RESEARCH LETTER

Characterization of temperature-sensitive and lipopolysaccharide overproducing transposon mutants of *Pseudomonas putida* CA-3 affected in PHA accumulation

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Abstract

A library of 20 000 transposon (Tn5) mutants of the gram-negative bacterium *Pseudomonas putida* CA-3 was generated and screened for adverse affects in polyhydroxyalkanoates (PHA) accumulation. Two mutants of interest were characterized phenotypically. CA-3-126, a mutant disrupted in a stress-related protein Clp protease subunit ClpA, demonstrated greater decreases in PHA accumulation compared with the wild type at reduced and elevated temperatures under PHA-accumulating growth conditions. CA-3-M, which is affected in the aminotransferase class I enzyme, accumulated reduced levels of PHA relative to the wild type and had lower growth yields on all carbon sources tested. Mutant CA-3-M produced up to 10-fold higher levels of lipopolysaccharide relative to the wild type and exhibited 1.2-fold lower aminotransferase activity with phenylalanine as a substrate compared with the wild-type strain. The composition of the lipopolysaccharide produced by the mutant differed from that produced by the wild-type strain. Growth and PHA accumulation by CA-3-M was the same as the wild type when the nitrogen concentration in the medium was increased to 265 mg N L^{-1}.

Introduction

Polyhydroxyalkanoates (PHA) are a class of optically active biodegradable polyesters accumulated by numerous bacterial species as intracellular granules in response to inorganic nutrient limitation, such as nitrogen or phosphorous, in the presence of excess carbon (Lageveen *et al.*, 1988; Anderson & Dawes, 1990; Madison & Huisman, 1999). Because of their biodegradability and biocompatibility, these polymers have attracted considerable commercial interest in both the biomedical and the packaging fields (van der Walle *et al.*, 2001).

The metabolic pathways used for bacterial medium chain length PHA (mclPHA) biosynthesis have been well documented, with two major routes found in *Pseudomonas putida* (Huijberts *et al.*, 1994; Rehm *et al.*, 1998; Klinke *et al.*, 1999). β-Oxidation is the primary pathway used when substrates chemically similar to PHA such as alkanoic (e.g octanoic acid) or phenylalkanoic fatty acids are utilized as carbon sources (Huijberts *et al.*, 1994). When carbon sources unrelated to the chemical structure of PHA (e.g. glucose) are utilized, 3-hydroxy-acyl-ACP moieties are generated via the *de novo* fatty acid synthesis pathway (Eggink *et al.*, 1992; Huijberts *et al.*, 1994). These 3-hydroxyl-acyl-ACP-molecules are converted to their Co-A equivalents by 3-hydroxy-acyl ACP:CoA transacylase (PhaG) (Rehm *et al.*, 1998; Fiedler *et al.*, 2000), and polymerized by PHA polymerase (PhaC) to form PHA and free CoA (Rehm & Steinbüchel, 1999). In *P. putida* CA-3, two class II mclPHA synthases (*phaC1* and *phaC2*) are present (O'Leary *et al.*, 2005). They are organized in a *pha* operon also containing the *phaZ*, which codes for the PHA depolymerase enzyme (Rehm & Steinbüchel, 1999; O'Leary *et al.*, 2005). PhaZ catalyses the release of carbon from PHA granules once the limiting nutrient has been replenished (Jendrossek & Handrick, 2002). PhaF, a protein that negatively regulates the expression of the genes in the $phaC_1ZC_2D$ operon, was identified and located in a downstream position of the *pha* operon in *P. putida* U (Prieto *et al.*, 1999). This granule-associated protein plays a putative role in granule structure

formation (Prieto *et al.*, 1999). While the study of genes and their products directly related to mclPHA biosynthesis (e.g. PHA polymerase, PHA depolymerase) has been reported (Klinke *et al.*, 1999; Jendrossek & Handrick, 2002; Luengo *et al.*, 2003), there is a dearth of information on the role of other genes and gene products involved in secondary pathways such as nitrogen metabolism and stress responses.

In this study, we report on the generation and characterization of *P. putida* CA-3 transposon (Tn5) mutants disrupted in non-mclPHA genes that are affected in PHA accumulation.

Materials and methods

Chemicals

Styrene, phenylacetic acid, DL-β-phenylalanine (3-amino phenylpropionic acid) and Coomassie blue and Congo red dyes were purchased from Sigma Aldrich (Dublin, Ireland). Media components (peptone, agar and tryptone) were purchased from Difco (BD Diagnostics).

Bacterial strains

Pseudomonas putida CA-3 (NCIMB 41162) was isolated from a bioreactor containing styrene (O'Connor *et al.*, 1995). The mini Tn5 derivative pUT Km-1 was hosted in *Escherichia coli* CC118λpir. The suicide plasmid has the R6K origin of replication and encodes resistance to kanamycin and ampicillin (de Lorenzo *et al.*, 1990). The plasmid pRK600 (Cm^r) was hosted in *E. coli* HB101 and was used as a helper in the triparental mating experiment as it encodes the *tra* functions facilitating pUT-Km1 mobilization.

Culture media and growth conditions

Pseudomonas putida CA-3 wild-type cultures, as well as the *P. putida* CA-3 mutants 126 and CA-3-M, were grown in shake flasks (250-mL Erlenmeyer flasks) containing 50 mL of E2 medium (Vogel & Bonner, 1956) at 30 °C with shaking at 200 r.p.m. The growth medium for transposon mutants contained kanamycin at a concentration of 50 $\mu g\,mL^{-1}$. For the purpose of PHA-accumulation experiments, the inorganic nitrogen source sodium ammonium phosphate was supplied at a concentration of 0.25 $g\,L^{-1}$ (65 $mg\,N\,L^{-1}$). Except where otherwise stated, carbon substrates were included in the medium at a concentration of 20 mM. Styrene was supplied to the growth medium in the vapour phase as described previously (Ward *et al.*, 2005). Bacterial cultures were grown for 48 h.

Random mini Tn5 mutagenesis

The pUT–Km1 plasmid was introduced into *P. putida* CA-3 by means of triparental mating involving *E. coli* CC118λ*pir* (pUT-Km1) as the transposon donor strain, *P. putida* CA-3 as the recipient and *E. coli* HB101 (pRK600) as the helper strain as described previously (Vilchez *et al.*, 2000; O'Leary *et al.*, 2005).

Approximately 20 000 transconjugant mutant colonies were generated. These colonies were screened for transposition events, resulting in a decrease in PHA accumulation using an opacity screen (O'Leary *et al.*, 2005). Fifty colonies showing a decrease in colony opacity (generally associated with PHA accumulation) were subcultured for further studies. These strains were grown in shake flasks, under nitrogen limitation (65 $mg\,N\,L^{-1}$), with styrene as the sole source of carbon and energy for 48 h, after which PHA extraction was performed to determine which mutants were impaired in PHA-accumulating ability.

Identification of the transposon (Tn5) insertion site

Genes disrupted by Tn5 were identified using two consecutive rounds of PCR with primers based on the mini transposon sequence as described previously (Espinosa-Urgel *et al.*, 2000). The resulting PCR products were resolved by electrophoresis using 1% (w/v) agarose gels and visualized using ethidium bromide staining. Bands were excised and purified from the gel using a Qiaex II gel extraction kit (Hilden, Germany). The purified product was then cloned into pCR 2.1-TOPO (Invitrogen) before sequencing. Sequencing was performed by GATC biotech (Hamburg, Germany). Sequence comparative analysis was performed with the GenBank database using the BLAST program (Altschul *et al.*, 1997).

PHA monomer analysis

The 3-hydroxyalkanoic acid methyl esters were assayed by GC using a Hewlett-Packard HP6890 chromatograph equipped with a BP21 capillary column (25 m × 0.25 mm and 0.32-μm film thickness; SGE Analytical Sciences) and a flame ionization detector (FID) as described previously (Nikodinovic *et al.*, 2008).

Aminotransferase activity assay

Aminotransferase activity was determined using a whole-cell assay with DL-β-phenylalanine as the D-amino acid donor and pyruvate as the amino acid acceptor as described previously (Hwang & Kim, 2004). Wild-type and mutant CA-3-M strains were grown in E2 medium containing nitrogen at either 65, 165, or 265 $mg\,L^{-1}$ with phenylacetic acid as the carbon source. The assay was carried out using cell suspensions with an $OD_{540\,nm}$ of 5 in each case. The molar extinction coefficient of alanine was elucidated previously (Hwang & Kim, 2004). Absorbance was read at

540 nm using a microplate reader SpectraMax 340 (Molecular Devices Corp., Sunnyvale).

Exopolysaccharide detection, isolation and analysis

For the assessment of lipopolysaccharide production, colony colour and morphology, *P. putida* strains were grown on tryptone agar plates (tryptone, 10 g L^{-1}; agar, 10 g L^{-1}) supplemented with Congo red (40 μg mL^{-1}), Coomassie brilliant blue (20 μg mL^{-1}) and kanamycin (50 μg mL^{-1}) (for the mutant strain). Cells were plated by spotting and spreading 1 μL of bacterial starter culture, resulting in colonies arising from single cells. The plates were incubated at 30 °C for 4–7 days.

Lipopolysaccharide isolation was carried out as described previously (Fett *et al.*, 1995). Strains were cultured in 25 mL (250-mL Erlenmeyer flask) of King's medium B with increased glycerol concentration (peptone, 20 g L^{-1}; $MgSO_4 \cdot 7H_2O$, 1.5 g L^{-1}; K_2HPO_4, 1.5 g L^{-1}; and glycerol, 50 mL L^{-1}) or minimal E2 medium (Vogel & Bonner, 1956) containing 65 mg N L^{-1}, at 25 °C for 4 days with shaking (200 r.p.m.). Proteinase K (Promega) was added to a final concentration of 0.005% (w/v) to inhibit degradation of the lipopolysaccharide produced. Bacterial cells were removed by centrifugation (9000 ***g*** for 30 min at 4 °C). The clear supernatant was collected and a solution of KCl (25%, w/v) was added to a final concentration of 1% (w/v). Methanol was added to precipitate the lipopolysaccharide (75 mL). The mixture was incubated at 4 °C for 16 h, after which the precipitated lipopolysaccharide was collected by centrifugation (10 000 ***g*** for 30 min at 4 °C), dissolved in distilled water (10 mL), recentrifuged, freeze dried and weighed.

Glycosyl composition analysis was performed at the Complex Carbohydrate Research Centre (University of Georgia, Athens, Georgia) by combined GC/MS of the per-*O*-trimethylsilyl (TMS) derivatives of the monosaccharide methyl glycosides produced from the sample by acidic methanolysis (York *et al.*, 1986; Merkle & Poppe, 1994). GC/MS analysis of the TMS methyl glycosides was performed on an HP 6890 GC interfaced to a 5975b MSD, using an EC-1 fused silica capillary column (30 m × 0.25 mm ID, All Tech).

Results

Twenty thousand *P. putida* CA-3 transposon mutants were generated and initially screened for deficiencies in PHA accumulation by means of an opacity screen (O'Leary *et al.*, 2005). Fifty colonies that appeared less opaque were subcultured on E2 solid media with styrene as the sole source of carbon and energy for further examination. Each of the 50 mutant strains was grown in liquid culture with styrene as the growth substrate, harvested and assessed for PHA accumulation (% cell dry weight) and PHA monomer composition (data not shown). Two mutants were chosen for further study based on decreased PHA levels and the location of the transposon in the chromosome of *P. putida* CA-3. Comparative BLAST GenBank analysis of the sequenced PCR product from the mutant CA-3-126 revealed a 98% homology with the ClpA protease gene from *P. putida* KT2440 (The Institute for Genomic Research-TIGR accession number: PP_4008). Mutant CA-3-M is disrupted in an amino transferase class I enzyme sharing 87% homology with aspartate aminotransferase in *P. putida* KT2440 (TIGR: PP_4692).

While mutant CA-3-126 was deficient, but not totally lacking PHA accumulation, mutant CA-3-M was incapable of PHA accumulation from styrene (Table 1). PHA accumulation by both mutants when grown on phenylacetic acid, an intermediate in styrene metabolism (Hartmans *et al.*, 1990; O'Connor *et al.*, 1995), glucose and octanoate, is decreased relative to the wild-type strain (Table 1). To further characterize these mutants, specific experiments were performed based on the identification of the genes disrupted by transposon mutagenesis.

Characterization of a ClpA mutant *P. putida* CA-3-126

ClpA is a subunit of the ClpAP complex. It is a chaperone-like ATPase associated with the proteolytic element ClpP. In *E. coli*, the ClpAP protease degrades larger proteins down to peptides 7–10amino acids in length and shows no apparent amino acid sequence specificity (Thompson *et al.*, 1994). For complete proteolytic activity, ClpP must associate with the ATPase ClpA or alternatively a smaller chaperone-like component ClpX (Thompson *et al.*, 1994). Clp proteases of *E. coli* play an important role in the regulatory mechanisms in nongrowing or slow-growing cells (Weichart *et al.*, 2003). In *Bacillus subtilis* ClpP is thought to play a role during heat shock as well as oxidative and salt stress, due to the increase in ClpP synthesis (Volker *et al.*, 1994). Biofilm formation, which by itself is a bacterial response mechanism to specific environmental triggers, is thought to be dependent on ClpP levels in *Pseudomonas fluorescens* (O'Toole & Kolter, 1998) and in *Staphylococcus epidermidis* (Wang *et al.*, 2007).

Table 1. Medium chain length PHA accumulation (% of cell dry weight) by *Pseudomonas putida* CA-3 and transposon mutants when grown on a variety of carbon substrates

Name	Identification*	Styrene	Phenylacetic acid	Glucose	Octanoate
CA-3	Wild type	25	28	33	30
CA-3-126	ClpA subunit	18	16	13	7
CA-3-M	Aminotransferase	0	4	16	14

*Gene disrupted or wild-type strain.

Mutant CA-3-126 accumulates 25% less PHA from styrene compared with the wild type at 30 °C (Table 1). However, the growth yield of mutant CA-3-126 is unaffected with either styrene, phenylacetic acid or glucose as the sole carbon and energy sources. A temperature of 30 °C is close to the optimal temperature for the growth of mesophilic organisms such as *P. putida* CA-3. A deviation above and below this temperature will contribute to increased stress to the organism and may thus challenge its ability to accumulate PHA. Given that bacteria accumulating polyhydroxybutyrate (PHB) are known to be under stress (Tessmer *et al.*, 2007), it is possible that ClpP protease may help the wild-type strain accumulate mclPHA to tolerate this stress better than the mutant. To avoid the effect of temperature on the partition of styrene in the growth medium and potentially the supply of carbon to the cells, phenylacetic acid, an intermediate in the styrene degradation pathway, was used as the growth substrate for these experiments. Wild-type CA-3 and mutant CA-3-126 exhibited a similar twofold decrease in PHA-accumulating ability when the growth temperature was increased from 30 to 33 °C. At 19 °C, the wild-type strain accumulates 2.2-fold less PHA compared with cells grown at 30 °C (Fig. 1). However, at 19 °C, the mutant strain accumulates PHA at only one-fifth of that accumulated at 30 °C. The wild type was severely inhibited in its ability to accumulate PHA at 15 and 37 °C (Fig. 1). However, mutant 126 was completely deficient in PHA accumulation at these temperatures (Fig. 1). A 2.7-fold decrease in PHA-accumulating ability was observed for both the wild-type and the mutant strain when grown on phenylacetic acid at 19 °C compared with 30 °C. However, a ninefold greater decrease in PHA accumulation was observed for the mutant compared with the wild-type strains grown with phenylacetic acid at 37 °C (Fig. 1b). A similar pattern was observed for PHA accumulation for cells grown on glucose, with mutant CA-3-126 accumulating lower levels of PHA compared with the wild-type strain at all the temperatures tested (data not shown).

Characterization of an aminotransferase class I mutant of *P. putida* CA-3-M

Aminotransferases play a central role in nitrogen metabolism through the transfer of amino groups. They are highly stable enzymes that exhibit broad substrate specificity (Taylor *et al.*, 1998; Hwang *et al.*, 2005). In *Pseudomonas* species, aminotransferases have been implicated in D-amino acid synthesis (Wiyakrutta & Meevootisom, 1997; Gu *et al.*, 1998); similarly, in *Bacillus* species, aminotransferases provide D-amino acids for the biosynthesis of peptidoglycan and other secondary metabolites (Taylor *et al.*, 1998).

While no PHA was accumulated by mutant CA-3-M from styrene, low levels of PHA were detected in cells grown on

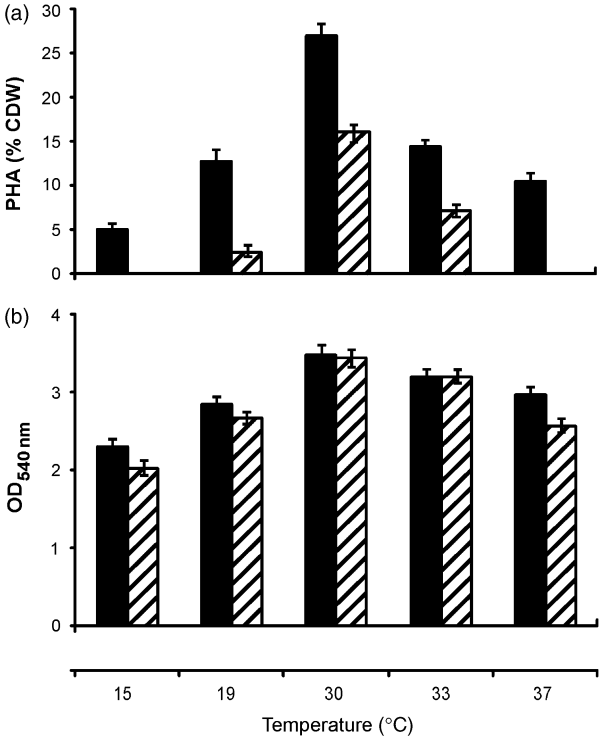

Fig. 1. PHA accumulation (a) and cell growth (b) of the Tn5 mutant CA-3-126 knocked out in the ClpA subunit of the ClpP protease protein (▨) and of the *Pseudomonas putida* CA-3 wild-type strain (■) grown on phenylacetic acid as the sole source of carbon and energy. Cells were grown at different temperatures.

phenylacetic acid and glucose using a limited nitrogen concentration (65 mg N L^{-1}) (Table 1). As aminotransferases may play a role in nitrogen recycling in the cell, the disruption of this gene could increase the demand for nitrogen by the mutant strain and affect PHA accumulation. Further experiments were carried out using growth medium containing a range of nitrogen concentrations (65 mg L^{-1}, 165 mg L^{-1} and 265 mg L^{-1}) to assess the ability of CA-3-M to grow and accumulate PHA from phenylacetic acid and glucose, respectively, at a constant starting concentration of carbon (1.92 g L^{-1}) (Fig. 2a).

PHA accumulation was 6.5-fold lower in mutant CA-3-M at 65 mg N L^{-1} relative to the wild type for phenylacetic acid-grown cells (Fig. 2a). There was a 2.4-fold higher growth yield for the wild type compared with the mutant strain under these growth conditions. The addition of higher concentrations of nitrogen (165 mg N L^{-1}) in the growth medium resulted in a further decrease in PHA accumulation by wild type and mutant cells and an even greater difference (16-fold) in PHA accumulation between the wild type and mutant CA-3-M. However, the difference in growth yield between the wild type and mutant CA-3-M decreased 1.8-fold. Further increases in the nitrogen concentration (265 mg N L^{-1}) resulted in the same growth yield from

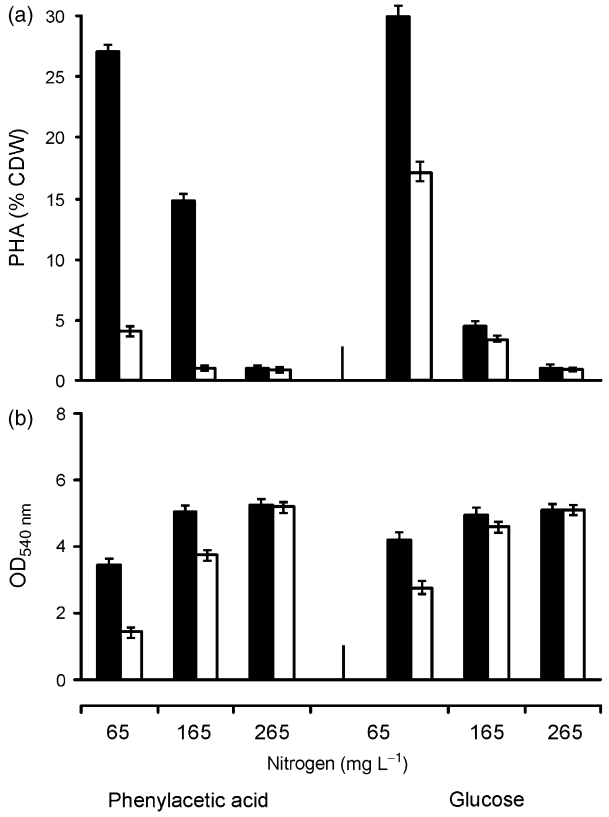

Fig. 2. The aminotransferase class I mutant CA-3-M (□) and *Pseudomonas putida* CA-3 wild-type strain (■) grown on various carbon sources at varying nitrogen concentrations. Values for PHA accumulation (a) and cell growth (b) are presented.

phenylacetic acid in both wild-type and mutant strains. Furthermore, they exhibited the same very low level of PHA accumulation.

A similar growth and PHA accumulation pattern is observed for growth on glucose, except that a lower nitrogen concentration (165 mg L^{-1}) is required to allow the mutant to achieve a similar growth yield and accumulate a level of PHA similar to the wild type (Fig. 2). The total amount of nitrogen utilized by the mutant strain (10.5 mg N in 50 mL E2 medium) was 1.5-fold higher than that utilized by the wild-type strain when either glucose or phenylacetic acid (1.92 g C L^{-1}) was supplied as the sole carbon and energy source with 265 mg N L^{-1}.

An aminotransferase whole-cell activity assay using DL-β-phenylalanine as a substrate was performed using wild type and mutant CA-3-M. When grown on E2 medium (65 and 165 mg N L^{-1}, respectively) with phenylacetic acid as the sole source of carbon and energy, CA-3-M cells exhibited a 1.25-fold lower aminotransferase activity compared with the wild-type cells (Table 2). Similarly, mutant cells grown on glucose showed a 1.2-fold lower activity relative to the wild

Table 2. Aminotransferase activity of *Pseudomonas putida* CA-3 and transposon mutant CA-3-M

Carbon substrate/ growth medium	Nitrogen concentration (mg L^{-1})	Activity (μmol min^{-1} mg^{-1} cell dry weight)	
		Wild-type CA-3	Mutant CA-3-M
Phenylacetic acid	65	131 ± 2	108 ± 3
	165	116 ± 3	99 ± 2
	265	108 ± 2	90 ± 1
Glucose	65	123 ± 4	105 ± 2
	165	105 ± 1	88 ± 3
	265	101 ± 2	86 ± 1

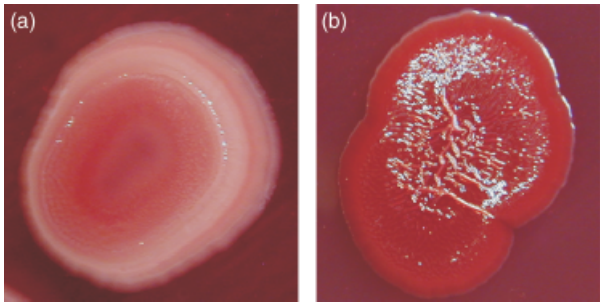

Fig. 3. The aminotransferase class I mutant CA-3-M bacterial colony (b) and the wild-type *Pseudomonas putida* CA-3 wild-type colony (a) following staining with Congo red dye.

type at nitrogen concentrations of 65 and 165 mg L^{-1}, respectively (Table 2).

CA-3-M exhibited a colony morphology very different from that of the wild type when grown on E2-solid medium. A halo was observed around the colonies that was sticky in texture, which is suggestive of extracellular polysaccharide production in the mutant strain. Wild-type and mutant CA-3-M colonies were stained with Congo red, which is known to bind extracellular polysaccharides (Arciola *et al.*, 2005). Mutant colonies stained a much deeper red than wild-type colonies; in addition, after staining, the entire mutant colony appeared to take up the stain, whereas the wild-type colony staining was restricted to the centre of the colony (Fig. 3), suggesting that the mutant CA-3-M is producing excess exopolysaccharide relative to the wild type.

In order to confirm the increased levels of lipopolysaccharide production in the aminotransferase mutant, lipopolysaccharide isolation was carried out on both wild type and mutant MCA-3 cells. Cells were grown on one of two growth media, Kings B or E2 (65 mg N L^{-1}, supplemented with either phenylacetic acid or glucose). Maximum lipopolysaccharide (158.5 mg) was isolated from mutant cells grown on King's B medium (Table 3). The total lipopolysaccharide isolated from wild-type CA-3 cells was nearly twofold lower than that isolated from mutant MCA-3 cells

Table 3. Exopolysaccharide (lipopolysaccharide) production in *Pseudomonas putida* CA-3 and the aminotransferase class I mutant *P. putida* MCA-3 grown in King's B medium, E2 medium supplemented with phenylacetic acid and E2 medium supplemented with glucose

	Total lipopolysaccharide weight (mg)		Lipopolysaccharide (mg mg^{-1} CDW)	
Medium	CA-3	MCA-3	CA-3	MCA-3
King's B	83.2	158.5	1.4	2.62
E2/PA	7	40	0.18	1.25
E2/glucose	5.9	47	0.08	0.79

The data shown are an average of three determinations.
CDW, cell dry weight.

on this growth medium. The total lipopolysaccharide isolated from cells grown on E2 medium supplemented with phenylacetic acid was fivefold greater in the mutant cells than that in the wild type; however, the greatest difference in the total lipopolysaccharide isolated was seen when cells were grown on E2 medium supplemented with glucose, where the total lipopolysaccharide isolated is 7.9-fold higher in the mutant compared with the wild-type strain (Table 3). The amount of lipopolysaccharide isolated (mg) was also expressed per mg of the cell dry weight achieved (Table 3). This shows a 10-fold greater lipopolysaccharide production in the mutant relative to the wild type when grown on E2 and glucose. Lipopolysaccharide isolated from MCA-3 and the wild type is predominantly composed of a lipid, with 0.2–0.5% of the lipopolysaccharide composed of carbohydrate (data not shown). Lipopolysaccharides from both the wild-type and the mutant strain contained traces of glutamic acid and were similar in carbohydrate composition. However, minor differences were observed because the carbohydrate fraction of lipopolysaccharide from wild-type strain contained glucose, mannose, *N*-acetyl-glucosamine, galactose and rhamnose in a 38 : 27 : 17 : 14 : 4 respective percentage ratio, while the carbohydrate fraction of lipopolysaccharide from the mutant strain contained glucose, *N*-acetyl glucosamine, galactose and mannose in a 37 : 22 : 21 : 20 respective percentage ratio. The lipopolysaccharide isolated from both strains contained a lipid component comprising mainly of hexadecanoic acid as determined by GC/MS (data not shown). The lipopolysaccharide accumulated by *P. putida* CA-3 is similar to that reported for other *Pseudomonas* strains (Wilkinson, 1983; Zdorovenko & Veremeichenko, 2001).

Discussion

The chemo-biotechnological conversion of polystyrene to PHA, as well as a process manipulation strategy to increase PHA yields in *P. putida* CA-3 have been reported previously by this laboratory (Ward *et al.*, 2006). We are currently aiming to understand more fully the factors affecting PHA biosynthesis in *P. putida* CA-3 in order to improve this process. We used a random transposon (Tn5) mutagenesis approach to generate mutants with decreased ability to accumulate PHA.

A ClpA subunit of the ClpP protease appears to have an impact on PHA biosynthesis in *P. putida* CA-3 as seen from phenotypic characterization of the ClpA knockout mutant CA-3-126. ClpP forms an active protease when bound to the chaperone-like ClpA subunit. ClpA is thought to function by binding target proteins and unfolding the protein to allow access of the protein to the active site of the ClpP subunit where proteolysis can occur (Wickner *et al.*, 1994). While a function for ClpP at higher temperatures has been shown in *B. subtilis*, where ClpP mutants were unable to grow at elevated temperatures (Msadek *et al.*, 1998), the effect of ClpP mutation on PHA accumulation has not been reported. PHA accumulation in the ClpA mutant CA-3-126 is severely affected relative to the wild-type strain at temperatures beyond those for optimal growth relative to the wild type, suggesting that the activity of the ClpP protease is important for PHA accumulation in *P. putida* CA-3. Bacteria accumulating PHA have been reported to express proteins that are produced in response to stress (Tessmer *et al.*, 2007; Kang *et al.*, 2008). The greater decrease in PHA accumulation in the *P. putida* CA-3-126 *clpA* mutant cells compared with the wild-type cells at lower and higher temperatures may reflect the inability of the cells to deal with additional stresses. Tessmer *et al.* (2007) reported large amounts of a heat shock protein (HspA) associated with the PHB granule in recombinant *E. coli* lacking the phasin PhaP1. The authors propose that HspA can act like a phasin protein, affecting PHA granule coalescence.

Limiting the nitrogen source in the growth media is commonly used to stimulate PHA accumulation in bacteria (Huisman *et al.*, 1989; Garcia *et al.*, 1999). We have shown that the supply of low levels of nitrogen to the growth medium dramatically increases the PHA accumulation by *P. putida* CA-3 (Goff *et al.*, 2007). CA-3-M, the aminotransferase class I mutant, accumulates reduced levels of PHA relative to the wild type at nitrogen concentrations of 65 and 165 mg L^{-1}. A decrease in aminotransferase activity reduced the ability to make nitrogen available to the cell, which

negatively affected PHA accumulation in *P. putida* CA-3. While the aminotransferase activity was decreased 1.25-fold relative to the wild type, the decrease in PHA levels was much more dramatic (six- and 16-fold). The increased demand for external nitrogen by mutant CA-3-M compared with the wild-type strain (Fig. 2) supports the suggestion that the role of the aminotransferase is to recycle nitrogen in the cell. These data, combined with complementation of the growth yields through addition of extra nitrogen to the growth medium, suggest a vital role for the aminotransferase both in the growth and in PHA accumulation in *P. putida* CA-3.

The change in colony morphology (lipopolysaccharide production) in the mutant strain indicates the complex effect of the aminotransferase mutation. An increase in the carbon to nitrogen ratio is known to trigger exopolysaccharide synthesis (Sutherland, 1972; Veiga *et al.*, 1997; Shenga *et al.*, 2006). If the aminotransferase is critical for nitrogen release from amino acids, then a decrease in activity would lead to a decrease in the nitrogen concentration in the cell. This could lead to an alteration in the carbon to nitrogen ratio and induce lipopolysaccharide production, thus diverting carbon away from PHA.

The disruption of genes encoding proteins associated with stress and nitrogen metabolism appears to affect the ability of *P. putida* CA-3 to accumulate mclPHA. The decrease in PHA concomitant with the increase in lipopolysaccharide production indicates that the metabolic pathway for other carbon polymers such as lipopolysaccharide competes with the mclPHA pathway for carbon. In addition, the temperature-sensitive nature of PHA accumulation indicates the important role of stress proteins in the complex mclPHA pathway.

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Authors' contribution

M.G. and J.N.-R. contributed equally to this work.

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