microbial biotechnology

Microbial Biotechnology (2011) 4(4), 533–547 doi:10.1111/j.1751-7915.2011.00257.x

Controlled autolysis facilitates the polyhydroxyalkanoate recovery in *Pseudomonas putida* KT2440

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Summary

The development of efficient recovery processes is essential to reduce the cost of polyhydroxyalkanoates (PHAs) production. In this work, a programmed self-disruptive *Pseudomonas putida* BXHL strain, derived from the prototype medium-chain-length PHA producer bacterium *P. putida* KT2440, was constructed as a proof of concept for exploring the possibility to control and facilitate the release of PHA granules to the extracellular medium. The new autolytic cell disruption system is based on two simultaneous strategies: the coordinated action of two proteins from the pneumococcal bacteriophage EJ-1, an endolysin (Ejl) and a holin (Ejh), and the mutation of the *tolB* gene, which exhibits alterations in outer membrane integrity that induce lysis hypersensitivity. The *ejl* and *ejh* coding genes were expressed under a XylS/*Pm* monocopy expression system inserted into the chromosome of the *tolB* mutant strain, in the presence of 3-methylbenzoate as inducer molecule. Our results demonstrate that the intracellular presence of PHA granules confers resistance to cell envelope. Conditions to control the cell autolysis in *P. putida* BXHL in terms of optimal fermentation, PHA content and PHA recovery have been set up by exploring the sensitivity to detergents, chelating agents and wet biomass solubility in organic solvents such as ethyl acetate.

Introduction

Human overpopulation combined with the current lifestyle urges the rational, efficient and sustainable use of natural resources to produce environmentally friendly plastic materials such as polyhydroxyalkanoic acids (PHAs), whose production/degradation cycle reduces undesirable wastes and emissions (Gavrilescu and Chisti, 2005). PHAs are optically active biopolyoxoesters composed of (*R*) 3-hydroxy fatty acids, which represent a complex class of storage polyesters. They are synthesized by some Archaea and a wide range of Gram-positive and Gram-negative bacteria in aerobic and anaerobic environments (Madison and Huisman, 1999). These biopolymers are accumulated as inclusions (PHA granules) in the bacterial cytoplasm in response to inorganic nutrient limitations, generally, when the microbes are cultured in the presence of an excess carbon source (Madison and Huisman, 1999). At present, PHAs are classified in two major classes: short-chain-length PHAs (scl-PHAs) with C4-C5 monomers and medium-chain-length PHAs (mcl-PHAs) with C6-C14 monomers. Mcl-PHAs are mainly produced by *Pseudomonas* species (revised in Prieto *et al.*, 2007). Because of structural differences, the physical properties of mcl-PHAs are generally quite different from the archetypal polyhydroxybutyrate (PHB) and other scl-PHAs (Gagnon *et al.*, 1992).

Using currently available technology, large-scale production of PHA is suitable with expenditures almost evenly divided between carbon source, fermentation process and separation process (30% each) (Sun *et al.*, 2007; Elbahloul and Steinbüchel, 2009). Because PHAs accumulate intracellularly, the development of an efficient recovery process is indispensable to reduce the total cost of PHAs production (Prieto, 2007). At present, different separation processes have been described, like filtration, froth flotation (van Hee *et al.*, 2006) and continuous centrifugation (Gorenflo *et al.*, 2001). Recovery procedures for mcl-PHA mainly resemble those developed for PHB (Ramsay *et al.*, 1994). PHB recovery using hypochlorite, SDS in an *in situ* extraction process (Thakor *et al.*, 2005), or an enzyme cocktail (de Koning *et al.*, 1997; Kellerhals *et al.*, 1999) have been reported. The disadvantages related to applications of hypochlorite and detergents are the severe reduction in polymer molecular weight and the requirement of extensive washing steps to get rid of detergent residuals respectively. Furthermore, the use of acetone, ethylacetate or hexane extraction of PHAs from

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Table 1. Bacterial strains, plasmids and oligonucleotides used in this study.

Strain	Relevant genotype	Reference
E. coli		
CC118λ*pir*	Δ(*ara-leu*) *araD*, Δ*lacX74*, *galE*, *galK*, *phoA20*, *thi-1*, *rpsE*, *rpoB*, *argE* (Am), *recA1* Rf^{r}, Sp^{r}, λ*pir*	Herrero *et al.* (1990)
P. putida		
KT2440	*hsdR1*	Nakazawa (2002)
KTHL	*P. putida* KT2440 with *ejh* and *ejl* genes in the chromosome, Km^{r}	This study
AX	*P. putida* KT2440 *tolA*::*xylE* (TolA shortened to 94 amino acids)	Llamas *et al.* (2000)
BX	*P. putida* KT2440 *tolB*::*xylE* (TolB shortened to 29 amino acids)	Llamas *et al.* (2000)
QX	*P. putida* KT2440 *tolQ*::*xylE* (TolQ shortened to 29 amino acids)	Llamas *et al.* (2000)
RX	*P. putida* KT2440 *tolR*::*xylE* (TolR completely removed)	Llamas *et al.* (2000)
AXHL	*P. putida* KT2440 *tolA::xylE* with *ejh* and *ejl* genes in the chromosome, Km^{r}	This study
BXHL	*P. putida* KT2440 *tolB::xylE* with *ejh* and *ejl* genes in the chromosome, Km^{r}	This study
QXHL	*P. putida* KT2440 *tolQ::xylE* with *ejh* and *ejl* genes in the chromosome, Km^{r}	This study
RXHL	*P. putida* KT2440 *tolR::xylE* with *ejh* and *ejl* genes in the chromosome, Km^{r}	This study
Plasmids	Description	Reference
pNM185	*xylS/Pm*,Ap^{r}, Km^{r}	Mermod *et al.* (1986)
pEDF12	pNM185 derivative with *ejh* and *ejl* genes	Díaz *et al.* (1996)
pCNB1	pUTmini-Tn5, *xylS/Pm*, Ap^{r}, Km^{r}	de Lorenzo *et al.* (1993)
pUC18Not	pUC18 with NotI sites flanking the polylinker, Ap^{r}	Herrero *et al.* (1990)
pUCHL	pUC18Not with *ejh* and *ejl* genes	This study
pCNBHL	pCNB1 with *ejh* and *ejl* genes	This study
Primers[a]	Sequence 5′→3′	Reference
V08	CTAGTCTAGAGGCCAACAACATTACCATAAT AGAA	This study
V09	CGCGGATCCGTCTTTCTATTTTGTCGTAATCA AGCCG	This study

a. Engineered endonuclease sites on the oligonucleotides are shown underlined.

dried biomass in combination with subsequent precipitation of the extracted polymer using non-solvents of PHAs, such as methanol, has been described (Williams *et al.*, 1999; Furrer *et al.*, 2007; Elbahloul and Steinbüchel, 2009).

Most bacteriophages accomplish lysis with a tandem, late transcriptional, two gene products: a holin, a small membrane protein that oligomerizes in the membrane to form non-specific lesions or ‘holes’ and a specific endolysin. At a ‘programmed’ time, the holes cause a permeabilization of the membrane that facilitates the action of the active endolysins, murein hydrolases that degrade the bacterial cell wall. As endolysins coding genes do not harbour secretory signal sequence they accumulate in a fully folded and active state in the cytoplasm during the vegetative cycle until they reach the peptidoglycan, hydrolyse it and lyse the cells (Wang *et al.*, 2000). The utilization of phage lysis genes to disrupt recombinant cells that produce PHB has been reported in *Escherichia coli* (Fidler and Dennis, 1992; Resch *et al.*, 1998; Yu *et al.*, 2000) and in *Bacillus megaterium* (Hori *et al.*, 2002), but such technologies have not been tested yet in bacteria producing mcl-PHAs.

In this work, a novel self-disruption extraction system was tested as a proof of concept in *Pseudomonas putida* KT2440, a paradigmatic strain in environmental microbiology (Nelson *et al.*, 2002), which produces mcl-PHAs (Huijberts *et al.*, 1992; de Eugenio *et al.*, 2010). This technology was based on a controlled autolysis that utilizes the endolysin Ejl and the holin Ejh from the pneumococcal bacteriophage EJ-1 (Díaz *et al.*, 1996). To improve the efficiency of the lytic system we have tested the system in different *P. putida tol-pal* mutant strains, which exhibit alterations in outer membrane integrity that induce lysis hypersensitivity (Llamas *et al.*, 2000). The combination of different lytic facilitators renders the cells more susceptible to become extractable by simplified procedures.

Results

Construction of a stable P. putida *KT2440 strain for controlling the expression of the* ejh-ejl *lytic cassette*

The *ejh-ejl* cassette isolated from the EJ-1 phage genome was previously cloned in the shuttle plasmid pNM185 rendering the recombinant plasmid pEDF12 (Table 1), as a multicopy XylS/*Pm* expression system, where transcription of *Pm* promoter is induced by the *xylS* gene product after its activation by effector molecules such as 3-methylbenzoate (3MB) (Díaz *et al.*, 1996). The simultaneous expression of the lytic cassette producing the Ejh holin and the Ejl endolysin reduced the viability of *E. coli* and *P. putida* cells. Moreover, the presence of nucleic acids into the culture medium suggested that cell lysis occurred (Díaz *et al.*, 1996). To investigate if this inducible lytic system could be applied into the PHA production process by facilitating the release of the PHA granules in a controlled downstream process, we first studied the effect of the expression of *ejh-ejl* genes on the growth profile of the strain *P. putida*

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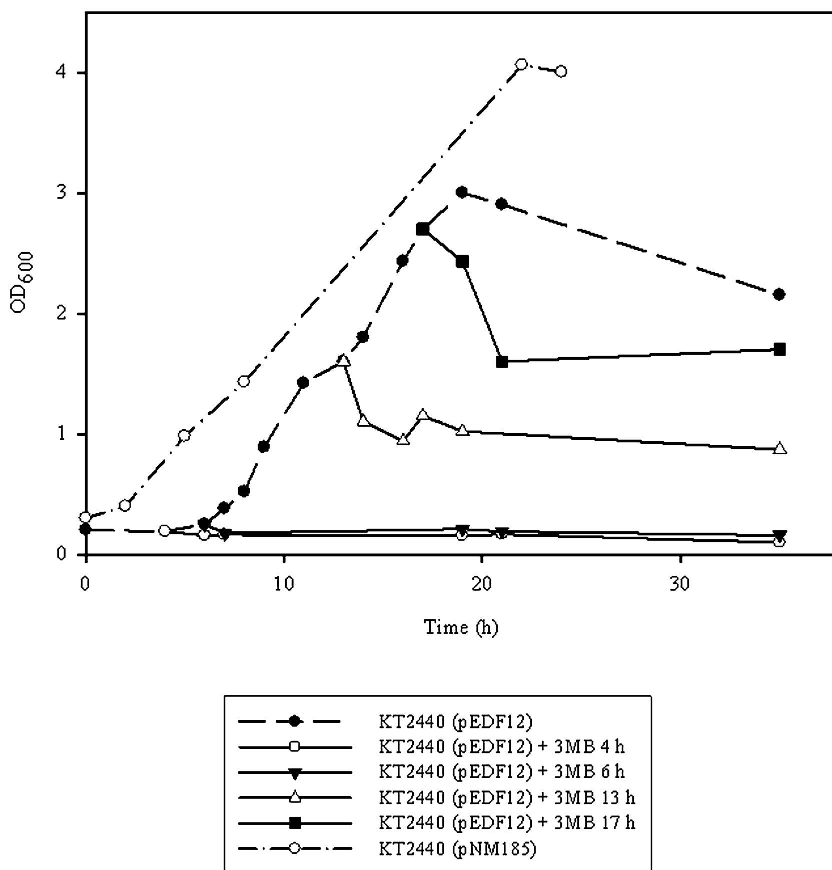

Fig. 1. Profiles of cell lysis in *P. putida* strains caused by expression of the *ejh-ejl* genes cloned in a multicopy plasmid under control of the 3MB inducible promoter. Strains were grown under one phase PHA production conditions and the bacterial growth was followed by measuring OD_{600} of cell cultures (see *Experimental procedures*). The effect of the *ejh-ejl* cassette expression after the addition of the inducer (3MB) was studied in *P. putida* KT2440 (pEDF12) at different times. 3MB was added at the indicated times during the incubation (4, 6, 13 and 17 h). The growth curve of the control strain *P. putida* KT2440 (pNM185) without the lytic cassette is also shown.

KT2440 (pEDF12), in optimal PHA production conditions. *P. putida* KT2440 (pEDF12) and *P. putida* KT2440 (pNM185) cells were cultured in 200 ml of M63 0.1N with 15 mM octanoic acid medium to promote the PHA production in one step fermentation system (see *Experimental procedures*). At different times, 40 ml aliquots were taken and the effect of the *ejh-ejl* cassette expression after the addition of 3MB inducer was studied (Fig. 1). In all aliquots tested, a growth inhibition was detected as soon as the inducer was added, indicating that the lytic system had been induced. However, a decrease in the turbidity was also observed in the absence of the inducer, when culture reached an optical density at 600 nm (OD_{600}) ~3. These results indicated that the gene expression system is not tightly controlled and some basal expression of the *ejh* and *ejl* genes caused a partial non-programmed cell lysis. Another disadvantage of this multicopy system is that *P. putida* KT2440 (pEDF12) strain did not reach the same OD_{600} level, related to the biomass, as the wild-type strain (Fig. 1).

The above results demonstrated the feasibility of the cell disruption strategy under PHA production conditions but also revealed the need for strain optimization to improve the process yield. To fully control the effect of the lytic system by reducing the XylS-independent *Pm* activity, we constructed a monocopy expression system by transferring a *xylS*/*Pm*::*ejh-ejl* cassette onto the chromosome of the wild type *P. putida* KT2440 strain, generating the strain *P. putida* KTHL (see *Experimental procedures* and Fig. 2 for details). We tested the ability of this strain for growing and producing PHA in one step fermentation system in minimal medium M63 0.1N containing 15 mM octanoic acid in the absence of the 3MB inducer. Table 2 compares the growth parameters and PHA production of *P. putida* KTHL *versus P. putida* KT2440 demonstrating that both strains were similar in terms of biomass production, PHA production and cell viability. However, the addition of 3MB to the medium during the fermentation reduced by one order of magnitude the viability of the KTHL cells but did not affect the survival of the control strain (Table 2). It is worth mentioning that when 3MB was added at different times along the fermentation process, the turbidity was not altered in *P. putida* KTHL. The lytic effect was exclusively detected when the inducer was added at the beginning of the culture (data not shown). In these growth conditions, biomass of induced *P. putida* KTHL cells was affected although the PHA production yield is similar to that of the wild-type strain (Table 2).

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Table 2. Growth parameters and PHA production of *P. putida* KTHL *versus P. putida* KT2440 cultured in one phase fermentation system.

P. putida strains	OD_{600}[a] (24 h)	Total PHA content (g l^{-1})	Biomass (g l^{-1})	Viability (10^{-7} cells ml^{-1})
KT2440	6.7 ± 0.21	0.9 ± 0.07	1.45 ± 0.01	2.31 ± 1.04
KT2440 + 3MB	6.5 ± 0.49	0.86 ± 0.03	1.38 ± 0.05	2.09 ± 0.28
KTHL	6.43 ± 0.02	1 ± 0.05	1.51 ± 0.06	2.65 ± 0.59
KTHL + 3MB	4.13 ± 0.05	0.89 ± 0.03	0.95 ± 0.04	0.21 ± 0.06

a. Cells were grown in nitrogen limited mineral medium plus 15 mM octanoic acid (one phase) with and without inducer 3MB (5 mM) for 24 h (see *Experimental procedures*).

Two steps culture fermentation system

As an alternative culture approach, we established a two steps culture fermentation system where cells are initially cultured in a rich medium, and afterwards transferred to the PHA production medium (see *Experimental procedures*). Table 3 shows the growth parameters concerning *P. putida* KT2440 and KTHL strains cultured in a two steps system. When 3MB was added to the culture medium at

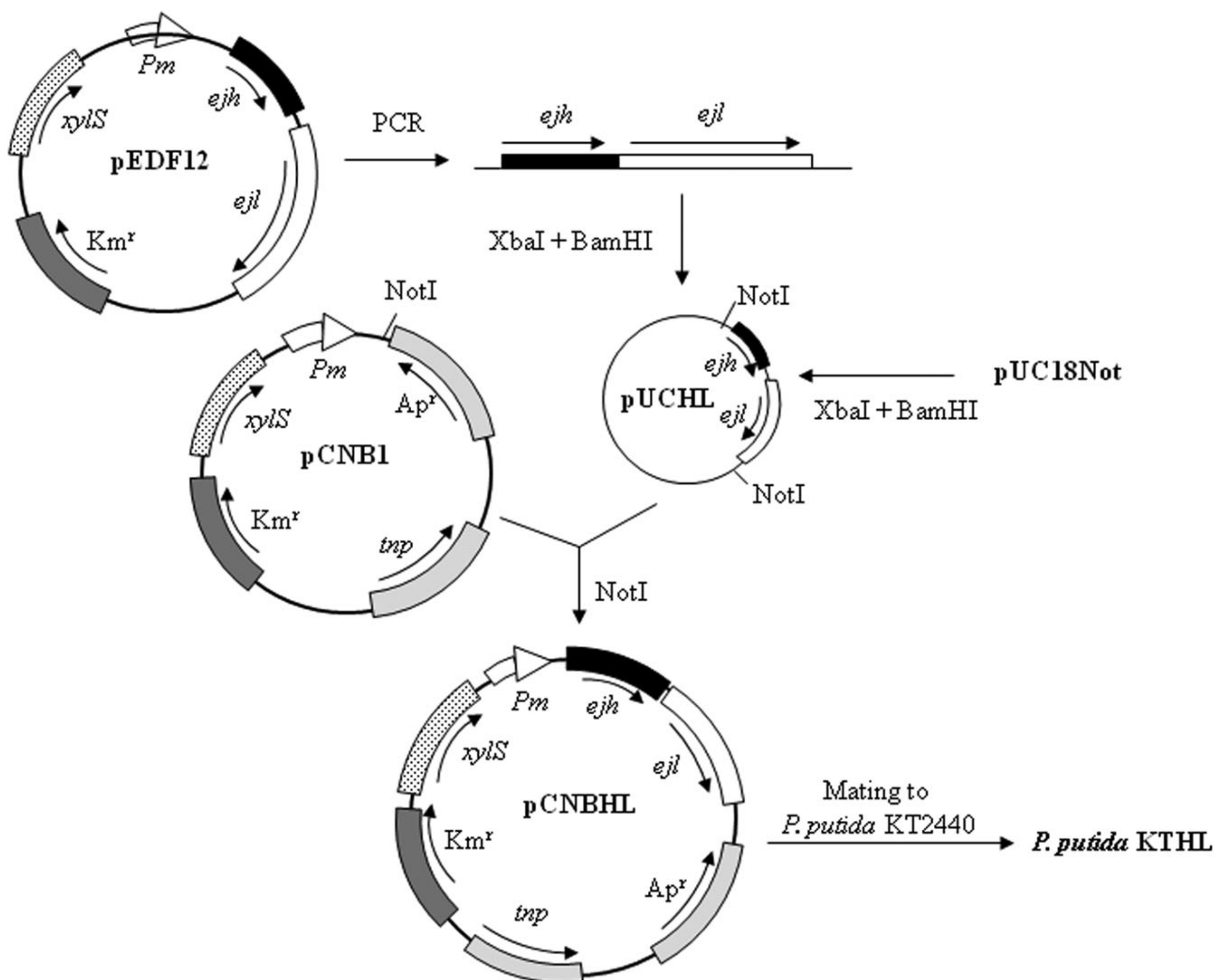

Fig. 2. Schematic representation of the subcloning of *ejh-ejl* cassette in the chromosome of *P. putida* KT2440 under transcriptional control of the positively regulated *Pm* promoter. Plasmids are drawn with the relevant elements and restriction sites indicated, as well as the direction of transcription of the genes. White arrows represent the *Pm* promoter of the *meta*-pathway operon of the TOL plasmid. The e*jh* and *ejl* genes are shown as black and white blocks boxes respectively. *xylS* gene, encoding the cognate regulator of *Pm*, is indicated as a dotted block. Dark grey blocks indicate the antibiotic resistance (Km^R indicates the gene conferring kanamycine resistance). The regions of the plasmids encoding the Tn5 transposase gene and the beta-lactamase gene are also indicated.

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Table 3. Growth parameters and PHA production of *P. putida* KTHL and BXHL *versus P. putida* KT2440, cultured under two phases fermentation system.

P. putida strains	OD_{600} (24 h)	Total PHA content (g l^{-1})	Biomass (g l^{-1})	Viability (10^{-9} cells ml^{-1})	Recovery of PHA extracted from wet biomass with ethyl acetate (g l^{-1})
KT2440	7.52 ± 0.13	0.84 ± 0.05	2.38 ± 0.04	2.33 ± 0.04	0
KT2440 + 3MB	7.66 ± 0.37	0.78 ± 0.04	2.26 ± 0.02	3.16 ± 1.19	0
KTHL	7.71 ± 0.03	0.88 ± 0.03	2.45 ± 0.04	3.33 ± 0.82	0
KTHL + 3MB	6.53 ± 0.18	0.79 ± 0.15	2.28 ± 0.14	0.51 ± 0.02	0.13 ± 0.01
AX	Nd	1.02	2.49	Nd	Nd
BX	Nd	0.82	2.56	Nd	Nd
QX	Nd	1.03	2.55	Nd	Nd
RX	Nd	0.87	2.45	Nd	Nd
AXHL	4.63 ± 0.62	0.43	1.38	Nd	Nd
BXHL	7.83 ± 0.36	0.86 ± 0.09	2.00 ± 0.01	1.05 ± 0.03	0.1 ± 0.07
BXHL + 3MB	7.1 ± 0.67	0.68 ± 0.01	1.52 ± 0.1	0.1 ± 0.4	0.28 ± 0.08
QXHL	0.78 ± 0.02	0	0.57 ± 0.13	Nd	Nd
RXHL	2.7 ± 0.11	0.07 ± 0.003	1.30 ± 0.09	Nd	Nd

3MB inducer was added at 5 mM to the PHA production medium (see *Experimental procedures*).
Nd, not determined.

the beginning of the second step, the viability of the KTHL cells was significantly reduced (Fig. 3) but biomass and PHA yield reached similar levels to those obtained with the wild-type strain or with non-induced KTHL strain (Table 3). Therefore, the two steps fermentation strategy was selected as the optimal method for producing PHA in *P. putida* KTHL strain.

After fermentation, cultures were subjected to sucrose density step-gradient ultracentrifugation to check the presence of PHA granules in the culture supernatant because of the cell disruption induced in the *P. putida* KTHL strain (Fig. 4A). This method allows the separation of the PHA granules from the cell fraction (non-disrupted cells and cell debris) as PHA granules have a specific gravity lower than cell fraction, which sediments at the bottom of the tube (Moldes *et al.*, 2004). In the absence of inducer, PHA granules were not released into the medium and sedimented together with the cell fraction (Fig. 4A, tube 2). However, in the presence of the 3MB inducer, a PHA white band was visible at the sucrose step-gradient interface, indicating the release of the granules to the extracellular medium (Fig. 4A, tube 3). In the case of the wild-type strain, extracellular PHA granules were not detected even in the presence of 3MB (Fig. 4A, tube 1). Thus, it was clearly demonstrated that PHA granules were effectively released by the self-disruption system integrated into the recombinant *P. putida* KTHL strain, proving the potentiality of this system.

To study the efficiency of the lytic system in *P. putida* KTHL, the amount of self-released PHA was compared

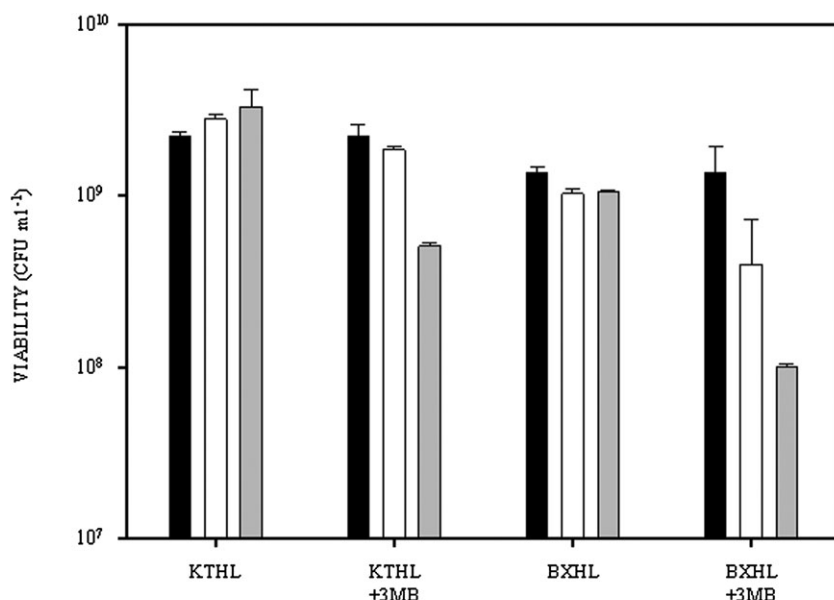

Fig. 3. Number of viable cells of *P. putida* KTHL and *P. putida* BXHL grown under two steps PHA production conditions. Strains were grown with and without 3MB (5 mM), added at the beginning of the second step (see *Experimental procedures*). Black bars, values at 0 time; white bars, values at 6 h; gray bars, values at 24 h.

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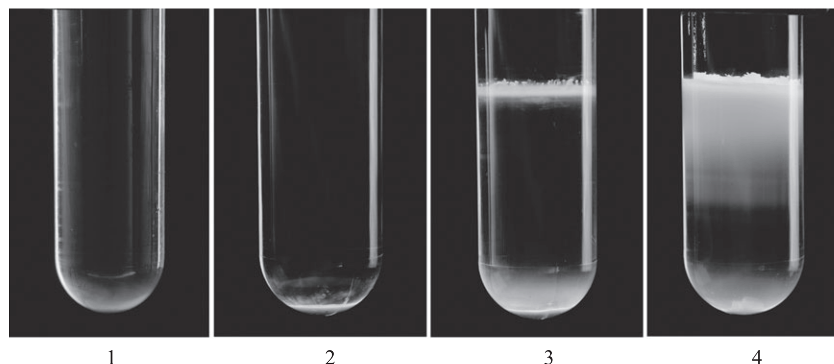

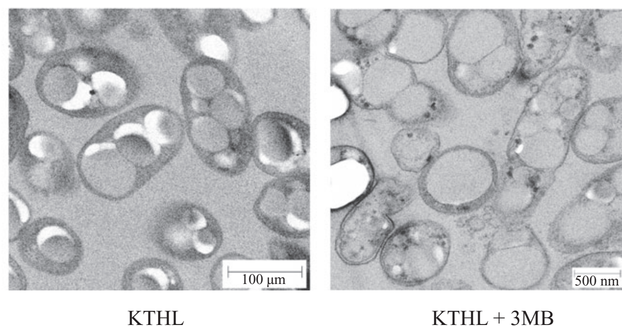

Fig. 4. Sucrose density step-gradient ultracentrifugation of *P. putida* strains. Cells were grown under two steps PHA production conditions, with or without 3MB (5 mM) as inducer (see *Experimental procedures*).
A. Tube 1, *P. putida* KT2440 wild type grown with 3MB; tube 2, *P. putida* KTHL grown without 3MB; tube 3, *P. putida* KTHL grown with 3MB; tube 4, *P. putida* KTHL grown with 3MB broken by a fourfold passage through a French press. The white band located at the interfaces of tubes 3 and 4 corresponds to the free PHA granules released to the extracellular medium.
B. Transmition electronic microscopy views of mcl-PHA accumulating *P. putida* KTHL cells. Samples were taken after 24 h growing under the two phases fermentation system in the presence (right) or absence (left) of 3MB inducer.

with the total PHA obtained after breaking the cells through the French press (Fig. 4A, tube 4). The PHA content detected extracellularly by the French press standard procedure was 84.9% of CDW, while only 7.6% of CDW was detected in the case of KTHL strain grown in the presence of 3MB. The wide white band clearly visible at the sucrose step-gradient interface after the French press cell disruption procedure demonstrated that the lytic system was indeed active in *P. putida* KTHL strain, but the efficiency was still low. The incomplete autolytic effect of the *xylS*/*Pm*::*ejh-ejl* cassette induction, when 3MB was added to the medium, was monitored by transmition electronic microscopy (Fig. 4B), demonstrating that, although cell envelopes were considerably disturbed, most granules maintained their intracellular location.

Sensitivity of P. putida *KTHL strain to detergents and chelating agents*

Cell viability and biomass production data derived from the results described above were in agreement with those obtained in *P. putida* KTHL cells growing in rich medium (Luria–Bertani, LB) in the presence of 3MB, which showed, under the phase contrast microscope, a majority of 'ghost cells' (Fig. 5). Morphology of these 'ghosts' is a typical consequence of holin action, where the cells are

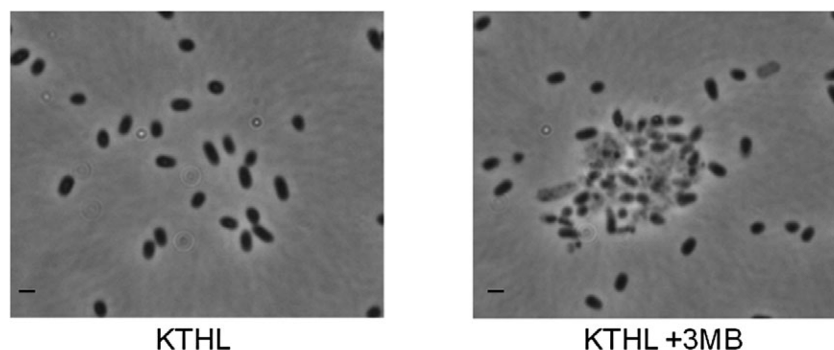

Fig. 5. Phase contrast microscopy of *P. putida* KTHL ghost cells as consequence of the expression of the lytic cassette. Cells were grown in rich medium (LB) with (right) and without (left) 3MB for 24 h. Black bars correspond to 2 μm.

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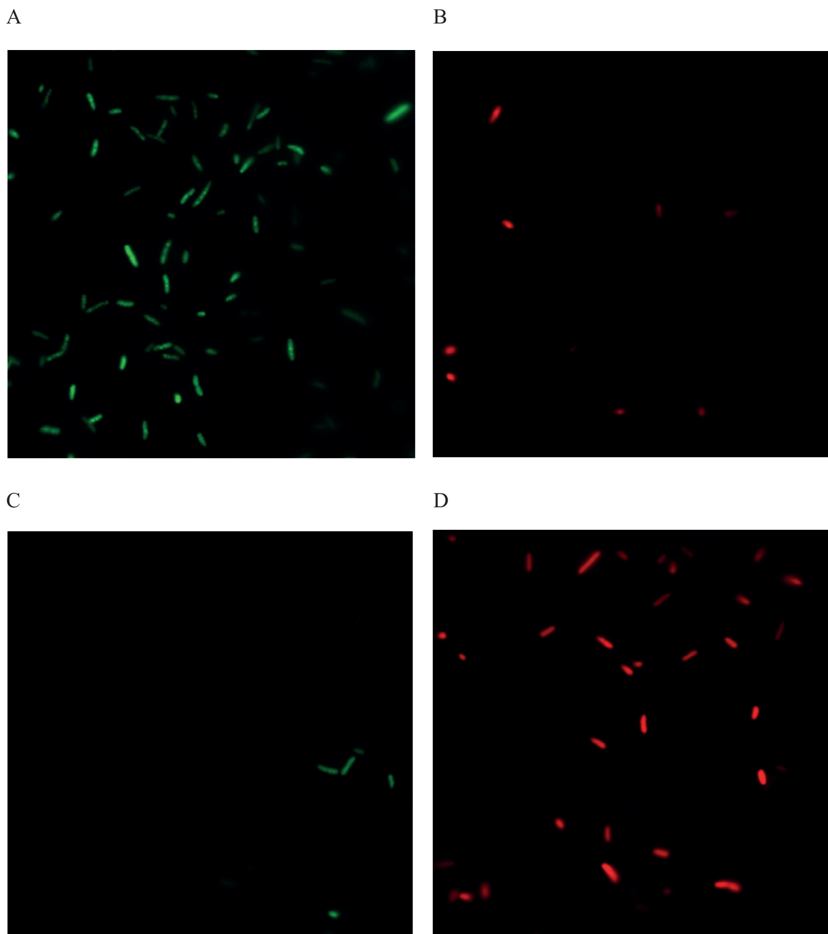

Fig. 6. Monitoring the viability of *P. putida* KTHL population as function of the membrane integrity. LIVE/DEAD bacterial viability assay of KTHL cells grown in LB in the presence (panels C and D) and absence (panels A and B) of 3MB.

still keeping their shape but lack their cytoplasmic content and, consequently, there is a significant drop in viability when 3MB is added to the medium. This was demonstrated by the viability analyses as function of membrane integrity by staining with the LIVE/DEAD *Bac*Light bacterial viability kit (Fig. 6). In this assay, cells with a compromised membrane, which are considered to be dead or dying, stained red (Fig. 6B and D), whereas cells with an intact membrane stained green (Fig. 6A and C). Figure 6 showed a drastic effect on the green/red cell ratio after the addition of the lytic inducer. The cell viability was also affected in the presence of 3MB when cells were grown under PHA production conditions (Tables 2 and 3). These findings confirmed that when the *ejh-ejl* cassette was expressed, it caused the death of most of the cells but they were not totally disrupted, probably as a result of an intact outer membrane or a reduced efficiency of the phage endolysin. Thus, to improve the yield of the extraction procedure we tested the sensitivity of the strains to detergents or chelating agents in rich medium. To this aim, *P. putida* KTHL and wild-type cells were incubated for 18 h at 30°C in LB medium and in LB medium supplemented with 0.075% (w/v) deoxycholate (DOC) (Fig. 7), 0.01% SDS or 0.2 mM EDTA (data not shown). At these concentrations, the 3MB induced *P. putida* KTHL cells were sensitive to the chemical agents, while no effect was observed on the growth of the wild-type strain or the non-induced recombinant strain. In addition, we have determined that DOC, SDS and EDTA did not alter the growth of the 3MB induced *P. putida* KTHL cells when they were added to LB medium at final concentrations lower than 0.01%, 0.005% and 0.1 mM respectively. Besides, we also determined that the addition of concentrations higher than 0.2% DOC and 0.4 mM EDTA changed the growth of both, the wild type and the non-induced KTHL strains. All the above results clearly indicated that when the cells grow in a rich medium the cell envelope of the *P. putida* KTHL mutant strain is significantly altered as a result of the expression of the Ejh and Ejl proteins.

Conversely, when cells were cultured under two steps PHA production conditions with 3MB and in the presence of these chemical agents for 18 h, the release of the granules into the extracellular medium did not increase in *P. putida* KTHL strain compared with similar experiments performed in the absence of chemical agents (7.6% of

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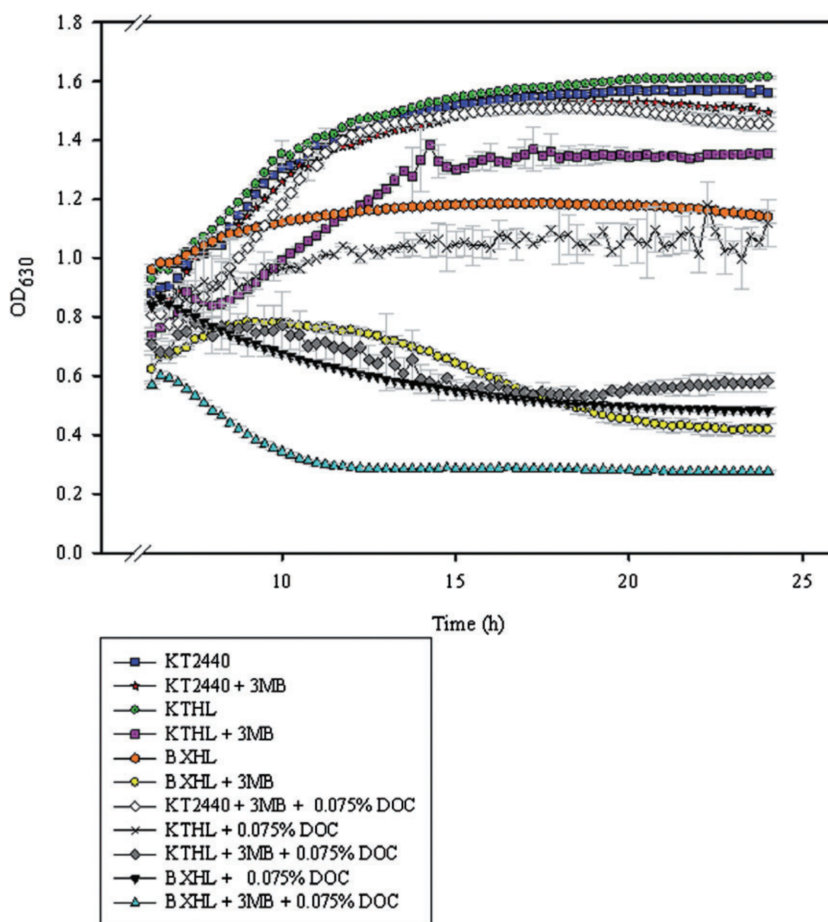

Fig. 7. Growth curves in LB medium of *P. putida* strains in the presence of DOC. *P. putida* KT2440, KTHL and BXHL strains were grown in LB medium with and without 3MB (see *Experimental procedures*). After 6 h of incubation at 30°C, aliquots of each culture were supplemented with 0.075% (w/v) DOC. The ability for growing in the presence of the detergent was determined by measuring the OD_{630} of the cultures during 18 h in 96-microwell plates (see *Experimental procedures* for details). As control, aliquots of the cultures with no added agent were also analysed. Error bars represent standard deviation found in three different experiments.

CDW). Even at high concentrations of EDTA (10 mM) and SDS (0.1%) simultaneously added to the medium, the detected PHA at the extracellular medium was 9.2% of CDW. This result suggested that the intracellular presence of PHA granules renders the cells more resistant to cell disruption.

Expression of the lytic cassette in P. putida tol-pal *mutant strains*

Our previous results indicated that cells expressing the *ejh-ejl* cassette were more sensitive to outer membrane disturbing agents, such as chelating agents and detergents, when growing in rich medium than under optimal PHA production conditions in nitrogen limited minimal medium. Therefore, to improve the cell disruption for recovering PHA granules we tested a different strategy to alter the stability of the cell envelope. With this aim, we used *tol-pal* mutants of *P. putida* KT2440 as recipients of the lytic system instead of wild-type strain, because these mutants exhibit severe alterations in outer membrane integrity (Table 1). A comprehensive scheme of the Tol/Pal membrane proteins is depicted in Fig. 8.

First, we tested the growth and PHA production capacities in the two steps culture fermentation system of the four *tol-pal* mutant strains AX, BX, QX and RX, mutated in the *tolA, tolB, tolQ* and *tolR* genes respectively. Gas chromatography-mass spectrometry (GC-MS) analyses confirmed that the four mutants were able to grow and produce PHA under these fermentation conditions and that the quantity of PHA accumulated by the four strains was similar to that of the wild-type strain (Table 3). Afterwards, the lytic expression system was transferred, through a transposition-mediated mechanism, to the chromosome of mutant strains rendering the recombinant AXHL, BXHL, QXHL and RXHL strains (Table 1). When we tested the ability of these four new strains for growing and producing PHA in the two steps culture fermentation system, in the presence and absence of 3MB, we observed that *P. putida* BXHL was the only strain that reached similar OD_{600} values than those of the wild type, while the other mutants grew very poorly under these conditions (Table 3). In addition, the intracellular PHA contents of AXHL, RXHL and QXHL strains were lower than 0.4 g l^{-1}, while the PHA content of BXHL was 0.86 g l^{-1}. Interestingly, cell viability of BXHL was reduced by one

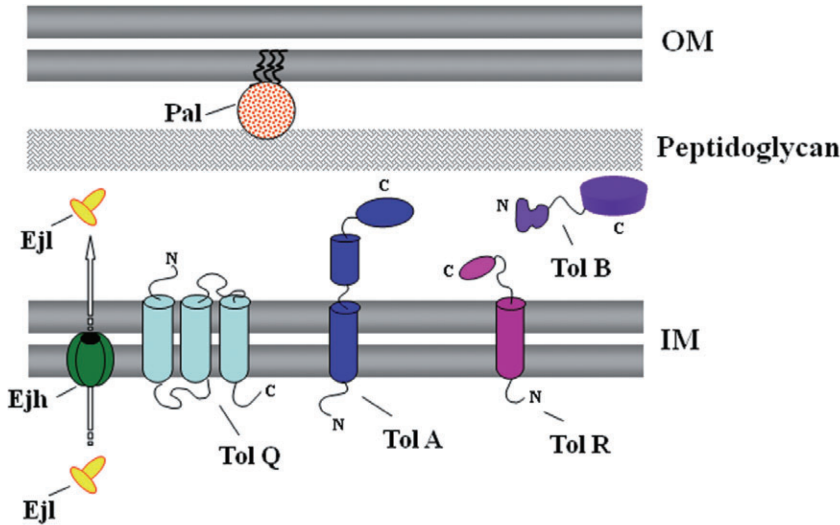

Fig. 8. Schematic localization of the Ejh holin, the Ejl endolysin and the Tol–Pal proteins in the cell envelope of *P. putida* strains. TolQ, TolR and TolA are cytoplasmic membrane proteins that form a complex in the cytoplasmic (inner) membrane (IM). TolQ is an integral inner membrane protein containing three transmembrane domains with two cytoplasmic regions. TolR and TolA are anchored to the cytoplasmic membrane leaving most of the protein exposed to the periplasm. Pal is an outer membrane (OM) peptidoglycan-associated lipoprotein and forms a complex with TolB, which is a periplasmic protein. Ejh holin is a small membrane protein that oligomerizes in the IM to form 'holes' that allow the translocation of the Ejl endolysin to the peptidoglycan, where it degrades the cell wall and lyses the cell.

order of magnitude when the cells were cultured in the presence of the 3MB lytic inducer (Table 3, Fig. 3). Taking into account the above results, *P. putida* BXHL was selected as the best candidate strain to apply the autolytic disruption strategy.

Pseudomonas putida BXHL strain was monitored by transmition electronic microscopy (Fig. 9) showing that, similarly to KTHL strain, the addition of 3MB generated deep alterations in the cell envelopes but most of the granules remained inside the cells. Remarkably, when we tested the susceptibility of *P. putida* BXHL strain grown in rich medium to the effect of membrane disrupting agents such as DOC, SDS and EDTA, we observed that this strain was even more sensitive than *P. putida* KTHL mutant, suggesting that *tolB* mutation contributes to alter the stability of cell envelope. As an example, Fig. 7 shows the growth curves of *P. putida* strains in LB medium with and without inducer and in the presence or absence of 0.075% DOC. It can be observed that induced *P. putida* BXHL is up to three times more sensitive to the detergent than induced *P. putida* KTHL. In addition, we compared the PHA granule release in *P. putida* wild type and BXHL mutant strains after simultaneous incubation with 10 mM EDTA and 0.1% SDS. In this experiment, both strains were cultured using the two steps culture fermentation system with 3MB. After 24 h of incubation in the PHA production conditions media (this is, the end of second step), the cultures were centrifuged and resulting sediments were suspended in distilled water supplemented with 10 mM EDTA and 0.1% SDS. After 8 h of incubation at room temperature, cultures were subjected to sucrose density step-gradient ultracentrifugation for analysing the presence of PHA granules in the culture supernatant (Fig. 10). It is worth to mention that none viable cell was detected at this condition. *P. putida* BXHL induced to self-disruption and incubated with the chemical agents (Fig. 10A, tube 2) showed a much thicker white band when compared with that of the wild-type strain (Fig. 10A, tube 1). These findings indicated the release of almost all granules to the extracellular medium. To confirm these results, the intracellular PHA content of the cell fraction present in the sediments after the ultracentrifugation step

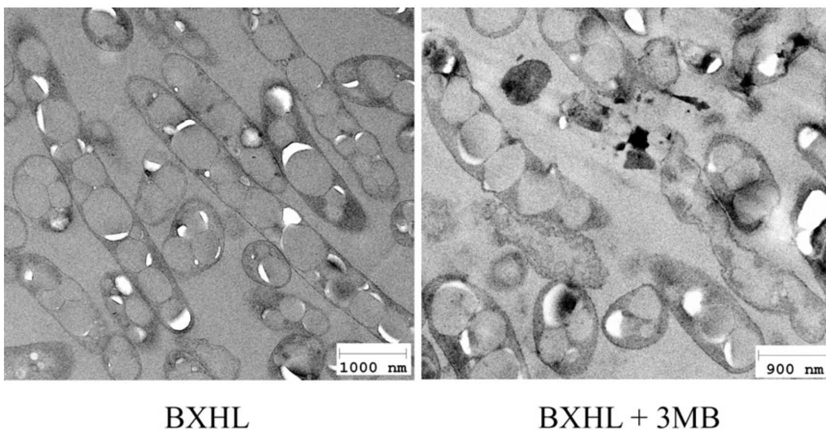

Fig. 9. Transmition electronic microscopy views of mcl-PHA accumulating *P. putida* BXHL cells. Cell samples were taken after 24 h growing under the two phases fermentation system in the presence or absence of 3MB inducer. *P. putida* BXHL grown in the presence of 3MB showed disturbed membranes.

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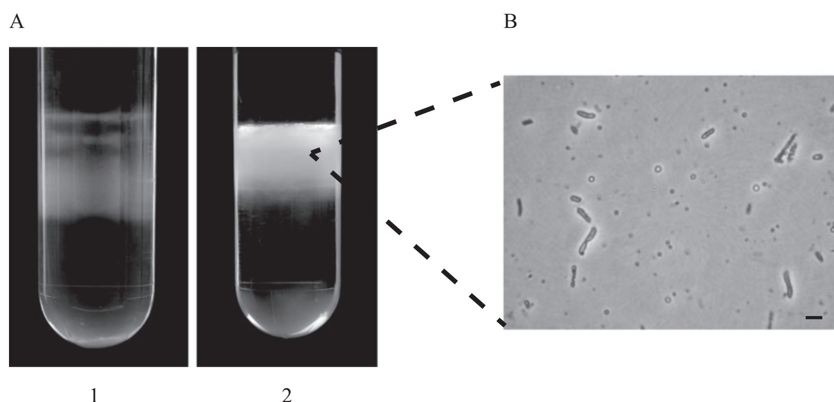

Fig. 10. PHA granule release analysis by sucrose density step-gradient ultracentrifugation of *P. putida* BXHL strain. Cells were grown under two steps PHA production conditions in the presence of the inducer 3MB for 24 h (see *Experimental procedures* for details).
A. Tube 1, cells of *P. putida* KT2440 wild type; tube 2, cells of *P. putida* BXHL. The white band in the interface of tube 2 corresponds to the free PHA granules released to the extracellular medium.
B. Phase contrast microscopy of the white band obtained in tube 2, where free PHA granules secreted to the extracellular medium and some cells can be observed. Black bar corresponds to 2 μm.

of the 3MB induced *P. putida* BXHL strain incubated with and without the chemical agents was quantified by GC-MS (see *Experimental procedures*). When no chemical agents were added, the PHA content present in the culture sediment was 0.7 g l^{-1}, similar to that found in the total culture (0.86 g l^{-1}). However, PHA was not detected at all in the sediment of cells incubated with 10 mM EDTA and 0.1% SDS, suggesting that this sediment is only composed of cell debris and that most of PHA (93% of CDW) was released to the extracellular medium, as shown in Fig. 10B.

Direct cell extraction of PHA by organic solvents

In addition to the advantage that the lytic systems could simplify the self-release of PHA from the cells to the medium, the alterations of the cell envelope might also be useful to facilitate the direct cell extraction of PHA by organic solvents. To analyse this assumption, we compared the PHA recovery in the wild-type, KTHL and BXHL strains, cultured in the presence or absence of the lytic inducer. PHA was extracted from the cells with organic solvents just after centrifugation or filtration (wet biomass) without additional treatments such as lyophilization (dried biomass) or physical cell disrupting methods plus separation, which are time- and energy-consuming processes. Direct solvent extraction of PHA from wet biomass could be beneficial, but has been demonstrated inoperative for wild-type cells (Table 3).

Cultures of 24 h of *P. putida* strains grown in the two steps culture fermentation system with and without 3MB were directly suspended after centrifugation in several organic solvents such as methylene chloride, ethyl acetate or acetone (see *Experimental procedures* for details). Then, dissolved PHA was precipitated by adding methanol. Remarkably, only ethyl acetate gave positive results when wet biomass was used for direct PHA extraction (Table 3). As expected, wild-type strain grown with and without 3MB inducer did not render polymer after precipitation whereas 0.13 g l^{-1} of PHA was obtained from the 3MB induced KTHL cells. BXHL cells induced with 3MB yielded higher recovery rates (0.28 g l^{-1} of PHA) with a purity of 97.65% ± 2.16, indicating that this strain has been improved to facilitate the direct solvent extraction of PHA from wet biomass. Although the PHA direct recovery in organic solvents is still far from being useful at industrial scale, these experiments demonstrated the possibility of improving the wet biomass direct solvent extraction of PHA by modifying the characteristics of the bacterial cell envelope and the properties of the solvents.

Discussion

Polyhydroxyalkanoate is the paradigmatic example of a bacterial biopolymer that accumulates only in the cytoplasm (de Smet *et al*., 1983; Madison and Huisman, 1999). Depending on the organism, PHA production can reach levels as high as 90% of the cell dry weight (Olivera *et al*., 2001a). The cytoplasm space limits the amount of polymer that can be produced by a microbial cell, and the yield per volume is limited by the number of cells and the biopolymer fraction in the biomass. This limitation increases the complexity of the production and downstream processes for preparing purified PHA, implying the use of cell disruption procedures as well as processes for extracting PHA from crude lysates (Zinn *et al*., 2001). Metabolic engineering approaches have succeeded in improving microorganisms for PHA production and in fact, a considerable variety of strategies have been designed to increase the yield of mcl-PHA production in homologous and heterologous microorganisms (Qi *et al*., 1998; Prieto *et al*., 1999; Ren *et al*., 2000; Olivera *et al*., 2001b). However, only one example has been reported to date for engineering *Alcanivorax borkumensis* in order to overproduce mcl-PHA and facilitate its extraction as the polymer was deposited extracellularly by an unknown mechanism (Sabirova *et al*., 2006). Several attempts have been carried out to mimic such process by using phage lysis genes to disrupt recombinant cells that produce PHB (Fidler and Dennis, 1992; Resch *et al*., 1998; Yu *et al*.,

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2000; Hori *et al*., 2002), but a similar approach in mcl-PHA producing strains remained to be explored.

In this study, we report the construction of a programmed self-disruptive *P. putida* BXHL strain that facilitates the release of the mcl-PHAs granules to the extracellular medium. The engineered cell disruption system is based on two proteins from the pneumococcal bacteriophage EJ-1, Ejh holin and Ejl endolysin (amidase), which have been transferred to the chromosome of a *tolB* mutant of *P. putida* KT2440 to increase the strain stability by reducing the gene copy number. The coupled expression of phage genes involved in host lysis is a transcriptional late event, in the phage physiological cycle, which has been shown to have only a reduced specificity for the bacterial host, i.e. the phage lytic systems are effective when transferred to other bacteria different than their specific phage hosts (Young, 2005). In this sense, phage holin proteins are able to oligomerize in different heterologous bacteria to form holes in the inner membrane (Wang *et al*., 2000). Moreover, some phage murein hydrolases can degrade the peptidoglycan of non-host bacteria, although its optimal enzymatic activity might depend on specific structural requirements (López and García, 2004). In our case, combined results of growth curves, viability data and microscopic observations strongly suggest that Ejh holin is fully functional in *P. putida*, although the Ejl amidase appears to be less efficient than in pneumococcus because of its specific requirement for choline-containing cell walls (López and García, 2004). Remarkably, the low activity of Ejl is certainly an advantage for our engineered system as it allows us to diminish the toxicity of the lytic system and facilitates its control. In fact, in spite of the low hydrolytic activity we have observed a high toxicity of the lytic system when it was expressed in multicopy plasmids. Therefore, it seems extremely important to tightly control the lytic systems and partially reduce their activities when used for biotechnological purposes. In this context, it would appear surprising that not all the cell population of the same culture could be lysed at the same time and with the same efficiency, but because of need of balancing the efficiency and toxicity of the lytic system it is assumable that not all the cells reach the instability threshold required to become lysed.

It is worth to mention the unexpected lytic resistance phenotype of *P. putida* KTHL observed even in the presence of chemical agents when the cells were cultured in minimal medium under PHA production conditions, suggesting that accumulation of PHA granules renders the cells more resistant to cell lysis. Although the mechanism supporting this resistance is not yet well understood, we can speculate on several possible reasons: one could be a reduced production of the lytic proteins in the nitrogen limited minimal medium; another could be related to the presence of large amounts of lipid monolayers in the PHA granules that could trap holin monomers and thus, disturb the oligomerization of the holin in the membrane lipid bilayer. We cannot discard also that the own synthesis of PHA granules could interfere with hole formation and endolysin secretion or that the presence of granules could result in a more rigid cell structure. Whether the structure of the cell envelope of PHA producing cells is reinforced to support the pressure generated by the presence of the PHA granules in the cytoplasm rendering these cells more resistant to membrane perturbations could merit to be investigated.

The advantage of the self-disruptive *P. putida* BXHL strain to fulfil the proposed task is based on the introduction of a mutation in the TolB protein, which forms part of the Tol–Pal protein system (Llamas *et al*., 2000; Godlewska *et al*., 2009) (Fig. 8). In *P. putida*, like in the majority of Gram-negative bacteria, the Tol–Pal complex forms a membrane-spanning multiprotein system composed of five core proteins: TolQ, TolR, TolA, TolB and Pal (Llamas *et al*., 2003) (Fig. 8). As documented in *E. coli*, the Tol-Pal system is organized into two protein complexes: a cytoplasmic membrane complex composed of the TolQ, TolR and TolA proteins, which interact with each other via their transmembrane domains (Derouiche *et al*., 1995; Koebnik, 1995; Germon *et al*., 1998; 2001; Journet *et al*., 1999) and an outer membrane complex made up of TolB and Pal, which also interact with Lpp, OmpA, and the peptidoglycan layer (Bouveret *et al*., 1995; Koebnik, 1995; Clavel *et al*., 1998) (Fig. 8). The Tol–Pal complexes appear to confer stability to the cell envelope because the Tol mutants present some envelope defects that render the cells more sensitive to lysis (Llamas *et al*., 2000). Therefore, the combination of Tol mutations with the engineered lytic system provided a novel approach to investigate the possibility to induce a controlled cell lysis under PHA producing conditions or, at least, to produce PHA containing cells that were more susceptible to lytic treatments at the end of the fermentation process in order to facilitate PHA extraction. In a few words, we have demonstrated that PHA extraction by detergents or organic solvents can be significantly facilitated in the BXHL strain (Fig. 10 and Table 3). In fact, we have demonstrated that a significant fraction of highly pure PHA generated in lytic induced cells can be extracted, without additional treatments, with ethyl acetate directly from wet biomass. This work opens new prospects for using lytic facilitators not necessarily to release PHA to the medium but for altering the stability of cell envelope to simplify the extraction of pure polymers without using other expensive and low environmentally friendly procedures. The different behaviour of the *tol-pal* mutants revealed the importance of finding the critical equilibrium between cell viability/stability, PHA production and facilitation of PHA extraction.

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Although other solvents should be tested, ethyl acetate is a friendly chemical as a result ot its low cost, low toxicity and nice odour, avoiding the halogenated solvents that are frequently used for PHA extraction (Thakor *et al.*, 2005). The combination of different lytic facilitator tools appears to be very useful to fine tune the cell suitability for optimizing the PHA extraction process providing a wide range of different conditions that probably cannot be reached using a single lytic system.

Although additional experiments should be performed to optimize the use of self-disruptive systems for scaling up conditions, this proof of concept settles the bases to demonstrate that engineering cells for facilitating PHA extraction is feasible, showing new perspectives to satisfy the needs for an environmentally friendly and economical production process of bioplastics.

Experimental procedures

Bacterial strains, plasmids and growth conditions

The bacterial strains, plasmids and oligonucleotides used are listed in Table 1. Unless otherwise stated, *E. coli* and *P. putida* strains were grown in LB medium (Sambrook and Russell, 2001) with shaking at 37°C and 30°C respectively. The appropriate selection antibiotics, kanamycin (50 μg ml^{-1}) or ampicillin (100 μg ml^{-1}) were added when needed. Growth was monitored with a Shimadzu UV-260 spectrophotometer at 600 nm (OD_{600}). Solid media were supplemented with 1.5% (w/v) agar. Depending on the experiments, cells could be cultured in liquid media in one or two phases fermentation systems: (i) one step culture system, where the strains are grown in one step under PHA production conditions in order to reach as much cell biomass/PHA yield as possible, and (ii) two steps culture system, where cells are initially cultured in an undefined rich medium (LB) and afterwards transferred to the PHA production medium to fill up of storage biopolymer. For mcl-PHA accumulation in one phase fermentation process, *P. putida* strains were cultured in minimal medium 0.1 N M63 (de Eugenio *et al.*, 2010) using 15 mM octanoic acid as carbon source in a 500 ml flask. In the second strategy by using two steps fermentation procedure, 100 ml of LB were inoculated with 0.5 ml of each overnight preculture of *P. putida* strains. After 14 h of incubation at 30°C with shaking (250 rpm), the cultures were centrifuged and resuspended in 200 ml of 0.1 N M63 mineral medium with 15 mM octanoic acid as the carbon source, in 500 ml flask. The strains were cultivated at 30°C with shaking for 24 h. Unless otherwise stated, the 3MB inducer was added at 5 mM to the PHA production medium. Biomass in different cultures was determined as previously reported (de Eugenio *et al.*, 2010).

DNA manipulations and plasmid constructions

DNA manipulations and other molecular biology techniques were essentially performed as described previously (Sambrook and Russell, 2001). Plasmid pEDF12 is a pNM185 derivative (Mermod *et al.*, 1986) that contains a SacI-SacII restriction fragment encoding holin (Ejh) and lysin (Ejl) proteins from the genome of the pneumococcal bacteriophage EJ-1. The DNA fragment containing e*jh* and *ejl* genes was isolated by PCR using as template the pEDF12 vector and the oligonucleotides V08 and V09 (Table 1). For PCR amplifications, we used 2 U of AmpliTaq DNA polymerase (Perkin-Elmer Applied Biosystems, Norwalk, CT, USA), 0.1 μg of DNA template, 0.5 μg of each deoxynucleoside triphosphate and 2.5 mM of $MgCl_2$ in the buffer recommended by the manufacturer. Conditions for amplification were chosen according to the G + C content of the corresponding oligonucleotides. The PCR product was digested with appropriate endonucleases and cloned in pUC18Not, producing the plasmid pUCNotHL. The *ejh-ejl* cassette was transferred as NotI fragment to pCNB1 plasmid (pUT/mini-Tn*5 xylS*/*Pm*) (Fig. 2), for stable insertion in the chromosome of *P. putida* KT2440 under transcriptional control of the *Pm* promoter. The resulting strain is called *P. putida* KTHL (Fig. 2). All these constructions were confirmed by sequencing using an ABI Prism 3730 DNA Sequencer. Nucleotide sequences were determined directly with the same oligonucleotides used for cloning.

Granule isolation and quantification

Granule fraction was essentially isolated as described (Moldes *et al.*, 2004). Briefly, cultures were centrifuged for 1 h at 31 000 *g* to separate culture supernatants from cells and the resulting pellets were resuspended in phosphate buffer, pH 8.0. Fifty percent of each sample was lyophilized and analysed by GC-MS for total PHA quantification of the culture, as previously reported (de Eugenio *et al.*, 2007). The other 50% of each sample was placed on sucrose gradient layers preformed by overloading 11 ml of 20% and 15% sucrose solutions (Moldes *et al.*, 2004). After centrifugation at 120 000 *g* for 15 h, the resulting bands were collected and observed under an optical microscope for granule visualization. The intracellular PHA content was calculated by quantifying the PHA content of the sediment after ultracentrifugation. The extracellular PHA content was determined indirectly as the difference between the total PHA content of the culture and the intracellular PHA content.

Transmission electron microscopy

Cells were harvested, washed twice in PBS, and fixed in 5% (w/v) glutaraldehyde in the same solution. Afterwards, cells were incubated with 2.5% (w/v) OsO_4 for 1 h, gradually dehydrated in ethanol solutions [30, 50, 70, 90 and 100% (v/v); 30 min each] and propylene oxide (1 h). Finally, they were embedded in Epon 812 resin. Ultrathin sections (thickness 70 nm) were cut with a microtome using a Diatome diamond knife. The sections were picked up with 400 mesh cupper grids coated with a layer of carbon and subsequently observed in a Jeol-1230 electron microscope (JeolLtd. Akishima, Japan).

Cell viability calculation

To calculate cell viability, serial dilutions from 10^{-1} to 10^{-7} were made in saline solution (0.9% NaCl). 10 μl of each dilution

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from 10^{-2} to 10^{-7} was plated on LB solid medium and colony-forming units were counted. For each strain, three different experiments were carried out. A LIVE/DEAD *Bac*Light bacterial viability kit l-13152 (Invitrogen-Molecular Probes) was also used for monitoring the viability of bacterial populations as function of the membrane integrity of the cell according to the instruction of the manufacturers.

Cell sensitivity to detergents and chelating compounds

To determine the bacterial sensitivity to SDS, DOC and EDTA, overnight cultures of each strain were diluted in fresh LB medium with and without 3MB at an OD_{600} of 0.5. After 6 h of incubation at 30°C in an orbital shaker at 250 rpm, a 10 ml aliquot of each culture was taken and supplemented with 0.075, 0.01 or 0.2% (w/v) DOC; 0.01 or 0.005 (w/v) SDS; 0.1, 0.2 or 0.4 mM EDTA. The ability for growing in the presence of each agent was determined by measuring the OD_{630} of the cultures during 18 h in 96-microwell plates with 200 μl of the cultures in each well. Plates were incubated at 30°C during 24 h, with 2 min of orbital shaking every 15 min (Multiskan Ascent, Thermo). The growth rates shown are the mean values from three replicates. As controls, cultures with no added agent were also analysed.

Facilitated PHA extraction analysis

Thirty five ml of each *P. putida* strain grown for 24 h under two steps PHA producing conditions with and without 3MB were centrifuged and the resulting sediments were directly resuspended in 0.5 ml of different organic solvents (methylene chloride, ethyl acetate or acetone) and incubated for 24 h at room temperature under mild stirring. Solvent phases were collected, evaporated at room temperature to reach a final volume of 0.1 ml and precipitated at −20°C by adding 10 times the volume of methanol. Finally, the polymer was dried under vacuum for 10 min. The resulting polymers were analysed by GC-MS for determination of PHA content and purity.

Acknowledgements

We thank Dr E. Díaz for helpful discussions. We are indebted to E. Duque and J. L. Ramos for sending the *tol* mutants of *P. putida* KT2440. The technical works of A. Valencia and V. Morales are greatly appreciated. This work was supported by grants from the Comunidad Autónoma de Madrid (P-AMB-259-0505), the Ministry of Science and Innovation (BIO2007-67304, BIO2010-21049, CSD2007-00005) and by European Union Grants (GEN 2006-27750-C5-3-E and NMP2-CT-2007-026515).

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