indicated the capacity of *Bdellovibrio* to hydrolyse mcl-PHA.

**Prey size affects *B. bacteriovorus* HD100 yield**

Previous works from our lab demonstrated that *P. putida* cell size depends on the ability to accumulate PHA under high carbon/nitrogen (C/N) ratios (de Eugenio *et al*., 2010; Escapa *et al*., 2012). Under such growth conditions, the PHA-deficient mutant *P. putida* KT42C1 shows a considerably lower total biomass (PHA plus the other cellular components) than the wild type (de Eugenio *et al*., 2010). This is due to the difference in PHA content between the strains, as in the wild type the PHA-free biomass (residual biomass thereafter) was nearly identical to that of the mutant strain. Moreover, PHA accumulation had a strong influence on population abundance, since, at similar residual biomass levels, the cell number of the *P. putida* KT42C1 mutant was 10-fold higher than that of the wild-type strain after 24 h of growing, correlating with the considerably smaller cell size (de Eugenio *et al*., 2010; Escapa *et al*., 2012). A direct consequence of these differences in cell morphology was the largely dissimilar sizes of the cells used as prey (i.e. KT2442 versus KT42C1). This in turn, may affect the population size achieved by the predator. Kessel and Shilo (1976) using different strains of *Escherichia coli* observed an increase in the number of *Bdellovibrio* progeny with increased prey cell size. This raises the possibility that a similar relationship is observed when *Bdellovibrio* predate upon KT2442 or KT42C1. As the initial prey cell number was similar in both cultures, the sixfold increase in predator population size may be due either to the prey’s cell size or to its intracellular PHA content. In fact, the calculated residual biomass at the onset of predation (time zero) was almost 10-fold higher in the parental strain than in the KT42C1 mutant (0.50 g l⁻¹ and 0.057 g l⁻¹ respectively).

To examine the relationship between prey cell dimensions, residual biomass and number of *Bdellovibrio* progeny, we established a procedure to control the *P. putida* cell size, by keeping carbon concentration fixed and varying the C/N ratio in the PHA defective strain, *P. putida* KT42C1. This strain was cultured under two growth regimes yielding cultures with two different average cell sizes: (i) optimal PHA production conditions in an unbalanced medium with a C/N ratio of 12.50 and (ii) a balanced medium with a C/N ratio of 1.25 (see *Experimental procedures* for details). After 24 h of growth, the viable cell number was similar for both cultures (1.9 ± 0.26 10⁹ and 1.4 ± 0.43 10⁹ cfu ml⁻¹, for the high and low C/N ratio respectively). However, the mean size of the cells cultured in the balanced medium was significantly higher (Fig. 3). Mean cell length of KT42C1 grown at 12.50 C/N ratio was of 1.1 ± 0.2 µm (hereafter named small-KT42C1). Conversely, mean cell length of KT42C1 grown at a 1.25 C/N ratio was almost twofold higher (hereafter named large-KT42C1), standing at 2 ± 0.5 µm. This increase in cell size in the large-KT42C1 population was correlated to an increment in the culture’s biomass (0.93 ± 0.02 g l⁻¹ and 0.56 ± 0.11 g l⁻¹, for large- and

![Fig. 3. Differences in cell size of *P. putida* KT42C1 cultured under different C/N ratios.](image)

A. Phase-contrast microscopy of small-KT42C1 cells (grown under unbalanced medium with C/N ratio of 12.50 as for optimal PHA production conditions).

B. Phase-contrast microscopy of large-KT42C1 cells (grown under a balanced medium with C/N ratio of 1.25). White bars correspond to 2 µm.

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Influence of prey PHA content on B. bacteriovorus HD100 fitness

The results presented above strongly support the ability of *Bdellovibrio* to hydrolyse PHA during predation on *P. putida* cells, but they do not determine that the predator derives a fitness advantage from this capability. Growth parameters of the predator linked to predatory fitness such as motility and predation efficiency were measured after infection on cells containing or devoid of PHA. For these predation experiments, KT2442, KT42C1 and KT42Z prey cell suspensions containing equal residual biomass were infected with *Bdellovibrio*, as described before. Under these conditions, the PHA content was different in each prey (Table 2). Motility of *Bdellovibrio* cells growing on the different prey strains for 24 h, was recorded by phase-contrast microscopy (see Movies S1–S3 in Supporting information). Mean swimming speed was significantly higher in *Bdellovibrio* preying on the KT2442 or on the KT42Z strains (50 μm s⁻¹) than on the KT42C1 strain (< 5 μm s⁻¹). Despite these huge differences in motility between predators growing on prey with and without PHA, it is worth to remark that *Bdellovibrio* progeny numbers after 24 h was similar in all the co-cultures (1.8·10⁹ pfu ml⁻¹).

To ascribe these differences in the predator motility to the prey PHA content, a culture of *Bdellovibrio* preying on KT42C1 cells was supplied with 0.8 g l⁻¹ of a suspension of PHA latex. The external addition of the PHA polymer suspension to *Bdellovibrio* growing on KT42C1 restored the predator’s swimming close to that achieved on the wild-type strain, reaching 37 μm s⁻¹ (see Movie S4 in Supporting information). Alterations in predator motility were

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**Table 3.** Progeny of *B. bacteriovorus* HD100 preying on small-KT42C1 or large-KT42C1 *P. putida* cells.

<table>
<thead>
<tr>
<th>Strain culturesa,b</th>
<th>Prey cell count 24 h (10⁵ cfu ml⁻¹)</th>
<th>Bdellovibrio cell count 24 h (10⁴ pfu ml⁻¹)</th>
<th>Prey biomass 0 h (g l⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Small-KT42C1 (Bd⁻)</td>
<td>1.9 ± 0.15</td>
<td>–</td>
<td>0.057 ± 0.0</td>
</tr>
<tr>
<td>Small-KT42C1 + Bd</td>
<td>0.001 ± 0.0</td>
<td>2.6 ± 0.4</td>
<td>0.057 ± 0.0</td>
</tr>
<tr>
<td>Large-KT42C1 (Bd⁻)</td>
<td>2.3 ± 0.35</td>
<td>–</td>
<td>0.13 ± 0.01</td>
</tr>
<tr>
<td>Large-KT42C1 + Bd</td>
<td>0.001 ± 0.0</td>
<td>6.4 ± 0.85</td>
<td>0.13 ± 0.01</td>
</tr>
</tbody>
</table>

a. Small-KT42C1 and large-KT42C1 cells were previously grown under unbalanced medium with C/N ratio of 12.50 and in a balanced medium with C/N ratio of 1.25 respectively.
b. Viable prey cell numbers were adjusted to a mean value of 3·10⁸ cfu ml⁻¹ at time zero of predation.
c. Control cultures of the prey without predator.
correlated to its energetic state, as measurement of the intracellular ATP concentrations of filtered attack phase predators after 24 h of predation on KT2442 and KT42C1 strains showed by using a bioluminescence assay. Intracellular ATP levels of *Bdellovibrio* preying on KT2442 were almost twofold higher than those of *Bdellovibrio* preying on the KT42C1 mutant (2.13 ± 0.16 pmol and 1.20 ± 0.08 pmol respectively), demonstrating that the ability to hydrolyse PHA confers the predator an energetic gain in the form of ATP that can be used to achieve faster swimming speeds.

To investigate if these differences in *Bdellovibrio* motility affected predator fitness, *Bdellovibrio* was first grown on prey containing or devoid of PHA (Fig. 4A). Then, predators from each culture were mixed with wild-type *P. putida* KT2442 grown on NB (devoid of PHA) (Fig. 4B). Co-cultures with *Bdellovibrio* derived from KT42C1 infection exhibited an 8 h lag compared with those that were previously grown on the PHA accumulating strains (Fig. 4B). Nevertheless, no significant difference was observed in the final *Bdellovibrio* yield after 24 h of predation (Fig. 4B). These results indicated that *Bdellovibrio* motility is a key factor for efficient encounters with the prey in liquid environments, although we do not exclude that it might be a temporary benefit. Even more, this result demonstrated that preying on a PHA producer strain may confer ecological advantages enhancing *Bdellovibrio* predation efficiency.

**Discussion**

Although PHA intracellular hydrolysis has been traditionally considered as part of the carbon source assimilation machinery for producing biomass under carbon starvation conditions (Jendrossek and Handrick, 2002), it is nowadays assumed that PHA metabolism confers some other advantages to the producer bacterium. Our recent studies on *P. putida* PHA metabolism demonstrated that intracellular PHA turnover brought about by synthesis and degradation processes (the PHA cycle) also plays a key role in synchronizing global metabolism to the availability of resources in PHA producing microorganisms (de Eugenio et al., 2010; Escapa et al., 2012). PhaZ depolymerase plays a fundamental function in bacterial carbon metabolism by maintaining PHA turnover, and this simultaneous PHA accumulation/mobilization cycle allows robust growth during transient nutrient conditions (de Eugenio et al., 2007; 2010; Ren et al., 2009). Moreover, the intracellular PHA cycle improves bacterial fitness, enhancing the ability to survive, tolerate or alleviate various stresses, which favours the establishment of PHA-producing bacteria in competitive environments, such as soil, water and rhizosphere (Madison and Huisman, 1999; Kadouri et al., 2005; Zhao et al., 2007; Castro-Sowinski et al., 2010).

This observation has been ascribed to a reduced susceptibility to adverse factors linked, somehow, to PHA content, or to the ability to mobilize the reserve storage carbon along time (Dawes and Senior, 1973; Matin et al., 1979).

The ability to degrade extracellular scl-PHA is widespread among bacteria in comparison with that of mcl-PHA. Therefore, many extracellular scl-PHA depolymerases have been characterized in depth over the last decade and a considerably high number of genes have been identified. From an ecological point of view, extracellular degradation of PHA is an example of a microbial metabolic benefit for using exogenous carbon and energy sources, awarded from PHA producers to maintain the carbon balance of the bacterial community (Jendrossek and Handrick, 2002). In this context, *B. bacteriovorus* HD100, an obligate predator bacterium that develops in the periplasm of other Gram-negative bacteria, including PHA producers such as *P. putida* may also fill an unsuspected ecological role. The existence of a mcl-PHA depolymerase in this predator prompted us to investigate its ability to degrade prey PHA during the predatory cycle, and the potential advantages it may draw from the degradation of this extra carbon and energy source.

Our previous studies demonstrated a periplasmic location of the mcl-PHA depolymerase from *B. bacteriovorus* HD100 (PhaZbd) when produced in *P. putida* prey (Martínez et al., 2012). The current findings raise a still open question about the natural cellular location of the enzyme, since the extracellular medium for the predator is either the periplasm of the prey or the culture medium itself, depending on the life cycle stage (intraperiplasmic growth phase or attack phase respectively). Expression studies by quantitative-PCR for phaZbd depolymerase gene, revealed that it was constitutively expressed throughout the life cycle of *Bdellovibrio* growing on PHA accumulating *P. putida* KT2442 cells. In addition, PhaZbd depolymerase activity detection in *Bdellovibrio* cells developed on PHA accumulating *P. putida* KT2442Z cells, demonstrated the ability of *Bdellovibrio* cells to hydrolyse mcl-PHA. These results further support the idea that the PhaZbd enzyme is active along the predator developmental cycle, degrading the prey PHA, which may then be assimilated as a source of carbon and energy by *Bdellovibrio*.

Therefore, to address these possibilities, we studied the impact of prey PHA content on the predator fitness in terms of number of progeny, motility and predation efficiency of new prey, and the potential advantages for preying on a PHA producer. Our results demonstrated that the number of *Bdellovibrio* progeny depends on the prey biomass and not on the viable prey cell number or the PHA content. The prey size has been reported to be a key factor in *Bdellovibrio* predation, although there are very few reports describing this effect (Kessel and Shilo,
available biomass. Moreover, \textit{P. putida} predation experiments upon these large and small cells. In this way, the prey’s cell size was controlled and in the mineral medium of the PHA cycle defective strain, \textit{P. putida} KT42C1, generating large- and small-KT42C1 cells. In this way, the prey’s cell size was controlled and predation experiments upon these large and small \textit{P. putida} cells confirmed that, for equal cell numbers, the number of predator progeny depends on the prey’s total available biomass. Moreover, \textit{Bdellovibrio} reached a similar number of progeny when preying upon equal residual biomass of PHA producers and non-PHA producers \textit{P. putida} strains. Thus, the residual biomass provides other essential nutrients and determined the yield of predator progeny cells. However, we have observed interesting differences between the predators grown on the different prey, probably due to their ability to metabolize PHA. The presence of mcl-PHA-hydrolysed products (monomers, dimers and trimers) in the culture supernatant after 24 h of predation confirmed the ability of \textit{Bdellovibrio} to degrade prey PHA. The difference in total PHA content before and after predation suggests that the predator uses part but not all the monomers and oligomers during the time span of the experiment. Early work on \textit{Bdellovibrio} reported that fatty acids from the prey can be directly incorporated by the predator (Kuenen and Rittenberg, 1975). Other authors reported that the lipids for the lipopolysaccharide are not imported from the prey (Schwudke et al., 2003). According to the genome data, \textit{Bdellovibrio} is able to transform fatty acids, including PHA monomers, into acetyl-CoA (Rendulic et al., 2004; Martinez et al., 2012). This key metabolite enters the tricarboxylic acid (TCA) cycle, contributing to the energy supply, which could explain the higher intracellular ATP levels detected in \textit{Bdellovibrio} preying upon PHA accumulating KT2442. However, PHA hydrolysis during the predatory cycle would not cause an increase of predator progeny, most likely due to an incomplete glyoxylate shunt in \textit{Bdellovibrio} metabolism (http://www.genome.jp/kegg-bin/show_pathway?baa00630), which would link the catabolism of fatty acids such as octanoic acid to the generation of biomass (Garcia et al., 1999; Escapa et al., 2012). Since PHA produced by KT2442 is mainly composed of hydroxyoctanoic acid, the derived monomers can be used to obtain energy, but they could not be transformed into predator biomass in the absence of an active glyoxylate shunt. Catabolism of odd mcl-PHAs in bacteria yields acetyl-CoA and propionyl-CoA, which could be further catabolized through several pathways that might be directed to gluconeogenesis (Textor et al., 1997; Horswill and Escalante-Semerena, 1999; Upton and McKinney, 2007). However, similar results were obtained when \textit{Bdellovibrio} preying on \textit{P. putida} KT2442-acculminating polyhydroxynonanoate (PHN) (data not shown), suggesting that \textit{Bdellovibrio} might not be able to transform propionyl-CoA into biomass in our assay conditions.

An unexpected finding of this study was the huge difference in motile speed of attack phase \textit{Bdellovibrio} grown on equal residual biomass of PHA producer and non-producer \textit{P. putida} strains, after 24 h. The predator’s motility was significantly lower when growing on the PHA non-accumulating strain. Moreover, the external addition of the PHA polymer (latex suspension) to \textit{Bdellovibrio} growing on a PHA minus mutant KT42C1, restored high motility, which reached swimming speeds of approximately 75% of that of the \textit{Bdellovibrio} cells preying on the PHA producing strain. This finding leads us to assume that PHA was a key prey component to sustain predator motility.

High speeds observed in \textit{Bdellovibrio} preying on the PHA producing strain were correlated to the high intracellular ATP levels found in these predatory cells. It has been previously reported that attack phase \textit{Bdellovibrio} swims at high speeds (35–160 \(\mu\)m s\(^{-1}\)) (Lambert et al., 2006b) with motility generated by rotation of a single, polar flagellum that is surrounded by a continuous sheath (Seidler and Starr, 1968; Thomashow and Rittenberg, 1985). The role of flagellar motility in predation has been reported, highlighting the importance for efficient encounters with prey in liquid environments (Lambert et al., 2006b; Morehouse et al., 2011). Our results revealed a more efficient predation of \textit{Bdellovibrio} grown on the PHA producers, confirming the importance of \textit{Bdellovibrio} motility to seek further prey. Even more, this evidence would support our premise that preying on a PHA producer strain confers ecological advantages enhancing \textit{Bdellovibrio} motility and predatory efficiency.

Moreover, \textit{Bdellovibrio} requires attachment to the prey cell to perform its predatory life cycle. Attachment to the prey is always achieved at the non-flagellated pole and requires a significant force to penetrate into the prey periplasm and overcome the opposing force of the prey cell’s inner osmotic potential (Lambert et al., 2008). This sort of force could be generated by type IV pili, which are made up of polymeric monomers of pilin forming a polar filament anchored to the inner membrane and associated with ATPases that drive the addition or subtraction of monomers to the fibre. Type IV pili are essential for the infection process by \textit{Bdellovibrio}, since the interruption of the fibre PilA protein, rendered non-predatory \textit{Bdellovibrio} cells (Evans et al., 2007). In this sense, the higher intracellular ATP levels observed in \textit{Bdellovibrio} preying on the PHA producing strain might be also correlated to the enhanced predation efficiency found in these predatory cells.

We suggest that the ability for PHA degradation could make \textit{Bdellovibrio} more robust to seek for further prey and...
to become adapted to unfavourable nutrient conditions as mean of low number of potential prey cells. This work provides new insights into the critical role of the PHA cycle in the physiology of bacterial predators.

*Bdellovibrio bacteriovorus* and like organisms (BALOs) are considered as potential therapeutic living agents and as source of hydrolytic enzymes (biocatalysts) for industrial applications (Rendulic *et al*., 2004; Sockett, 2009; Martínez *et al*., 2012). In the present study, we have demonstrated that *Bdellovibrio* preying upon PHA producers is able to release the prey’s PHA and the hydrolysed enantiopure monomers and oligomers (Fig. 1A, Table 2). These findings pave the way to design new industrial applications for processing intracellular bioproducts such as PHA and its derivatives. *Bdellovibrio* is here highlighted as a potent and innovative lytic system to facilitate bacterial polyester extraction.

**Experimental procedures**

**Bacterial strains and growth conditions**

The bacterial strains used are listed in Table 1. *Pseudomonas putida* strains were grown in nutrient broth (NB) medium (Difco) and in LB medium (Sambrook and Russell, 2001) at 30°C. Kanamycin (50 μg ml⁻¹) was added when needed. Growth was monitored with a Shimadzu UV-260 spectrophotometer at 600 nm (OD₆₀₀). Solid media were supplemented with 1.5% (w/v) agar. For mcl-PHA production, *P. putida* strains were grown in 0.1 N M63, a nitrogen-limited minimal medium supplemented with 1 mM MgSO₄ and a solution medium [13.6 g of KH₂PO₄ l⁻¹, 0.2 g of (NH₄)₂SO₄ l⁻¹, 0.5 mg of CaCl₂·2H₂O and 3 mM MgCl₂·3H₂O, pH 7.8] with the prey.

Assays for predatory capability of *B. bacteriovorus* HD100 preying on *P. putida* KT2442 strains containing PHA

Predator cell suspension was prepared as described above. For predation ability experiments in the presence or absence of PHA, *P. putida* KT2442 prey suspensions were adjusted (i) to equal viable cell number (3·10⁸ cfu ml⁻¹) or (ii) to equal residual biomass (0.45 ± 0.02 g l⁻¹), and subsequently infected with 3·10⁷ pfu ml⁻¹ *Bdellovibrio*. Co-cultures were developed in 250 ml flasks in a final volume of 30 ml with Heps buffer.

**Predation efficiency assays of *B. bacteriovorus* HD100 after preying on *P. putida* KT2442 strains containing PHA**

For the predatory efficiency assays in liquid media after infecting cells containing or devoid of PHA adjusted to equal residual biomass, 100 μl aliquots of each co-culture at 24 h were used to infect 30 ml of *P. putida* KT2442 wild type pre-grown on rich NB medium (7·10⁸ cfu ml⁻¹). Co-cultures were incubated at 30°C for additional 24 h and prey and predator viabilities were determined.

**B. bacteriovorus HD100 and *P. putida* KT2442 prey strains viability calculation**

*Bdellovibrio* and prey strain viabilities were calculated from a co-culture containing both strains; serial dilutions of the co-culture from 10⁻¹ to 10⁻⁷ were made in dilute nutrient broth (DNB) liquid medium, consisting of 0.8 g l⁻¹ NB supplemented with 2 mM CaCl₂ and 3 mM MgCl₂. To calculate *Bdellovibrio* viability 0.1 ml of the appropriate dilution was mixed with additional 0.5 ml of prey cell suspension of *P. putida* KT2442 pre-grown in NB and prepared in Heps buffer at OD₆₀₀ 10, vortexed and plated on DNB solid medium by using the double overlay method (Lambert *et al*., 2003). Predators were counted as pfu developing on the lawn of *P. putida* KT2442 after 48 h of incubation at 30°C. To calculate prey strain viability, 10 μl of each dilution was placed on LB solid medium and cfu were counted. For each strain, three different experiments were carried out.

**Biomass calculation**

Cell densities, expressed in grams of the cell dry weight (CDW) per litre, were determined gravimetrically by using tared 50 ml Falcon tubes. Thirty millilitres of culture medium was centrifuged for 45 min at 3800 g and 4°C. Cell pellets were freeze-dried for 24 h in a lyophilizer and weighed. Biomass free of PHA (residual biomass) is defined as total dry mass minus PHA mass, and has been used for the analysis of the predatory capabilities of *Bdellovibrio* growing on PHA producers.

**PHA depolymerase assay**

A quick and simple qualitative procedure for estimating the mcl-PHA depolymerase activity was performed by spot test on indicator plates. A homogeneous latex suspension was obtained as previously described (Martinez *et al*., 2012). PHA agar plates were prepared by adding 1.5% (w/v) agar to the PHA latex suspension (6 mg ml⁻¹) in 50 mM phosphate buffer (pH 8). After solidification, 20 μl of an infection mixture solution was dropped onto 5-mm-diameter holes made in the
PHA agar plates, and the plates were incubated at 37°C for 4 days. The resulting clearing zones indicated the depolymerase activity.

The infection mixture solution was prepared from synchronous co-cultures of \textit{B. bacteriovorus} HD100 growing on PHA accumulating \textit{P. putida} KT42Z incubated for 20 h. Two millilitres of the co-culture were centrifuged and resuspended in 2 ml of 50 mM phosphate buffer (pH 8). Cells were broken by sonication treatment and centrifuged at 15 000 g. The resulting supernatant was stored for further analysis.

**Intracellular ATP measurements**

Intracellular ATP was determined by means of an ATP bioluminescence assay kit (ATP Biomass Kit HS, BioThema, Sweden) according to the manufacturer instructions. \textit{Bdellovibrio} cells were filtrated twice through a 0.45 µm filter (Sartorius) for separation from the remaining prey. For each strain, three different experiments were carried out.

**Gas chromatography analysis for PHA content determinations**

For total PHA quantification, cultures were lyophilized and analysed by gas chromatography-mass spectrometry (GC-MS) as previously reported (de Eugenio et al., 2007; 2010).

**HPLC-MS analysis for identification of the products released**

To identify the degradation products, co-culture supernatants were analysed by HPLC-MS as previously reported (Martínez et al., 2012). Briefly, lyophilized supernatants of the infection mixtures were resuspended on methanol and 5 µl were injected in the chromatographic system. Separation of the hydrolysis products was carried out on a Finnigan Surveyor (Thermo Electron) pump coupled with a Finnigan LXQ TM (Thermo Electron) ion trap mass spectrometer.

**Transmission electron microscopy**

Co-cultures of \textit{Bdellovibrio} growing for 24 h on \textit{P. putida} KT2442 accumulating PHA were harvested, washed twice in PBS and fixed in 5% (w/v) glutaraldehyde in the same solution, as previously described (Martinez et al., 2011). Briefly, cells were incubated with 2.5% (w/v) OsO4 for 1 h, gradually dehydrated in ethanol solutions and propylene oxide and finally embedded in Epon 812 resin. Ultrathin sections were cut and observed in a Jeol-1230 electron microscope (Jeol Ltd, Akishima, Japan).

**Phase-contrast microscopy**

Cultures were routinely visualized with a 100× phase-contrast objective and images were taken with a COLLPPIX camera. \textit{Pseudomonas putida} strains cell length was calculated by measuring all the cells in at least three different fields in two separate experiments.

**Microscopic analysis of \textit{B. bacteriovorus} HD100 swimming behaviour**

\textit{Pseudomonas putida} KT2442 strains adjusted to equal residual biomass were infected by \textit{Bdellovibrio} and tracked 18 h after infection. Cultures were observed using a 100× phase-contrast objective on a microscope (Leica AF6000 LX) connected to a video camera (Hamamatsu C9100-02). Microscopic images were acquired over 40 s at room temperature every 40 ms. Speeds of moving \textit{Bdellovibrio} bacteria growing on each strain were measured by determining the path of specific cells using ImageJ free software (NIH). Data on mean run speeds were collected from a minimum of 50 cells of \textit{Bdellovibrio} from each experiment.

**RNA isolation and real-time RT-PCR assay**

For real-time RT-PCR analysis, synchronization of \textit{B. bacteriovorus} HD100 cultures was obtained by mixing 2·10^7 cfu ml⁻¹ of \textit{P. putida} KT2442 with 10⁶ pfu ml⁻¹ of the predator in Hepes buffer (Dori-Bachash et al., 2009). Samples were collected at 15, 45, 120 and 180 and 240 min post infection. Total RNA was isolated using the RNeasy mini kit (Qiagen, Germany), including a DNase I treatment according to the manufacturer’s instructions, precipitated with ethanol, washed and resuspended in 40 µl of RNase-free water. DNA traces were removed with Turbo DNA-free (Ambion, UK), as confirmed by PCR without the reverse transcription step. The concentration and purity of the RNA samples were measured by using a NanoPhotometer™ Pearl (Implen, Germany). Synthesis of total cDNA was carried out with 20 µl of reverse transcription reactions containing 1 µg of RNA, 0.5 mM dNTPs, 200 U of SuperScript II Reverse Transcriptase (Invitrogen, USA) and 5 µM random hexamers as primers, in the buffer recommended by the manufacturer. Samples were initially heated at 65°C for 5 min and then incubated at 42°C for 1 h, terminated by incubation at 70°C for 15 min. The cDNA obtained was purified using Gene cleanTurbo kit (MP Biomedicals, USA) and the concentration was measured using a Nanophotometer™ Pearl (Implen, Germany). For the analysis of the transcripts levels target cDNAs (0.5 and 5 ng) and reference samples were amplified three times in separate PCR with 0.2 mM each of target primers by using the iQ5 Multicolour Real-Time PCR Detection System (Bio-Rad, USA). Target primers for \textit{phaZBd} were VF and VR (Table 1). Real-time PCR was performed using SYBR Green technology in an ABI Prism 7000 Sequence Detection System (Applied Biosystems, UK). Samples were initially denatured by heating at 95°C for 4 min, followed by 30 cycles of amplification (95°C, 1 min; test annealing temperature, 58°C, 1 min; elongation and signal acquisition, 72°C, 30 s). For quantification of the fluorescence values, a calibration curve was made using dilution series from 5·10⁻⁷ to 5 ng of \textit{P. putida} KT2442 genomic DNA sample. RT-PCR procedures were performed with two independent biological experiments. Negative control with \textit{P. putida} KT2442 RNA as template was carried out and gave no significant amplification. Expression of the \textit{phaZBd} detected was presented relative to expression of the housekeeping gene \textit{Bd2400}, as previously described (Dori-Bachash et al., 2009). Real-time RT-PCR was performed
with triplicate samples from three independent biological experiments.

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