microbial biotechnology

Open Access

A polyhydroxyalkanoate-based encapsulating strategy for 'bioplasticizing' microorganisms

Erika González,[1,2] Cristina Herencias[1,2] and M. Auxiliadora Prieto[1,2]*

[1]*Polymer Biotechnology Group, Microbial and Plant Biotechnology Department, Centro de Investigaciones Biológicas, CIB-CSIC, Ramiro de Maeztu 9, 28040, Madrid, Spain.*

[2]*Interdisciplinary Platform for Sustainable Plastics towards a Circular Economy-Spanish National Research Council (SusPlast-CSIC), Madrid, Spain.*

Summary

Over the past few decades, considerable interest has been shown in developing nano- and microcarriers with biocompatible and biodegradable materials for medical and biotechnological applications. Microencapsulation is a technology capable of enhancing the survival rate of bacteria, providing stability in harsh environments. In the present paper, we developed a technology to encapsulate microorganisms within polyhydroxyalkanoate (PHA)-based microcapsules (MPs), employing a modified double emulsion solvent evaporation technique, with *Pseudomonas putida* KT2440 as a biotechnological model strain. The resulting MPs display a spherical morphology and an average particle size of 10 μm. The stability of the MPs was monitored under different conditions of storage and stress. The MPs remained stable for at least 24 days stored at 4°C in a water suspension. They exhibited greater tolerance to stress conditions; encapsulated cells remained viable for 2 h in alkaline solution and after 24 h of H_2O_2 exposure at 10 and 20 mM. Results suggested the potential of MPs as a microcontainer of bacterial cells, even for biotechnological applications requiring high alkaline conditions and oxidative stress. We validated the potential applicability of the PHA-based microencapsulation method in other microorganisms by encapsulating the predatory bacterium *Bdellovibrio bacteriovorus*.

Received 2 July, 2019; revised 16 September, 2019; accepted 17 September, 2019.

*For correspondence. E-mail auxi@cib.csic.es; Tel. (+34) 918373112 (ext.4228) Fax (+34) 915360432

Microbial Biotechnology (2020) **13**(1), 185–198
doi:10.1111/1751-7915.13492

Funding information

The authors thank for the financial support from the Spanish Ministry of Science, Innovation and Universities (BIO2014-61515-EXP, BIO2017-83448-R) and the Community of Madrid (P2013/MIT2807, P2018/NMT4389).

Introduction

The use of encapsulated bacterial cells now constitutes a highly useful technology for a wide range of biotechnological applications such as microbial fuel cells, food systems, bioremediation, fermentation reactors and regenerative medicine (Sipailienė and Petraitytė, 2018). Successful encapsulation enhances cell survival and biocatalytic functionality during a particular biotechnological process by providing living bacterial cells a physical barrier against adverse conditions and harmful external stresses. This usually results in greater stability during production, storage and handling (Arslan *et al*., 2015; San Keskin *et al*., 2018). The optimal size and physicochemical properties of the particles depend, among other factors, upon the substance to be encapsulated. For example, it has previously been suggested to specifically explore the encapsulation of bacterial cells presenting an average size of 1–4 μm and an optimal particle diameter ranging from 3 to 200 μm (O'Riordan *et al*., 2001; Sipailienė and Petraitytė, 2018). The materials used for encapsulation also influence the properties of the particles, the efficacy of the encapsulation and the viability of the encapsulated microorganisms (Khem *et al*., 2016). In this context, the polymeric material and the microencapsulation process are crucial factors which determine the effectiveness, as well as the protective device, of microorganism viability. Aside from this protection, the device must withstand certain physicochemical insults whilst allowing control of the interfacial properties of the bacterial cells with the surrounding matrix (Sipailienė and Petraitytė, 2018). Several biodegradable polymeric matrices have been tested for bacteria encapsulation, such as alginate (Shi *et al*., 2013; Albadran *et al*., 2015), starch systems, carboxymethyl cellulose (Ke *et al*., 2014), poly-lactic (PLA) and poly-lactic-*co*-glycolic acid (PLGA) (Della Porta *et al*., 2012; Sarioglu *et al*., 2017), chitosan (D'Orazio *et al*., 2015), and proteins and polysaccharide mixtures (Ranadheera *et al*., 2015; San Keskin *et al*., 2018).

The bacterial polyesters polyhydroxyalkanoates (PHAs) have recently emerged as biodegradable polymers for

developing microcarrier devices (Lizarraga-Valderrama *et al*., 2016). They are polyesters of (*R*)-3-hydroxyalkanoic acids accumulated intracellularly by different microorganisms as carbon and energy storage sources. PHAs are found to be particularly attractive for application in drug delivery due to their properties, such as optical activity, biocompatibility, non-toxicity, thermoplasticity and biodegradability (Kniewel *et al*., 2017). These properties depend on the monomer composition; most short-chain-length PHAs (sclPHAs) like polyhydroxybutyrate (PHB) are mechanically fragile and have a high melting temperature, whereas medium-chain-length PHAs (mclPHAs) containing 6-14 carbon atom monomers are highly elastomeric by nature, presenting a low glass transition and a low melting temperature. PHAs have been widely studied in the field of tissue engineering and in the development of medical devices (Lim *et al*., 2017), and special attention should be paid to the biocompatibility of these polymers with regard to their potential in controlled release formulations (Zhang *et al*., 2018). In recent times, PHAs have been used to immobilize bacteriophages (Wang *et al*., 2016,), but to date they have not been described to encapsulate bacteria. Herein, we developed a double emulsion solvent evaporation method (water/oil/water) to generate mclPHA-coated microcapsules (MPs) with the capacity to protect biotechnologically relevant bacteria. We selected *Pseudomonas putida* KT2440 as a model bacterium in biotechnology (Nikel and de Lorenzo, 2018), and we employed the predatory bacterium *Bdellovibrio bacteriovorus* HD100 (Socket, 2009) as a potential living antibiotic agent, to encapsulate the strains and to test the capacity of the MP devices to protect against different physicochemical insults.

Results

Optimizing an encapsulation method by means of a double emulsion system based on mclPHA solubility and tolerance of P. putida *to solvents*

The encapsulation method based on the double emulsion solvent evaporation method (water/oil/water, hereafter $W_1/O/W_2$) has recently been the focus of great interest due to its ability to encapsulate and release hydrophilic or solid substances as a drug delivery system in cosmetics and food products (Della Porta *et al*., 2012; Iqbal *et al*., 2015). The present paper aims to investigate the possible use of the $W_1/O/W_2$ technology for bacterial cell encapsulation within mclPHA microdevices for biotechnological purposes. Selection of the organic solvent involves a challenge, since solvents can negatively affect cell viability and microcapsule size. We first investigated the feasibility of generating microcapsules (MPs) with the $W_1/O/W_2$ technique using the natural copolyester poly(3-hydroxyoctanoate-*co*-3-hydroxyhexanoate) (hereafter mclPHA) dissolved in different solvents (see below). Furthermore, we explored the effect of these solvents upon microcapsule size, particle morphology and cell viability. In these experiments, the *P. putida* KT2440 strain was selected as a model bacterium due to its stress tolerance (Nikel and de Lorenzo, 2018).

Organic solvent selection. The $W_1/O/W_2$ protocol established in the present research consists of four steps (see Experimental Procedures for details) (Fig. 1): (i) primary emulsification: a buffered solution (inner aqueous phase, W_1, containing bacterial cells) is emulsified into an organic solution containing mclPHA (organic phase, O); (ii) secondary emulsification: the primary emulsion (W_1/O) is further emulsified into a second aqueous phase containing the surfactant polyvinylalcohol (PVA) as a stabilizer (external aqueous phase, W_2) in order to obtain a $W_1/O/W_2$ emulsion; (iii) solidification of the MPs: the organic solvent (O) is removed by evaporation and solid microparticles are produced; and (iv) isolation of the MPs by centrifugation.

Ethyl acetate (EtOAc), dichloromethane (DCM) and chloroform ($CHCl_3$) were selected as potential organic solvents for the double emulsion technique based on their appropriate properties with regard to developing emulsions; these attributes include the high solubility of mclPHA, their low or partial solubility in water (Log $P_{o/w}$ values) and their low boiling point, which enables their evaporation in conditions of low pressure or room temperature (Table S1) (Song *et al*., 2006). We dissolved mclPHA at 100 mg ml^{-1} at room temperature and prepared MP formulations using each solvent, first in the absence of bacteria, in accordance with the methodology described in the Experimental Procedures (Fig. 1).

The external morphology and shape of the resulting MPs were observed by random scanning with phase-contrast optical microscopy (Fig. 2A); this revealed relatively spherical smooth particles in all conditions. The use of polyvinilalcohol (PVA) at 4.0% (w/v) as a stabilizer in the external aqueous phase (W_2) and of EtOAc as an organic solvent provided a homogenous particle-size distribution with a polydispersity index (PI) of 0.43 established by means of dynamic light scattering and a diameter size of 10.9 $\pm$ 4.75 μm. In contrast, DCM and $CHCl_3$ showed a more heterogeneous distribution, with a PI of 0.76 and 0.82 respectively (Fig. 2B). Due to this high heterogeneity in size distribution, we discarded these solvents (DCM and $CHCl_3$) in the preparation of MPs for subsequent evaluations, selecting EtOAc instead. Moreover, EtOAc exhibits the lowest Log $P_{o/w}$ value of the solvents tested (Table S1), which inversely correlates with cell toxicity; solvents with Log $P_{o/w}$ values

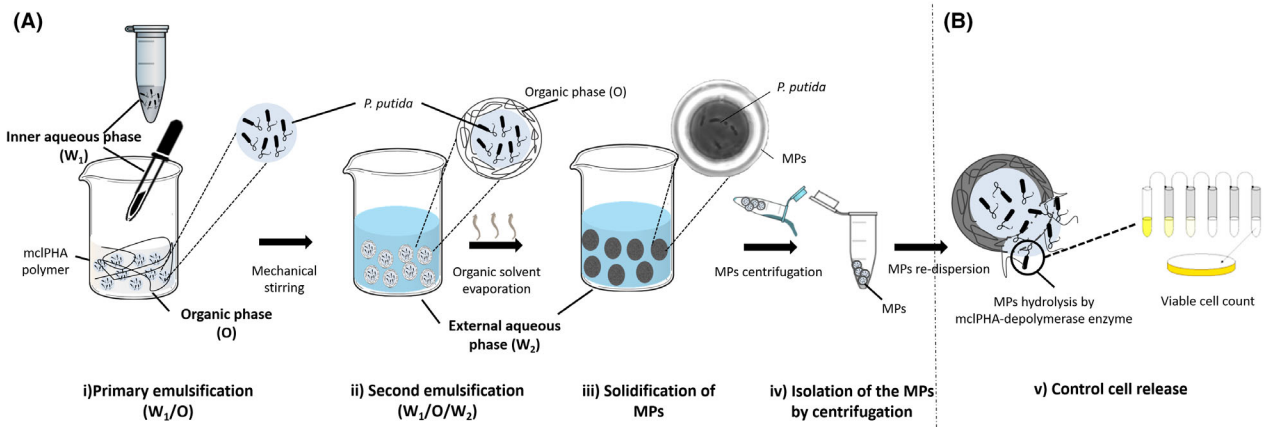

Fig. 1. Scheme of the strategy followed in this work for encapsulating microorganisms.
A. Double emulsion solvent evaporation (W_1/O/W_2) method: (i) primary emulsification: an inner aqueous suspension containing the microorganism is emulsified into an organic phase of mclPHA dissolved in organic solvent (W_1/O); (ii) secondary emulsification: W_1/O is further emulsified into an external aqueous phase (W_2) containing PVA (polyvinilalcohol) as surfactant to form the W_1/O/W_2 emulsion; (iii) solidification of the MPs: organic solvent evaporation at room temperature (iv) Isolation of the MPs by centrifugation.
B. Cell viability: (v) controlled cell release; the MPs are biodegraded by enzymatic hydrolysis and the viability of cells was monitored. See details of the protocol in Experimental Procedures.

between 1.5 and 4.0 have been reported to be highly toxic to microorganisms, due to their degree of partitioning into the aqueous cell suspension and to the high lipid membrane bilayer of the cells (Heipieper *et al.*, 2007; Ramos *et al.*, 2015).

Organic tolerance of P. putida *KT2440 to solvents.* Once we had selected EtOAc as the most suitable solvent for preparation of the MPs, we investigated the native tolerance of the model bacterium *P. putida* KT2440 strain (hereafter KT40wt) to this solvent versus the other ones considered in our research. We performed the analyses by testing cell survival by means of viable cell counting following direct exposure to the organic solvent at different concentrations (0.5–4.0% v/v) for 3 h at 30°C in LB, a rich medium (Fig. 2C), mimicking the conditions in

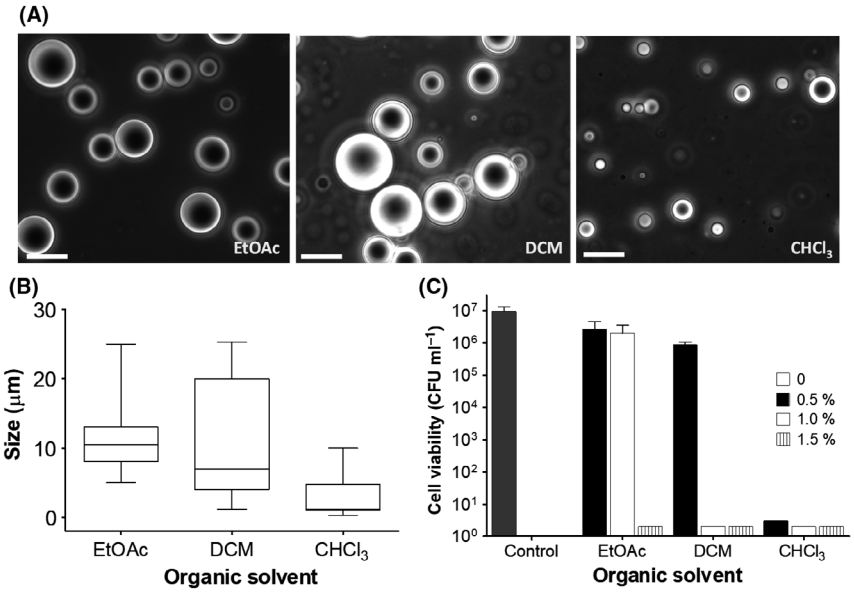

Fig. 2. Organic solvent effect on particle morphology, size distribution and *P. putida* cells viability.
A. Optical microphotography of the MPs produced by W_1/O/W_2 encapsulation method using ethyl acetate (EtOAc), or dichloromethane (DCM), or chloroform ($CHCl_3$). The scale bars represent 10 μm.
B. Particle-size distribution measured by dynamic light scattering. The population within the squares was used to calculated the polydispersity index (PDI); PDI_{EtOAc}: 0.43, PDI_{DCM}: 0.76 and PDI_{CHCl3}: 0.82. The lines in the squares represent the mean of the distribution.
C. Cell viability of *P. putida* KT2440 after 3 h of exposure at 30°C in LB rich medium supplemented with different concentrations of EtOAc, DCM or $CHCL_3$. The error bars represent the standard deviation of three different replicates.

which the cells would be exposed to the solvent during microcapsule preparation (steps i, ii and iii, Fig. 1). It was found that the KT40wt cells (bacteria at 1×10^7 CFU ml^{-1}) could tolerate EtOAc at concentrations of up to 1.0% (v/v) (Fig. 2C). No viable cells survived after exposure to higher concentrations. Interestingly, KT40wt viability showed a decrease (1 log) in the presence of DCM at 0.5% (v/v) and was totally decimated at higher concentrations (1.0–4.0% v/v). All concentrations of $CHCl_3$ tested strongly affected the integrity of the cells, completely preventing bacterial cell viability (Fig. 2C). These results confirmed EtOAc at 0.5% and 1.0% v/v as the most promising organic solvent due to the natural tolerance of the KT40wt strain, as well as its suitability for application in pharmaceutical formulations (Shivani Sujitha, 2015).

To further enhance the natural resistance of *P. putida* to EtOAc, we evolved the parental KT40wt strain by means of an adaptive laboratory evolution (ALE) approach, a frequent method for rapidly evolving strains to endow them with adaptive advantages during selection under specified growth conditions (Dragosits and Mattanovich, 2013; Sandberg *et al*., 2017). KT40wt was cultivated for several weeks in the LB medium supplemented with increasing concentrations of EtOAc (for details see Experimental Procedures). The concentration of EtOAc was increased gradually by 0.5% up to a final concentration of 1.5%. Further attempts to increase this concentration resulted in bacterial no growth. The strain evolved (hereafter KT40AE) displayed a significantly enhanced tolerance up to 1.5% v/v of EtOAc and was selected and applied in the following experiments.

Encapsulation of KT40AE within MPs. Despite the short exposure of cells to organic solvents during the encapsulation process, cell viability could be affected. The effect of the encapsulation process on KT40AE viability was analysed with the use of the optimal encapsulation conditions described above. Strikingly, the shape and size distribution of the MPs were not affected by the presence of encapsulated cells inside the capsules (Fig. 3A).

We quantified the viability of the encapsulated cells by means of enzymatic hydrolysis. We successfully established a turbidimetry assay based on the activity of the mclPHA depolymerase ($PhaZ_{Sex2}$) of *Streptomyces exfoliatus* capable of hydrolysing a suspension of PHA particles (see Experimental Procedures).

Complete hydrolysis of microcapsules was observed under 180 minutes when empty MPs and the encapsulated KT40AE strain (MPKTAE formulation) were used, because the optical density of suspensions showed a radical decrease in both cases (Fig. S1). When the MPKTAE formulations were incubated in the absence of an enzyme, no degradation was observed to occur (control sample Fig. S1). These results confirmed the ability of $PhaZ_{Sex2}$ depolymerase to hydrolyse MPs, even in the presence of bacteria, thus showing that it can be used as an appropriate releasing methodology. We subsequently monitored the *in vitro* release of *P. putida* from MPKTAE for 180 min. The MPKTAE formulation was seen to present a gradual viable cell delivery, reaching 40% of released bacteria at 60 min and 100% at 180 min. Percentage refers to the amount of viable cells released at each point over the total viable cells obtained after 180 min (Fig. 3B).

KT40AE viability was monitored in three different steps during the encapsulation process (Fig 3C): (1) a suspension of 3.5×10^5 CFU ml^{-1} of non-encapsulated cells in MOPS 25 mM pH7 at the beginning of the process (referenced as 'Free cells' in Fig. 3C), (2) a suspension of MPKTAE following solidification of the MPs (referenced as 'non-purified MPKTAE' in Fig. 3C) and (3) a suspension of purified MPKTAE after the separation and purification steps, in which non-encapsulated cells are discarded in the supernatant (referenced as 'Encapsulated Cells' in Fig. 3C).

MPKTAE exhibited a noteworthy increase in tolerance to EtOAc; the former is able to maintain cell viability at 1.18×10^5 CFU ml^{-1} (non-purified MPKTAE) after the encapsulation process, as compared with encapsulated KT40wt (2.6×10^2 CFU ml^{-1}, Fig. S2). Finally, 10% of the cells were efficiently encapsulated within MPKTAE (1.41×10^4 CFU ml^{-1}; 'Encapsulated Cells' in Fig. 3C).

According to the results shown above, it can be concluded that this protocol is capable of encapsulating KT40AE, whilst maintaining the morphology of the resulting particles. However, aside from the physicochemical properties and the immediate applicability of cell-containing MPs, successful development of a microencapsulation process relies on the maintenance of their functionality in terms of both microparticle stability and cell viability, especially under harsh conditions. To investigate the effect of the exposure to different physicochemical conditions (pH and oxidative stresses), stability over time of MPKTAE was evaluated (see below). To this end, the MPKTAE was centrifuged, washed and finally suspended in fresh medium containing the target stress solution. We then estimated cell viability by means of viable cell counting, as described in 'Experimental Procedures'.

Physical stability and cell viability of the MPKTAE aqueous suspension

The particle-size distribution of MPKTAE was analysed over 24 days with the use of dynamic light scattering. A stable profile was observed, which presented no significant shape or size differences at a storage temperature

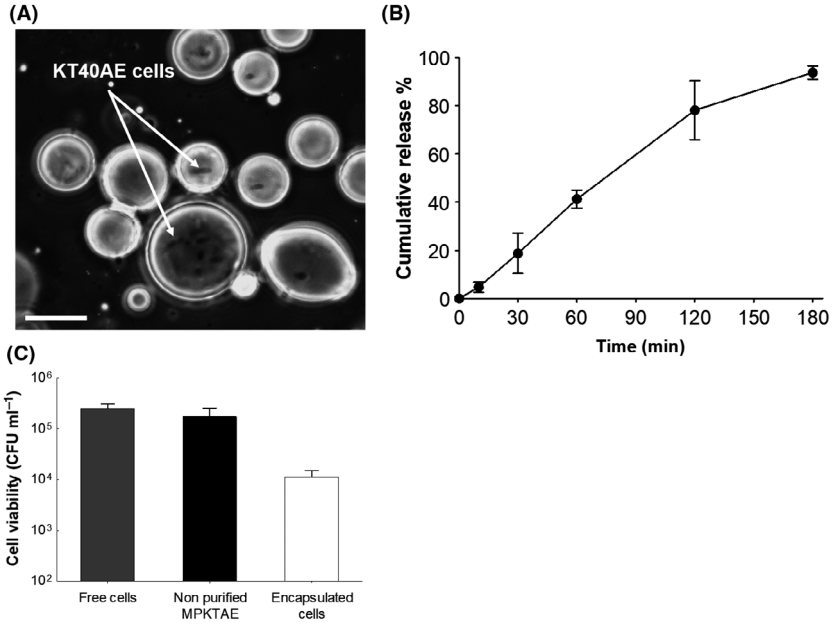

Fig. 3. Encapsulation of KT40AE strains into mclPHA-based microcapsules.
A. Optical microphotography of MPKTAE obtained by double emulsion solvent evaporation using EtOAc 1.5% v/v as organic solvent. The scale bar represents 5 μm.
B. *In vitro* release of KT40AE cells from MPKTAE by enzymatic hydrolysis using $PhaZ_{Sex2}$ depolymerase enzyme in Tris HCl 25 mM pH 7.0 during 180 min. 100% means the viable free cells after depolymerase treatment.
C. Cell viability of KT40AE cells during different steps in the encapsulation process: 'Free cells' at the beginning of the protocol, 'Non-purified MPKTAE' suspension after organic solvent evaporation (encapsulated and non-encapsulated cells) and the 'Encapsulated cells', means the viable cell number after purification. The error bars represent the standard deviation of three replicates.

of 4°C (Fig. 4A). Moreover, the empty MPs remained stable for at least 3 years under similar conditions (data not shown). Secondly, the bacterial cell viability of MPKTAE aqueous suspension was monitored over 3 weeks whilst stored at 4°C (for details see Experimental Procedures). As a control, data were compared with the conventional glycerol stock storage system (free KT40AE cells frozen at −80°C with glycerol as a cryoprotectant). Fig. 4B reveals that both storage systems provided similar viability profiles, thus highlighting the MPKTAE suspension as a suitable storage method; its functionality was preserved, both in terms of cell viability and MP stability, a fact that significantly enhances the biotechnological applicability of this formulation, as it can be stored and used when required.

Influence of pH on the physical stability and cell viability of MPKTAE. Throughout the present research, we have highlighted the advantageous use of encapsulated microorganisms as devices for protection against detrimental environmental factors, such as variations in pH. The *P. putida* strain presents an optimal pH range of 6.0–8.0, typically encountered in soil and in the rhizosphere. Since this model bacterium is considered to be an ideal candidate for biotechnological processes, as well as for bioremediation and agricultural applications (Nikel and de Lorenzo, 2018), establishing whether MP formulation protects cell viability under non-optimal environmental conditions such as acid or alkaline pH constitutes a primary objective.

MPKTAE was exposed to different pH conditions for 2 h, and viability was subsequently analysed (free KT40AE suspension was used as the control experiment). As expected, no significant decrease in cell viability was observed in MPKTAE or free KT40AE exposed to pH 7.4. In contrast, cell viability was prevented under acidic conditions, both in free KT40AE and in encapsulated cells. However, MPKTAE significantly preserves the viability of cells under alkaline conditions compared with the control culture of free cells, which completely lost their cellular integrity (Fig. 5A). These results demonstrated the potential of this MPKTAE formulation as a reservoir of bacterial cell viability for biotechnological applications requiring high alkalinity. In addition, results showed a good correlation between cell viability and MP stability. Whilst no significant changes in terms of morphology and particle-size distribution were detected in empty MPs exposed to neutral (pH 7.4) and alkaline conditions (pH 9.3), exposure to acidic conditions (pH 2.5) ostensibly modified particle stability, resulting in irregular capsules characterized by the presence of wrinkles on the surface (Fig. 5B). These data strongly

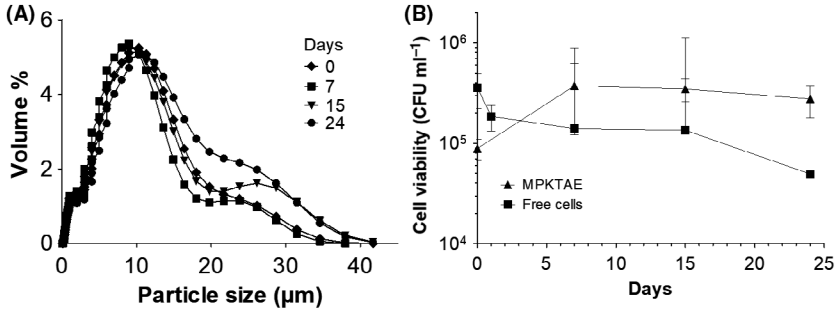

Fig. 4. Physical stability and cell viability of the MPKTAE aqueous suspension.
A. Particle-size distribution of MPKTAE stored at 4°C along 24 days, measured by dynamic light scattering at 7 (squares), 15 (inverse triangles) and 24 (circles) days. Control sample represent the MPs size distribution after purification (diamond). % Volume means the percentage that each size occupies of the overall suspension.
B. Viable cell number of MPKTAE (triangles) compared with free KT40AE standard glycerol stocks at 18% (v/v) stored at −80°C (squares, free cells). In each experiment, a glycerol stock aliquot was defrosted, and the cell viability was analysed. The procedure was repeated during three consecutive weeks. Each point was described as mean of three replicates. In parallel, the microcapsules were stored at 4°C and tested for viability by taking aliquots at the same time points than the frozen stocks. The error bars represent the standard deviation.

support the hypothesis that the protection observed under neutral and alkaline conditions is directly related to the presence of stable MPs and that it is thus a direct consequence of the microencapsulation process.

Physical stability and cell viability of MPKTAE under oxidative stress. Oxidative stress also constitutes an important environmental stress that hampers bacterial growth. Among the environmental applications of *P. putida*, the degradation of aromatic compounds has attracted much attention, but this metabolic mechanism causes bacterial cells to be exposed to different concentrations of reactive oxygen species (ROS). These components severely damage the cells and may inhibit the degradation pathways in specific biotechnological processes (Kim and Park, 2014; Nikel and de Lorenzo, 2018). In this context, we evaluated the effect of oxidative stress on cell viability. To this end, KT40AE and MPKTAE were exposed to increasing concentrations of H_2O_2, in which cell viability appears to be H_2O_2 dosage-dependent (Fig. 6A–D). Whilst no significant decrease in viability was observed at low concentrations (5 mM), high concentrations of H_2O_2 reduced the cell viability both of KT40AE and of MPKTAE. Strikingly, the encapsulated cells exhibited significantly enhanced oxidative tolerance when compared with free cells. Although exposure to 10 and 20 mM of peroxide completely prevented the viability of free cells after 24 h, the encapsulated cells remained significantly viable: 7.8×10^4 and 1.1×10^2 CFU ml^{-1} respectively. Nevertheless, polymeric aggregates appeared when the MPs were exposed to a concentration greater than 20 mM of hydrogen peroxide and experiments at higher concentrations were discarded.

Koskimäki and collaborators have reported a spontaneous H_2O_2 quenching mediated by PHA (Koskimäki *et al.*, 2016). To ascertain whether this putative protective effect was indeed exclusively due to the

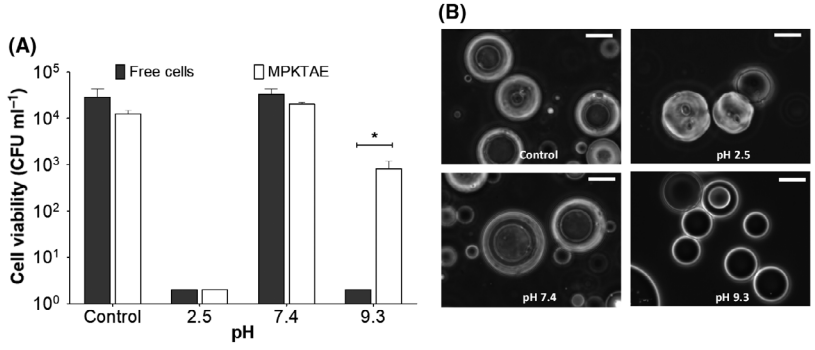

Fig. 5. Physical stability and cell viability of the MPKTAE at different pHs.
A. Effect of buffered solution at 25 mM with different pH on the viability of free KT40AE (grey bars) and MPKTAE (white bars). The MPKTAE were treated during 2 h at 30°C. Acetate, phosphate and carbonate buffers were used to prepare the different pH conditions, pH 2.5, 7.4 and 9.3 respectively. The error bars represent the standard deviation of three replicates.
B. Optical microphotography of the MPKTAE at different pH conditions after 2 h of exposure at 30°C. A water suspension of MPKTAE was used as control. The scale bars represent 10 μm.

encapsulation process, or whether it was merely a direct consequence of the presence of PHA, a free KT40AE cell suspension was exposed to increasing H_2O_2 concentrations in the presence of empty microcapsules (MPs + Free KT40AE). Cell viability quantification did not reveal any protective effects under these conditions (Fig. 6A–D), a fact that indicates that the enhanced tolerance to oxidative stress exhibited by MPKTAE resulted strictly from the encapsulation process, thus invalidating the quenching hypothesis.

Effect of lyophilization on MP stability and cell viability. As freeze-drying is a frequently used storage and preservation method (Da *et al*., 2017), we monitored the stability of MPKTAE formulation during this process. We first evaluated the integrity of empty MPs after the lyophilization process. To this end, sorbitol, fructose, glucose and sucrose at 10% w/v were tested as standard cryoprotectants (Strasser *et al*., 2009). The analyses of optical microscopy and particle-size distribution showed that the exclusive addition of sorbitol preserved the MPs from aggregation. The MPs could be effectively dispersed, resulting in similar size distribution and morphology compared with the non-treated MPs (referenced as control in Fig. S3A,B), but cell viability was drastically impacted after lyophilization; a progressive loss of cell viability was observed when MPKTAE was stored for 120 days at 4°C with sorbitol at 10% w/v as the cryoprotectant (Fig. S3C).

Extending the PHA- based encapsulation method toolbox to other microorganisms. In the present paper, we developed a PHA-based formulation to encapsulate *P. putida* cells, a model bacterium with several biotechnological applications. To verify and extend the applicability of the technology in other systems, we explored the encapsulation of other bacteria with promising biotechnological and clinical applications. *Bdellovibrio bacteriovorus* HD100 is a predatory bacterium included in the group of *Bdellovibrio* and like organisms (BALOs); it is capable of invading the periplasm of Gram-negative bacteria and of growing and replicating within this protective niche. Due to its interesting lifestyle and its ability to decimate bacterial cell populations including animal, plant and human pathogens (Sockett and Lambert, 2004; Atterbury *et al*., 2011), it has been highlighted as a potential living antibiotic, probiotic and lytic agent for biotechnological processes (Socket, 2009; Martínez *et al*., 2016). These characteristics gave rise to the idea of confining this predatory bacterium in microparticles for further application thereof or of studying its physiologic and metabolic capabilities within a controlled space.

The tolerance of *B. bacteriovorus* HD100 to different organic solvents was evaluated (Fig. 7A). This predator tolerated the EtOAc at concentrations of 0.5% and 1.0% (v/v) and the DCM at 0.5% (v/v), when cell viability after treatment decreased by 0.5–1 log respectively. After 3 h of exposure to $CHCl_3$, the predator cells did not survive

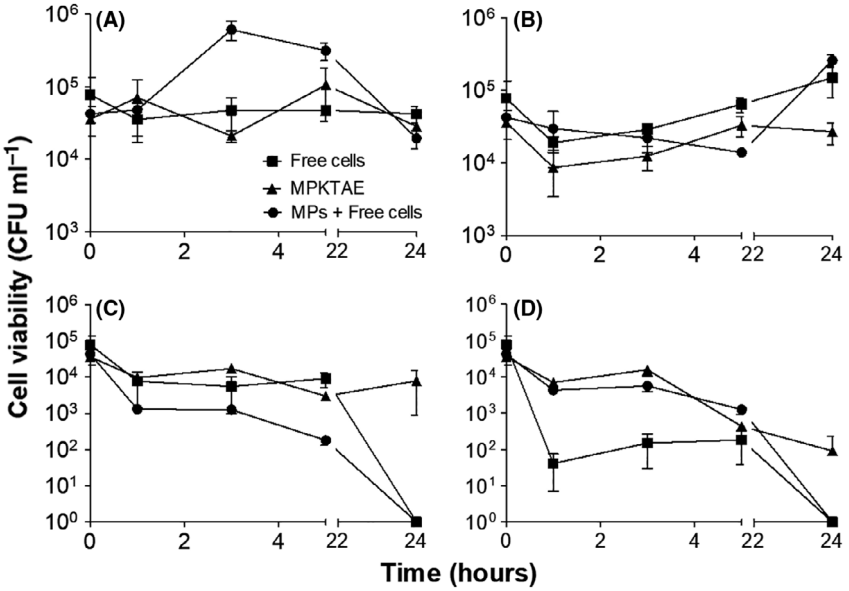

Fig. 6. Cell viability of MPKTAE under oxidative stress. Cell viability of free KT40AE (squares), MPKTAE (triangles) and free KT40AE in the presence of empty MPs (circles) exposed to different concentrations of H_2O_2 during 24 h at 30°C (A. 0 mM H_2O_2; B. 5 mM H_2O_2; C. 10 mM H_2O_2; D. 20 mM H_2O_2). The controls shown in panel A means that the samples were suspended in the absence of H_2O_2. Each point was described as mean of three replicates. The error bars represent the standard deviation.

any of the concentrations tested. Since EtOAc had previously been selected as the organic phase for the emulsification, the tolerance of *B. bacteriovorus* to this solvent prompted us to encapsulate the predator cells in accordance with the protocol developed herein (see Experimental Procedures). Figure 7B shows the cell viability of the predator at the beginning of the protocol and the encapsulated cells following purification of the MPs. The efficiency of the PHA depolymerase-based release system was confirmed; Fig. 7C reveals that the controlled release of the predatory cells reaches 70% at 30 min and almost 90% of the predatory cells at 60 min.

Discussion

Microbial biotechnology is limited by a large array of environmental stresses ultimately responsible for suboptimal industrial bioprocesses. Consequently, there is growing interest in developing both robust biocatalysts as well as improved bioprocess to ensure optimal performance of key industrial bacteria. Microorganism encapsulation represents a promising technology that protects microorganisms against different stressful physicochemical factors, thus paving the way for the development of more efficient industrial bioprocesses (Gotovtsev *et al.*, 2015).

As a proof of concept, in the present research we optimized a new mclPHA-based bacterial microencapsulation method by using the double emulsion solvent evaporation technique, which efficiently encapsulates bacterial cells whilst keeping the morphology of the resulting MPs intact. Our work demonstrates that (i) the double emulsion technology, when combined with mclPHA as encapsulation material, is an efficient method involving a long useful life for microbial encapsulation, (ii) the use of a biodegradable polymer such as mclPHA enables the controlled release of encapsulated bacteria by means of a specific enzymatic hydrolysis method, (iii) mclPHA-based microparticles effectively protect against some environmental stresses, increasing the applicability space of biotechnologically prominent bacteria such as *P. putida* KT2440 and (iv) the method and its protective effects can be extended to bacteria other than industrial microorganisms, such as the predator *B. bacteriov*orus HD100, thus increasing the applicability of the method to fields as diverse as living antibiotics.

The range of materials used for encapsulating bacterial living cells depends upon the application criteria and is primarily intended to preserve cell viability. Several parameters must be taken into account for successful encapsulation of a viable microbial cell, such as the optimal choice of biocompatible material, the organic solvent applied in the process and the use of an efficient encapsulation method. Several technologies have been tested for the production of bacteria-loaded microdevices, such as spray-drying, extrusion, phase separation and solvent

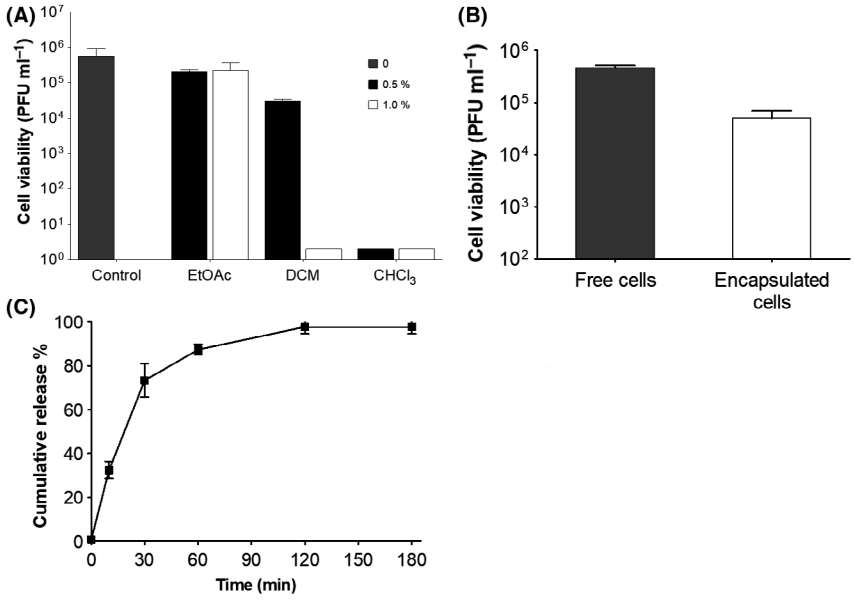

Fig. 7. Encapsulation of *B. bacteriovorus* HD100 strain into mclPHA microcapsules.
A. Organic solvent effect on *B. bacteriovorus* HD100 viability after 3 h of exposure at 30°C in DNB medium supplemented with different concentrations of EAC, DCM or $CHCl_3$ (0–1.0% v/v).
B. *B. bacteriovorus* cell viability (PFU ml^{-1}) of 'Free cells' and 'Encapsulated cells' in MPs.
C. *In vitro* release of *B. bacteriovorus* cells from MPs by mclPHA enzymatic hydrolysis using $PhaZSex_2$ enzyme in Tris HCl 25 mM pH 7.0 during 120 min. Each point was described as mean of three replicates. The error bars represent the standard deviation.

extraction/evaporation of emulsions. Almost all of these technologies were tested on probiotics bacteria (Shi *et al*., 2013; Ke *et al*., 2014; Sarioglu *et al*., 2017; San Keskin *et al*., 2018). There are many reasons for encapsulating microorganisms using the $W_1/O/W_2$ method, such as attempting to protect bacteria against stress conditions such as *Lactobacillus* spp. probiotics in contact with gastric juices. Recently, 3D microenvironments based on ionotropic hydrogels such as alginate or chitosan have been employed to contain and grow bacterial biofilms. However, these formulations swell uncontrollably and are chemically labile (Rodríguez-Huezo *et al*., 2014; El Kadri *et al*., 2015; van der Ark *et al*., 2017). Considering its biocompatible and biodegradable properties, mclPHA constitutes an attractive material for encapsulation, but the use of organic solvents during the double emulsion $W_1/O/W_2$ technology might impact cell viability in bacteria, as occurs in *P. putida*. In this sense, it has been reported that bacterial viability in the presence of organic solvents depends upon the inherent toxicity of the solvent and on the intrinsic tolerance of each particular bacterial strain (Torres *et al*., 2011; Hosseini *et al*., 2017). Indeed, the cell viability of KT40wt when encapsulated was highly compromised following the encapsulation process (Fig. S2) in comparison with the viability of *B. bacteriovorus* (Fig 7). However, the enhanced tolerance of the adapted *P. putida* KT40AE strain to EtOAc resulted in a significant increase in viability resistance after the encapsulation process when compared with the parenteral KT40wt strain (Fig. S2). These results confirmed that ALE strategies can be established for improving bacterial solvent tolerance, as previously described (Atsumi *et al*., 2010).

Regarding the encapsulation efficiency, previous studies on the encapsulation of microorganisms using alginates or chitosan have reported high encapsulation efficiencies (more than 80%) (Shi *et al*., 2013; D'Orazio *et al*., 2015). Nevertheless, very low cell viability (< 5% w/w) was monitored when PLGA polymer was used (Della Porta *et al*., 2012). Moreover, due to these formulations are chemically labile and swell uncontrollably, the microparticles stability and cell viability could be compromised when these polymeric microcarriers devices are stored in aqueous suspension. The method described in this work resulted in an encapsulation efficiency measured by mean of viable cells of 10% with respect to the loaded microorganisms. Overall, its functionality was preserved, both in terms of cell viability and MP stability, a fact that significantly enhance the biotechnological applicability of this formulation, as it can be stored and used when required during 3 weeks at least. Moreover, it is worth to highlight that empty mclPHA microcapsules remained stable for at least 3 years.

MPKTAE formulation preserves its functionality as a polymeric reservoir of bacterial cell viability over time, protecting cell viability in unfavourable environments, such as alkaline pH conditions and oxidative stress up to a certain level of exposure to H_2O_2 (Figs 5 and 6). Oxidative stresses are a well-known physicochemical insult with a negative impact upon bacterial-driven biotechnological processes. In this sense, our results pave the way for further developments focused upon *P. putida* as a biocatalyst for industrial biotransformations.

An encapsulated microorganism can be released from polymer-based MPs by many different means, including fracture by heat, solvent action, diffusion and pressure; the complete abiotic degradation of this compound often occurs over several months (Lim *et al*., 2017). When the encapsulating material is biodegradable, biotic delivery strategies come into play. The release of microorganisms from mclPHA-based microparticles can be performed by extracellular PHA depolymerases, such as $PhaZsex_2$ from *Streptomyces 17exfoliates* (Martínez *et al*., 2015), or even directly by the microorganisms producing these extracellular enzymes, when environmental applications are considered. Extracellular PHA depolymerases are widely distributed throughout different microorganisms and are ubiquitous in the environment. The ability to degrade extracellular PHA in the environment in order to use the released oligomers and monomers as carbon sources for growth constitutes an ecological advantage for the microorganism in this niche (Jendrossek, 2005). Interestingly, *B. bacteriovorus* HD100 possesses, as part of its hydrolytic arsenal, two extracellular PHA depolymerases, capable of degrading the capsule. Since *B. bacteriovorus* has been proposed as a 'living antibiotic', the use of encapsulated predators offers the possibility to use MPs for clinical applications (Martínez *et al*., 2012, 2013). Thus, the novelty of this release system based on enzymatic hydrolysis provides the possibility to develop autodelivery carrier systems based upon encapsulated microorganisms capable of controlled self-release from the PHA microparticles. In this sense, we have not observed autorelease capacity of *B. bacteriovorus* in the conditions assayed in this work suggesting that these strategies based on controlled self-release would need deep engineering of the predator. Moreover, precise studies to determine the porosity and permeability of the MPs would be necessary to identify potential molecules that could be used as inducer of those controlled expression systems. In summary, the present study highlights the potential use of mclPHA encapsulated bacteria in different biotechnological processes; in addition, our research highlights the applicability of this formulation in different fields, such as therapy or ecology.

Experimental procedures

Materials

MPs were prepared with the use of mclPHA, a heteropolymer of poly-(hydroxyoctanoate-*co*-hydroxyhexanoate) [PHO-*co*-HH) (Bioplastech, Dublin, Ireland) and stabilized with polyvinylalcohol (PVA, mol. wt.:30 000–55 000, Aldrich Chemical). All other reagents, such as EtOAc, DCM or $CHCl_3$, were of analytical grade and were used without further purification.

Strains and growth conditions

P. putida KT2440 and the evolved strain KT40AE were grown in lysogeny broth (LB) at 30°C with shaking at 250 rpm. Growth was monitored with a Shimadzu UV-260 spectrophotometer at 600 nm (OD_{600}). Solid media were prepared with agar at 1.5% (w/v). The *B. bacteriovorus* HD100 strain was routinely grown in coculture in Hepes buffer (25 mM Hepes amended with 2 mM $CaCl_2{\cdot}2H_2O$ and 3 mM $MgCl_2{\cdot}3H_2O$, pH 7.8) or DNB liquid medium (consisting of 0.8 g l^{-1} NB supplemented with 2 mM $CaCl_2$ and 3 mM $MgCl_2$), with *P. putida* KT2440 as prey, as previously described (Martínez *et al*., 2016). Prey cultures were prepared from cells grown in NB for 16 h and diluted to OD_{600} of 1 in Hepes buffer. After predation, the cocultures were filtered twice through a 0.45-μm filter (Sartorius) and the *B. bacteriovorus* cells were centrifuged at 14 000 *g*, 4°C, 15 min. This pellet was subsequently suspended in 1–2 ml of MOPS buffer and used in the encapsulation protocol.

P. putida *and* B. bacteriovorus *viability calculations*

To calculate cell viability, serial dilutions from 10^{-1} to 10^{-7} were made in saline solution. For the *P. putida* strains, three different spots of 10 μl of each dilution were plated on LB solid medium, and colony-forming units (CFU) were counted. We calculated the viability of *B. bacteriovorus* by counting the plate-forming units (PFU) following the double layer method previously described (Herencias *et al*., 2017). To calculate *B. bacteriovorus* viability, 0.1 ml of the appropriate dilution was mixed with an additional 0.5 ml of prey cell suspension of *P. putida* KT2440 pre-grown in NB, prepared in Hepes buffer at OD_{600} of 10, vortexed and plated on DNB solid medium based on the double agar overlay method. Predators were counted after 48 h of incubation at 30°C.

Encapsulation method

The MPs were prepared in accordance with the previously described double emulsion solvent evaporation method (González-Alvarez *et al*., 2013) adapted to mclPHA as an encapsulating polymer (Fig. 1). Briefly, the inner aqueous phase (W_1), consisting of 0.2 ml aqueous solution of MOPS (3-(N-morpholino) propanesulfonic acid) buffer at 25 mM pH 7.0 (in the presence or absence of suspended bacteria), was added to 12 mg of mclPHA previously dissolved in 0.2 ml of one of the selected organic solvents (organic phase, O). The W_1 was then emulsified with the organic phase by slow manual stirring (with a Pasteur pipette) for 1 min. The primary emulsion (W_1/O) was then immediately emulsified with 5 ml of external aqueous phase (W_2), with PVA (4.0% w/v) developing the double emulsion (W_1/O/W_2). The organic solvent was evaporated by mechanical stirring at 300 rpm for 180 min at room temperature in order to obtain an aqueous suspension of MPs. The MPs were then purified from the bulk aqueous phase by centrifugation at 4000 *g* (MICRO 185, Hettich Zentrifugen) for 5 min at room temperature and were washed twice with deionized water (Fig. 1). The organic solvents evaluated were DCM, $CHCl_3$ and EtOAc at 4% v/v final concentration.

For bacteria encapsulation, KT40wt, KT40AE or *B. bacteriovorus* HD100 strains at 1×10^5 CFU or PFU ml^{-1} were dispersed into the inner aqueous solution (W_1) and encapsulated following the methodology described in Fig. 1.

Characterization of the microparticles

Particle shape and morphology were evaluated by means of optical microscopy (phase-contrast microscopy, Nikon Instruments, model: OPTIFHOT-2). The polydispersity index (PDI) and microparticle size were estimated with dynamic light scattering which measures forward scattering (diffraction) of light from a single laser (wavelength of 750 nm) (Beckman Coulter/Small volume model plus LS230). Moreover, PDI was calculated by dividing standard deviation by average particle size.

Effect of organic solvents on bacterial cell viability

Bacteria were cultured as described above. Subsequently, cells of *P. putida* or *B. bacteriovorus* were harvested by centrifugation at 14 000 *g* for 3–10 min (Minispin eppendorf microfuge) and washed twice with saline solution (NaCl 0.85% (w/v)). The washed biomass was split and suspended in 10 ml of LB (or DNB medium for *B. bacteriovorus*) supplemented with different organic solvents (DCM, $CHCl_3$ and EtOAc) at concentrations of 0.5%, 1.0%, 1.5%, 2.5% and 4.0% v/v at 30°C, 250 rpm for 3 h. The initial optical density (OD_{600}) was set to 1.0 (9.5×10^6 CFU ml^{-1} for *P. putida* strains and 7×10^5 PFU ml^{-1} for the predator cells).

Adaptive laboratory evolution approach (ALE) involving P. putida *KT2440 for improving EtOAc tolerance*

We performed an adaptive laboratory experiment evolving the KT40wt strain by means of sequential serial passages cultivated for several weeks according to the previously described method (Pfeifer *et al*., 2017). The KT40wt strain was initially cultured in LB, a rich medium, until the stationary phase was reached. The culture was serially transferred from the stationary phase into fresh medium every 24 h for several passes, and the initial OD_{600} was adjusted to 0.1. We commenced the evolution experiments with two biological replicates in LB medium containing 1.0% (v/v) of EtOAc, gradually increasing this percentage to 4.0% v/v as the bacterial adaptation proceeded. In total, 28 serial transfers were performed during this ALE experiment. Samples were taken and stored as glycerol stocks after each transfer. We monitored the KT40wt evolution, measuring the increase in OD_{600}. To ensure that the adaptation phenomenon was the result of a genotypic change, aliquots of the evolved population culture were spread over plates containing agar LB medium and incubated at 30°C. Following 24 h of incubation, eight single colonies were picked and isolated. We performed three subsequent transfers on LB plates in the absence of EtOAc. Each strain was subsequently inoculated in liquid LB supplemented with EtOAc at 1.5% v/v for culturing. Potential culture contamination was discarded with rRNA16S PCR amplification and sequencing with the use of the primers: 16S1: 5′-AAGGAGGTGATCCAGCC-3′; 16S2: 5′-GAGASTTTGATCHTGGCTGCA-3′; 63F: 5′-CAGGCCTAACACATGCAAGTC-3′ and 1387R: 5′-GGGCGGWGTGTACAAGGC-3′. All the colonies tested (hereafter the KT40AE strain) were capable of growth in the presence of EtOAc at 1.5%. One single colony was stored and used for the remaining experiments.

Cell viability of bacteria encapsulated in MPs

We established a method for cell release from MPKTAE or microparticles carrying *B. bacteriovorus* based on the hydrolytic activity of the mclPHA depolymerase of *Streptomyses exfoliatus* K_{10} DSMZ 41693. The enzyme was purified as previously described, and its enzymatic activity was tested by means of measurements of the decrease in turbidity of a homogeneous MP suspension at 600 nm (Fisher Scientific Spectrophotometer) (Martínez *et al*., 2015). Although optimal reaction conditions require an alkaline pH, we modified the method to avoid cell damage, using for the reaction mixture a solution of Tris HCl 25 mM pH 7.0 and 2.5 mg ml^{-1} of mclPHA particles ($PhaZ_{Sex2}$/PHA ratio concentration of 3 μg mg^{-1}). The enzymatic reaction (1 ml) was started by the addition of 7.5 μg of pure enzyme and incubation at 30°C for 180 min. The decrease in turbidity at $OD_{600\ nm}$ was measured at 0, 5, 15, 30, 60, 120 and 180 min. Controls without the enzyme were performed in parallel to ascertain the non-enzymatic hydrolysis of MPs (data not shown). Following cell release from the microparticles, we counted cell viability, expressed as the mean ± standard deviation of three independent experiments. Control of the free cells incubated with the enzyme reveals a decrease in cell viability of 1 log both for KT40AE and for *B. bacteriovorus* HD100; thus, this loss of viability was taken into consideration for calculation of the encapsulated cells.

Stability of MPKTAE under different physicochemical conditions

The MPKTAE formulation was exposed independently to physicochemical stresses over time (Bojanovič *et al*., 2017). Thus, MPKTAE was centrifuged at 4000 *g* for 5 min, washed twice with deionized water and aliquoted separately in 1 ml of sterilized fresh medium under stress conditions.

To study the effect of pH upon microparticle stability and cell viability, MPKTAE and free KT40AE were dispersed into different buffers: sodium acetate 25 mM pH 2.5; sodium phosphate 25 mM pH 7.4, and sodium carbonate 25 mM pH 9.3 for 2 h at 30°C with 1 ml of volume. In order to evaluate the effect of oxidative stress, the MPKTAE and free cells were dispersed into 0.1 N M63 medium (13.6 g KH_2PO_4 l^{-1}, 0.2 g $(NH_4)_2SO_4$ l^{-1}, 0.5 mg $FeSO_4{\cdot}7H_2O$ l^{-1}, adjusted to pH 7.0 with NaOH), without a carbon source, and exposed separately to different concentrations of hydrogen peroxide (5, 10 and 20 mM). Cell viability counting was determined as described above.

To compare the viability of the MPKTAE formulation stored at 4°C versus glycerol frozen stocks at −80°C over the time, we prepared frozen aliquots of 18% v/v glycerol stocks from a single overnight cell culture of the evolved strain for ensuring the same number of cells in all aliquots. In each experiment, an aliquot (in triplicate) were defrosted, and the cell viability was analysed. The procedure was repeated during three consecutive weeks (Fig. 4B). In parallel, the microcapsules were stored at 4°C and tested for viability by taking aliquots at the same time points than the frozen stocks.

To analyse the effect of freeze-drying on microorganism viability and MP stability, cryoprotectants such as fructose, glucose, sucrose or sorbitol were added (10% w/v) to the external aqueous solution (W_2) during the formation of the second emulsion (Fig. 1). The MPKTAE formulations were then aliquoted and frozen by ultra-freezing at −80°C for 24 h. Subsequently, the dried

formulations were lyophilized for 24 h and stored at 4°C in a desiccator box following their evaluation.

Phase contrast microscopy

Cultures and MPs were routinely visualized with the use of a 100× phase-contrast objective, and photographs were taken with a Leica DFC345 FX camera.

Statistical analysis

We analysed the data sets with Prism 7 software (GraphPad Software, San Diego, CA, USA). Comparisons between the two groups were made with the use of the Student's *t* test. We compared multiple groups using the one-way or the two-way analysis of variance test, depending on whether one or two different variables were considered respectively.

Acknowledgements

The authors thank for the financial support from the Spanish Ministry of Science, Innovation and Universities (BIO2014-61515-EXP, BIO2017-83448-R) and the Community of Madrid (P2013/MIT2807, P2018/NMT4389). The contribution of Enrique Cubas in ALE experiments is much appreciated. E.G. wishes to acknowledge the 251952 financial postdoctoral scholarship number by CONACyT, México.

Conflict of interest

None declared.

References

Albadran, H.A., Chatzifragkou, A., Khutoryanskiy, V.V., and Charalampopoulos, D. (2015) Stability of probiotic *Lactobacillus plantarum* in dry microcapsules under accelerated storage conditions. *Food Res Int* **74:** 208–216.

van der Ark, K.C.H., Nugroho, A.D.W., Berton-Carabin, C., Wang, C., Belzer, C., de Vos, W.M., and Schroen, K. (2017) Encapsulation of the therapeutic microbe *Akkermansia muciniphila* in a double emulsion enhances survival in simulated gastric conditions. *Food Res Int* **102:** 372–379.

Arslan, S., Erbas, M., Tontul, I., and Topuz, A. (2015) Microencapsulation of probiotic *Saccharomyces cerevisiae* var. *boulardii* with different wall materials by spray drying. *LWT: J Food Sci Technol* **63:** 685–690.

Atsumi, S., Wu, T.Y., Machado, I.M., Huang, W.C., Chen, P.Y., Pellegrini, M., and Liao, J.C. (2010) Evolution, genomic analysis, and reconstruction of isobutanol tolerance in *Escherichia coli*. *Mol Syst Biol* **6:** 449.

Atterbury, R.J., Hobley, L., Till, R., Lambert, C., Capeness, M.J., and Lerner, T.R. (2011) Effects of orally administered *Bdellovibrio bacteriovorus* on the well-being and Salmonella colonization of young chicks. *Appl Environ Microbiol* **77:** 5794–5803.

Bojanovič, K., D'Arrigo, I., and Long, K. S. (2017) Global transcriptional responses to osmotic, oxidative, and imipenem stress conditions in Pseudomonas putida. *Appl Environ Microbiol* **83:** e03236–16.

Da, T., Kiyoshi, K., Shuto, M., and Yoshio, H. (2017) Stabilization of freeze-dried *Lactobacillus paracasei subsp. paracasei* JCM 8130T with the addition of disaccharides, polymers, and their mixtures. *Biosci Biotechnol Biochem* **81(4):** 768–773.

Della Porta, G., Castaldo, F., Scognamiglio, M., Paciello, L., Parascandola, P., and Reverchon, E. (2012) Bacteria microencapsulation in PLGA microdevices by supercritical emulsion extraction. *J Supercrit Fluids* **63:** 1–7.

D'Orazio, G., Di Gennaro, P., Boccarusso, M., Presti, I., Bizzaro, G., and Giardina, S. (2015) Microencapsulation of new probiotic formulations for gastrointestinal delivery: in vitro study to assess viability and biological properties. *Appl Microbiol Biotechnol* **99:** 9779–9789.

Dragosits, M., and Mattanovich, D. (2013) Adaptive laboratory evolution – principles and applications for biotechnology. *Microb Cell Fact* **12:** 64.

El Kadri, H., Overton, T., Bakalis, S., and Gkatzionis, K. (2015) Understanding and controlling the release mechanism of *Escherichia coli* in double W1/O/W2 emulsion globules in the presence of NaCl in the W2 phase. *RSC Adv* **5:** 105098–105110.

González-Alvarez, E., Castro, R., Luna, H.A., González, A., Galindo, S.A., and Chavez, A. (2013) Polymeric microparticles containing protein prepared using a controllable combination of diffusion and emulsification steps as part of the salting out procedure. *Afr J Pharm Pharmacol* **7:** 2849–2858.

Gotovtsev, P.M., Yuzbasheva, E.Y., Gorin, K.V., Butylin, V.V., Badranova, G.U., and Perkovskaya, N.I. (2015) Immobilization of microbial cells for biotechnological production: modern solutions and promising technologies. *Appl Biochem Biotech* **51:** 792–803.

Heipieper, H.J., Neumann, G., Cornelissen, S., and Meinhardt, F. (2007) Solvent-tolerant bacteria for biotransformations in two-phase fermentation systems. *Appl Microbiol Biotechnol* **74:** 961–973.

Herencias, C., Prieto, M., and Martínez, V. (2017) Determination of the Predatory Capability of *Bdellovibrio bacteriovorus* HD100. *Bio Protoc* **7(6):** 2177.

Hosseini, R., Kuepper, J., Koebbing, S., Blank, L.M., Wierckx, N., and de Winde, J.H. (2017) Regulation of solvent tolerance in *Pseudomonas putida* S12 mediated by mobile elements. *Microb Biotechnol* **10:** 1558–1568.

Iqbal, M., Zafar, N., Fessi, H., and Elaissari, A. (2015) Double emulsion solvent evaporation techniques used for drug encapsulation. *Int J Pharm* **496:** 173–190.

Jendrossek, D. (2005) Extracellular polyhydroxyalkanoate (PHA) depolymerases: the key enzymes of PHA degradation. In *Biopolymers Online*, Wiley-VCH, Weinheim, Germany, Steinbüchel, A. (ed.). pp. 42–75.

Ke, Y., Liu, G.S., Wang, J.H., Xue, W., Du, C., and Wu, G. (2014) Preparation of carboxymethyl cellulose based microgels for cell encapsulation. *Express Polym Lett* **8:** 841–849.

Khem, S., Small, D.M., and May, B.K. (2016) The behavior of whey protein isolate in protecting *Lactobacillus plantarum*. *Food Chem* **190:** 717–723.

Kim, J., and Park, W. (2014) Oxidative stress response in *Pseudomonas putida*. *Appl Microbiol Biotechnol* **98:** 6933–6946.

Kniewel, R., Revelles Lopez, O., and Prieto, M.A. (2017) Biogenesis of medium-chain-length polyhydroxyalkanoates. In *Biogenesis of Fatty Acids, Lipids and Membranes*. Geiger, O. (ed). Cham, Switzerland: Springer International Publishing, pp. 1–25.

Koskimäki, J.J., Kajula, M., Hokkanen, J., Ihantola, E.-L., Kim, J.H., and Hautajärvi, H. (2016) Methyl-esterified 3-hydroxybutyrate oligomers protect bacteria from hydroxyl radicals. *Nat Chem Biol* **12:** 332.

Lim, J., You, M., Li, J., and Li, Z. (2017) Emerging bone tissue engineering via Polyhydroxyalkanoate (PHA)-based scaffolds. *Mater Sci Eng C* **79:** 917–929.

Lizarraga-Valderrama, L.R., Panchal, B., Thomas, C., Boccaccini, A.R., and Roy, I. (2016) Biomedical applications of polyhydroxyalkanoates. In *Biomaterials from Nature for Advanced Devices and Therapies*. Reis, N.M.N.R.L. (ed). Hoboken, NJ: Society for Biomaterials Wiley, pp. 339–383.

Martínez, V., de la Peña, F., García-Hidalgo, J., de la Mata, I., García, J.L., and Prieto, M.A. (2012) Identification and biochemical evidence of a medium-chain-length polyhydroxyalkanoate depolymerase in the *Bdellovibrio bacteriovorus* predatory hydrolytic arsenal. *J Appl Environ Microbiol* **78:** 6017–6026.

Martínez, V., Jurkevitch, E., García, J.L., and Prieto, M.A. (2013) Reward for *Bdellovibrio bacteriovorus* for preying on a polyhydroxyalkanoate producer. *Environ Microbiol* **15:** 1204–1215.

Martínez, V., de Santos, P.G., García-Hidalgo, J., Hormigo, D., Prieto, M.A., Arroyo, M., and de la Mata, I. (2015) Novel extracellular medium-chain-length polyhydroxyalkanoate depolymerase from *Streptomyces exfoliatus* K10 DSMZ 41693: a promising biocatalyst for the efficient degradation of natural and functionalized mcl-PHAs. *Appl Microbiol Biotechnol* **99:** 9605–9615.

Martínez, V., Herencias, C., Jurkevitch, E., and Prieto, M.A. (2016) Engineering a predatory bacterium as a proficient killer agent for intracellular bio-products recovery: the case of the polyhydroxyalkanoates. *Sci Rep* **6:** 24381.

Nikel, P.I., and de Lorenzo, V. (2018) *Pseudomonas putida* as a functional chassis for industrial biocatalysis: from native biochemistry to trans-metabolism. *Metab Eng* **50:** 142–155.

O'Riordan, K., Andrews, D., Buckle, K., and Conway, P. (2001) Evaluation of microencapsulation of a *Bifidobacterium* strain with starch as an approach to prolonging viability during storage. *J Appl Microbiol* **91:** 1059–1066.

Pfeifer, E., Gätgens, C., Polen, T., and Frunzke, J. (2017) Adaptive laboratory evolution of *Corynebacterium glutamicum* towards higher growth rates on glucose minimal medium. *Sci Rep* **7:** 16780.

Ramos, J.-L., Sol Cuenca, M., Molina-Santiago, C., Segura, A., Duque, E., and Gómez-García, M.R. (2015) Mechanisms of solvent resistance mediated by interplay of cellular factors in *Pseudomonas putida*. *FEMS Microbiol Rev* **39:** 555–566.

Ranadheera, C.S., Evans, C.A., Adams, M.C., and Baines, S.K. (2015) Microencapsulation of *Lactobacillus acidophilus* LA-5, *Bifidobacterium animalis* subsp. l*actis* BB-12 and *Propionibacterium jensenii* 702 by spray drying in goat's milk. *Small Rumin Res* **123:** 155–159.

Rodríguez-Huezo, M.E., Estrada-Fernández, A.G., García-Almendárez, B.E., Ludeña-Urquizo, F., Campos-Montiel, R.G., and Pimentel-González, D.J. (2014) Viability of *Lactobacillus plantarum* entrapped in double emulsion during Oaxaca cheese manufacture, melting and simulated intestinal conditions. *LWT: J Food Sci Technol* **59:** 768–773.

San Keskin, N.O., Celebioglu, A., Sarioglu, O.F., Uyar, T., and Tekinay, T. (2018) Encapsulation of living bacteria in electrospun cyclodextrin ultrathin fibers for bioremediation of heavy metals and reactive dye from wastewater. *Colloids Surf B Biointerfaces* **161:** 169–176.

Sandberg, T.E., Lloyd, C.J., Palsson, B.O., and Feist, A.M. (2017) Laboratory evolution to alternating substrate environments yields distinct phenotypic and genetic adaptive strategies. *Appl Environ Microbiol* **83:** 410–417.

Sarioglu, O.F., San Keskin, N.O., Celebioglu, A., Tekinay, T., and Uyar, T. (2017) Bacteria immobilized electrospun polycaprolactone and polylactic acid fibrous webs for remediation of textile dyes in water. *Chemosphere* **184:** 393–399.

Shi, L.E., Li, Z.H., Li, D.T., Xu, M., Chen, H.Y., Zhang, Z.L., and Tang, Z.X. (2013) Encapsulation of probiotic *Lactobacillus bulgaricus* in alginate–milk microspheres and evaluation of the survival in simulated gastrointestinal conditions. *J Food Eng* **117:** 99–104.

Shivani Sujitha, H. (2015) Review article on microparticles. *IJPAR* **4(3):** 302–309.

Sipailienė, A., and Petraitytė, S. (2018) Encapsulation of probiotics: proper selection of the probiotic strain and the influence of encapsulation technology and materials on the viability of encapsulated microorganisms. *Probiotics Antimicrob Proteins* **10:** 1–10.

Socket, R.E. (2009) Predatory lifestyle of *Bdellovibrio bacteriovorus*. *Annu Rev Microbiol* **63:** 523–539.

Sockett, R.E., and Lambert, C. (2004) *Bdellovibrio* as therapeutic agents: a predatory renaissance? *Nat Rev Microbiol* **2:** 669.

Song, K.C., Lee, H.S., Choung, I.Y., Cho, K.I., Ahn, Y., and Choi, E.J. (2006) The effect of type of organic phase solvents on the particle size of poly(d, l-lactide-*co*-glycolide) nanoparticles. *Colloids Surf A Physicochem Eng Asp* **276:** 162–167.

Strasser, S., Neureiter, M., Geppl, M., Braun, R., and Danner, H.S. (2009) Influence of lyophilization, fluidized bed drying, addition of protectants, and storage on the viability of lactic acid bacteria. *J Appl Microbiol* **107:** 167–177.

Torres, S., Pandey, A., and Castro, G.R. (2011) Organic solvent adaptation of Gram positive bacteria: applications and biotechnological potentials. *Biotechnol Adv* **29:** 442–452.

Wang, C., Sauvageau, D., and Elias, A. (2016) Immobilization of active bacteriophages on polyhydroxyalkanoate surfaces. *ACS Appl Mater Interfaces* **8:** 1128–1138.

Zhang, J., Shishatskaya, E.I., Volova, T.G., da Silva, L.F., and Chen, G.-Q. (2018) Polyhydroxyalkanoates (PHA) for therapeutic applications. *Mater Sci Eng C* **86:** 144–150.

Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig. S1. Monitoring the enzymatic hydrolysis of MPs. Depolymerization kinetics curves of empty MPs (squares) and the MPKTAE (circles) as substrates dispersed into Tris HCl 25 mM pH 7.0 during 180 minutes using $PhaZ_{sex2}$ depolymerase. Control sample was prepared in the absence of $PhaZ_{sex}$ (triangles). Optical density was measured at 600 nm.

Fig. S2. Efficacy of the encapsulation with KT40wt and KT40AE. Cell viability assay of the different steps of the encapsulation process using the wild type strain KT40wt (black bars) and the EtOAc tolerant KT40AE (white bars). Free cells were treated with mclPHA depolymerase, mimicking the MPs hydrolysis process. The error bars represent the standard deviation.

Fig. S3. Effect of lyophilization process on microcapsules stability and cells viability. A. Microphotographs of control and lyophilized MPs using different cryoprotectants into the external aqueous phase (W_2) during encapsulation method. The scale bars represent 10 μm. B. Particle size distribution of MPs lyophilized (squares) and without treatment (circles) using sorbitol at 10% w/v as protectant, measured by dynamic light scattering**.** % Volume means the percentage that each size occupies of the overall suspension. C. Cell viability of free KT40AE dispersed glycerol storage at −80°C (squares) and the MPKTAE (triangle) during 120 days stored at 4°C, after lyophilization process in the presence of sorbitol 10% w/v.

Table S1. Characteristics of organic solvents considered in this work.

