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ORIGINAL PAPER

Genetic organization of *pha* gene locus affects *phaC* expression, poly(hydroxyalkanoate) composition and granule morphology in *Pseudomonas corrugata*

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Abstract The complete sequence of the *pha* locus responsible for the biosynthesis of poly(hydroxyalkanoates) (PHAs) in *Pseudomonas corrugata* 388 was determined. As with the other known pseudomonad *pha* gene loci, the one in *P. corrugata* 388 also consists of *phaC1* (1,680 bps; PHA synthase 1), *phaZ* (858 bp; PHA depolymerase) and *phaC2* (1,683 bp; PHA synthase 2) genes. A BLAST search showed that the nucleotide sequences of these genes and the amino-acid sequences of their respective gene products are homologous to those of *P. corrugata* CFBP5454 and *P. mediterranea* CFBP5447. A putative intrinsic transcription terminator consisting of a dyad symmetry (24 bp; $\Delta G = -41.8$ kcals) that precedes a stretch of dA residues was located in the *phaC1–phaZ* intergenic region. *P. corrugata* mutant-clones XI 32-1 and XI 32-4 were constructed in which this intergenic region was replaced with a selectable kanamycin-resistance gene. These mutant clones when grown on oleic acid for 48 h showed 4.7–to 7.0-fold increases of *phaC1* and *phaC2* relative expression in comparison to the initial inoculants, whereas the parental strain showed only 1.2- to 1.4-fold increases. Furthermore, in comparison to parental *P. corrugata* with only a few large PHA inclusion bodies, the mutants grown on oleic acid produce numerous smaller PHA granules that line the periphery of the cells. With glucose as a substrate, XI 32-1 and XI 32-4 clones produce mcl-PHA with a high content (26–31 mol%) of the monounsaturated 3-hydroxydodecenoate as a repeat-unit monomer. Our results show for the first time the effects of the *phaC1–phaZ* intergenic region on the substrate-dependent temporal expression of *phaC1* and *phaC2* genes, the repeat-unit composition of mcl-PHA, and the morphology of the PHA granules.

Keywords *Pha* genes · PHA synthase · PHA depolymerase

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Introduction

Poly(hydroxyalkanoates) (PHAs) are microbial polyesters produced by many microorganisms. There are three major groups of PHAs based on their repeat unit compositions: the short-chain-length (scl-) PHAs where the repeat units are hydroxy fatty acids (HFAs) of 3–5 carbon chain length (C3–C5), the medium-chain-length (mcl-) PHAs with repeat units of C6 and longer, and the scl-*co*-mcl-PHAs such as NODAX™ (Procter & Gamble, Cincinnati, OH, USA) that contain primarily 3-hydroxybutyrate but also some ≥C6 HFAs as their repeat units. In general, the scl- and scl-*co*-mcl-PHAs are thermoplastics with various degrees of crystallinity, and the mcl-PHAs are amorphous and have elastomeric and adhesive properties. PHAs have great potential to serve as ecologically sound substitutes for petroleum-derived polymers because they are biodegradable and can be produced by white-biotechnological process using renewable feedstocks. As such, research abounds to explore the use of PHAs in medical devices, foods, agriculture and consumer products [14, 20, 29]. To

this end, there is a continuous need to expand the repertoire of PHA varieties having novel compositions to improve their material properties, and to develop cost-effective production systems to reduce the price of the products. Metabolic engineering is a powerful means to achieve this goal [1], and the understanding of the structure–function relationship of PHA biosynthesis gene locus is in turn important for the carrying out of the metabolic engineering of PHA-producing organisms.

Pseudomonas corrugata is a versatile mcl-PHA producing organism [12, 23]. It is a potentially useful agricultural biocontrol agent because it can propagate in the root system of plants where it is a good competitor against other organisms. Various *P. corrugata* strains have also been found to produce valuable microbial products such as lipid A that could function as biosurfactants [7]; exopolysaccharides [9]; bioactive lipodepsipeptides such as cormycin A [19] and corpeptins [8]; and a lipopeptide siderophore, corrugatin [18]. The prospect of coproducing mcl-PHA with one of these valuable microbial products is attractive because it can lead to a partial recovery of the production costs of the biopolymer. In addition, *P. corrugata* is unique among most mcl-PHA-producing pseudomonads in that the biopolymer it produces has high molecular weight and that the production of PHA is not inhibited at 37 °C [23]. This latter property allows *P. corrugata* to better utilize animal fats as inexpensive substrates for mcl-PHA production. Overall, *P. corrugata* is an attractive target for further strain improvement via metabolic engineering to effect a viable mcl-PHA production system. In this paper, we describe our study to characterize the genetic organization of the *pha* locus of *P. corrugata*, and report for the first time the manipulation of the expression pattern of PHA synthase genes, the mcl-PHA composition, and the PHA granule morphology through the removal of an intergenic region of the *pha* locus.

Materials and methods

Bacteria, growth conditions, and plasmids

Pseudomonas corrugata 388, originally isolated from alfalfa roots by F.L. Lukezic (Pennsylvania State University, University Park, PA, USA) was obtained from Dr. W.F. Fett (Eastern Regional Research Center/ARS/USDA, Wyndmoor, PA, USA). *Escherichia coli* DH5α used in routine DNA subcloning and plasmid maintenance was purchased from Invitrogen (Carlsbad, CA, USA). We routinely used Luria medium (1% w/v tryptone, 0.5% w/v yeast extract, 0.5% w/v NaCl) or tryptic soy broth (TSB; Difco, Detroit, MI, USA) for growing *E. coli* (at 37 °C) and *P. corrugata* (30 or 37 °C as indicated). The solid media were prepared by adding agar (1.2–1.5% w/v) to the corresponding liquid broths before autoclaving. Kanamycin (Km, 35 μg/ml) and carbenicillin (Cb, 50 μg/ml) were added as needed to the growth media. The pCN51 [16] and pET-Blue-1 vectors were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA) and Novagen (Madison, WI, USA), respectively.

Compilation of the sequence of *pha* locus

We employed a chromosomal-walking method to sequence the entire *pha* locus containing *phaC1*, *phaZ* and *phaC2* genes of *P. corrugata* 388. A commercially available DNA Walking SpeedUp™ premix Kit (Seegene USA, Rockville, MD, USA) was used to carry out the chromosomal walk. Based on the previously determined sequences of the *phaC1* and *phaC2* fragments (*ΔphaC1* and *ΔphaC2*) of *P. corrugata* 388 [22], two target-specific primers (TSP1 and TSP2) were designed for use in a semi-nested PCR protocol to obtain the DNA fragments adjacent to each end of the *ΔphaC1* and *ΔphaC2* sequences according to the kit manufacturer's instruction. The sequences of the newly cloned PCR fragments were determined. Based on these sequences, new TSP1 and TSP2 primers were synthesized for a subsequent round of chromosomal walk. By repeating this chromosomal walk procedure several times, the entire *pha* locus containing *phaC1*, *phaZ* and *phaC2* of *P. corrugata 388* was obtained (Fig. 1). This sequence was deposited in GenBank under the accession number of EF067339. BLAST [2] search was performed using the web-based program of National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST/). Sequence comparative analysis was performed using the Two-Sequence Alignment (Global Comparison type) function of Clone Manager Professional Suite program (Scientific & Educational Software, Cary, NC, USA).

Construction of homologous-recombination plasmids

We had found [25] that homologous recombination can occur between a gene sequence on *Pseudomonas* genomic DNA and a homologous sequence carried on pETBlue-1 plasmid, an *E. coli* expression vector that cannot replicate in *Pseudomonas*. We therefore constructed pXI 22-1 and pXI 22-2 homologous-recombination vectors (Fig. 2) to use in the generation of *P. corrugata* mutants having their *phaC1–phaZ* intergenic region removed and replaced by a selectable kanamycin-resistance gene (KmR). To achieve this goal, a plasmid pX 196-3 (Fig. 2) that consists of a partial *pha* locus of *P. corrugata* 388 (nucleotide-numbers 1264–2867 of the *pha* sequence in GenBank EF067339) cloned into the pETBlue-1 vector at the *Eco*RV site was digested with restriction enzymes *Eco*RV and *Sph*I to

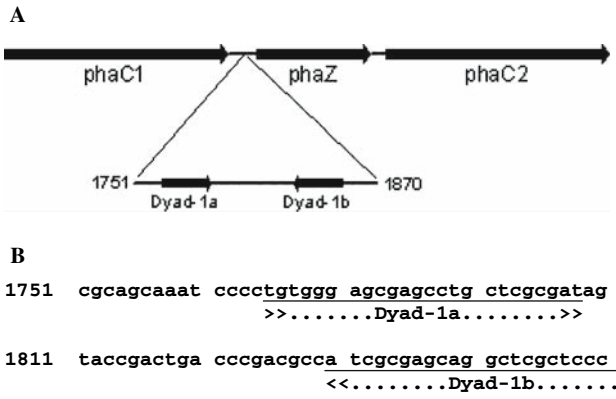

Fig. 1 The *pha* locus of *P. corrugata* 388. **a** Schematic diagram of the *pha* locus with the region containing the dyad symmetry expanded. Dyad 1a and Dyad 1b are the palindromic sequences of the dyad symmetry. **b** The nucleotide sequence of the DNA segment containing the dyad symmetry

remove the *phaC1–phaZ* intergenic region (0.2 kb) including the terminator signal. The resultant 4.9-kb DNA was rendered blunt-ended at both terminals by treating it with T4 DNA polymerase to remove the 3′-overhang of the *Sph*I cut-site. At the same time, a DNA segment (1.3 kb) containing KmR gene was excised from a pCN51 shuttle vector [16] using *Stu*I restriction endonuclease. (It should be noted that the pCN51 vector was purified from a previously transformed *P. resinovorans*. PCN51 purified from *E. coli* DH5α, a bacterial host routinely used in our laboratory to maintain plasmid DNAs, would have been methylated by the host's *dcm* and *dam* DNA methylation systems and thus is resistant to *Stu*I digestion.) The 4.9-kb blunt-ended pX 196-3 derivative was ligated with the KmR-containing DNA piece using T4 DNA ligase to yield pXI 22-1 and pXI 22-2 (Fig. 2).

Construction of *P. corrugata* mutants lacking *phaC1–phaZ* intergenic region

The plasmids pXI 22-1 and pXI 22-2 were introduced into *P. corrugata* 388 by an electroporation procedure described by Solaiman et al. [24]. The electroporated cells were spread on LB-agar containing Km (35 μg/ml) as selection marker. The successful replacement of the genomic *phaC1–phaZ* intergenic region by the KmR-containing DNA segment via a homologous recombination event in the surviving cells was confirmed by PCR amplification and subsequent sequence determination of the junction regions. For confirmatory screening of the KmR clones resulting from pXI 22-1 integration, primer pairs SQ11-50-C1F1 (5′-ACGACCTGATCTGGAACTAC-3′)/SQ11-50-C1R1-4 (5′-TCAGATCACGCATCTTCC-3′) and SQ11-50-ZF1-4 (5′-TTGACGGGACGGCGGCTTTGTTG-3′)/SQ11-50-C2 (5′-ACAACAGGTCCTTGCCGCGTAG-3′) were used with the expected sizes of the PCR amplicons as 0.7- and 1.3-kb, respectively (Fig. 3). For KmR clones from pXI 22-2, the primer pairs were SQ11-50-C1F1 (5′-ACGACCTGATCTGGAACTAC-3′)/SQ11-50-C1R1-3 (5′- GAGCATTACGCTGACTTGAC-3′) and SQ11-50-ZF1-3 (5′-GATCAG

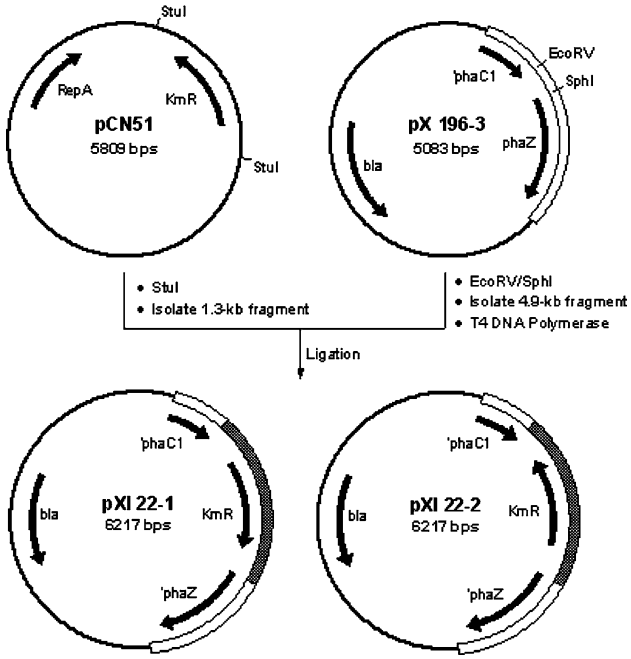

Fig. 2 Construction of homologous-recombination plasmids. *RepA* replication protein, *KmR* kanamycin-resistance gene, *bla* β-lactamase gene, *'phaC1* PHA synthase 1 gene (5′-truncated), *phaZ* and *'phaZ* PHA depolymerase gene and its 5′-truncated progeny, respectively

ATCACGCATCTTCC-3′)/SQ11-50-C2 (5′-ACAACAGGTCCTTGCCGCGTAG-3′) with expected PCR amplicons of 0.8 and 1.2 kb. These PCR products were directly used as templates for sequence determination using a Perkin-Elmer ABI Prism 3730 DNA Analyzer.

PHA isolation and compositional analysis

PHA was isolated and characterized as described previously [26]. Briefly, 25–50 mL of a seed culture was prepared in LB medium and grown overnight (18–20 h) at 30 °C with 200 rpm shaking. For the experiments with glucose as a substrate, the 25-mL seed culture was used to inoculate 2.5 L (in a 3-L fermentor vessel) of medium E* [4] supplemented with glucose (2% w/v; Sigma Chemicals)

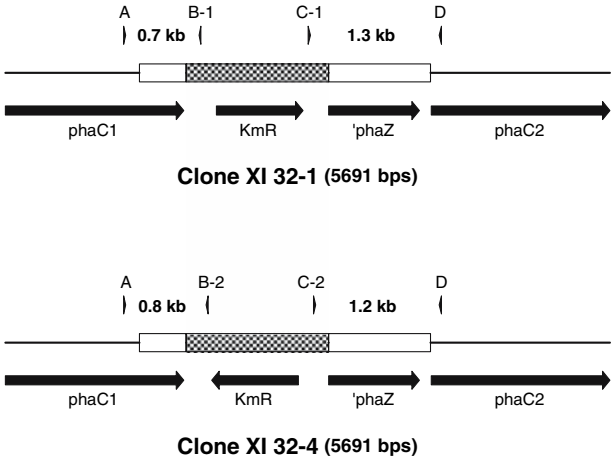

Fig. 3 Genetic organization of *phaC1–phaZ* intergenic regions of *P. corrugata* XI 32-1 and XI 32-4 mutants. *Boxed* region marks the sequence originated from plasmid pXI 22-1 or pXI 22-2 that has replaced the genomic sequence through a homologous recombination event. The sequences of PCR primers (A–D) are described in the text

and Km (35 μg/mL). The glucose-grown culture was incubated for 48 h in a BioFlo 3000 Fermentor (New Brunswick Scientific, Edison, NJ, USA) operated at 30 °C, 300 rpm and 2 L/min airflow. For the study with oleic acid as a substrate, 50 mL of seed culture was added to 5 L (in a 10-L fermenter jar) of E* medium containing oleic acid (2% v/v; Technical grade; Sigma Chemicals, St Louis, MO, USA) and Km (35 μg/mL). The oleic acid-culture was grown for 72 h in a BioFloIII Fermentor (New Brunswick Scientific) operated at 30 °C, 300 rpm and 2 L/min airflow. (Two different volumes were used for the glucose and oleic acid experiments because we wanted to fully utilize the two fermentors in our laboratory simultaneously to expedite the study.) Cells were harvested by centrifugation and lyophilized to dryness. The total weight of the lyophilized cells was recorded as the cell dry weight (CDW). A portion of the lyophilized cells (10–20 mg) was subjected to acid-catalyzed methanolysis [28] and silylation [3]. The remaining lypophilized bacteria were extracted with chloroform. The solvent was removed by using a roto-evaporator to a syrupy consistency, which constitutes the crude PHA preparation. The crude polymer samples were added drop-wise to cold methanol under constant stirring. Purified polymer collected on the stirring bar was scraped into a vial and dried under vacuum. The weight of the dried polymer was recorded for use in the calculation of the PHA productivity values (Table 2). The polymer (crude or purified) was subjected to methanolysis and silylation as described for the lyophilized cells. The silylated methyl esters of the 3-hydroxyalkanoate monomers of all samples were analyzed on a GC/MS using the ion selection mode at m/e 175 [13].

RNA extraction and cDNA synthesis

Total RNAs were prepared using a previously described procedure [6]. Briefly, RNA was prepared from *P. corrugata* strains by using a commercial RNA extraction kit (Purescript, Gentra) as recommended by the manufacturer. For this purpose, samples (1 mL) were removed from *P. corrugata* cultures (OD 600 nm ~0.2–0.4) grown overnight in LB medium at 30 °C (preinoculum or time 0) and from cultures grown in E* medium supplemented with oleic acid (0.5%, Sigma) or glucose (2%, Sigma) for 48 h at 30 °C and appropriate antibiotics. RNA samples were quantitatively analyzed by agarose gel electrophoresis. Following a DNase purification step by DNase I (Invitrogen), 200 ng of total RNA was used in each 20 μl reaction containing one unit of Superscript III reverse transcriptase (Invitrogen), 10 pmol random examer (Invitrogen) and 10 pmol dNTPs. Samples in which reverse transcriptase (RT) was not added were used as negative controls.

Quantitative PCR primers and methods

Primers and TaqMan probes sequences for *phaC1*, *phaC2* and 16S rDNA and real-time PCR conditions are as in Conte et al. [6] with a slight modification. One microliter aliquot of template DNA was used in a 25 μl reaction containing 1X iQTM Multiplex Powermix (Biorad) and 400 and 200 nM of primers and probe, respectively. PCR amplification of 40 cycles at 95 °C for 15 s and at 60° for 30 s was performed using Smart Cycler TD II System (Cepheid). Data were normalized to levels of 16S rRNA and analyzed by use of the comparative critical threshold (C_t) method [6]. The relative comparison method was used to compare the levels of expression of target genes in the preinoculum on LB medium and after 48 h on E* medium [6]. All results are expressed as the means ± SD for duplicate samples from two separate experiments.

Transmission electron micrography

Aliquots of cell cultures were removed for transmission electron micrographic imaging at the time of harvest. Cells in the culture medium were chemically fixed by addition of glutaraldehyde to a final concentration of 1%; the mixture was quickly centrifuged in 1.5 mL tubes into a cell pellet, which was then stored. For embedding and thin sectioning, the cell pellets were immersed in 2% osmium tetroxide in 0.1 M imidazole buffer solution (pH 7.0) for 2 h, then washed with the buffer and dehydrated for 1 h by exchange with a graded series of ethanol solutions, followed by

propylene oxide. Pellets were infiltrated with a 1:1 mixture of propylene oxide and an epoxy resin mixture overnight and finally embedded in epoxy resin and cured at 55 °C for 2 days. Thin sections of the embedded pellets were cut with a diamond knife, stained with uranyl acetate and lead citrate solutions, followed by imaging in a model CM12 transmission electron microscope (FEI Co., Hillsboro, OR, USA) operated at 80 kV in the bright field mode. Images were recorded on photographic film at 22,000×.

Results and discussion

Cloning and sequencing of *pha* locus

We had cloned and sequenced the gene segments (Δ*phaC1* and Δ*phaC2*) of the PHA synthase 1 (PhaC1) and 2 (PhaC2) genes of *P. corrugata* 388 using PCR methods that allow the cloning of type II PHA synthase genes [22]. In this study, we further employed a genomic DNA walking technique to complete the cloning and sequencing of the entire *phaC1-Z-C2* locus of *P. corrugata* 388. We had used the same approach to successfully achieve the sequence determination and subsequent cloning of the entire *pha* (i.e., *phaC1-Z-C2*) locus of *P. resinovornas* [21]. Accordingly, we designed in this present study several semi-nested *pha* gene-specific primers based on the sequence information of Δ*phaC1* (GenBank GI: 12659053) and Δ*phaC2* (GenBank GI: 12659051) to use in the DNA walking experiment. Once the immediate flanking sequences to Δ*phaC1* and Δ*phaC2* were obtained, another round of DNA walk was performed using gene-specific primers derived from these newly sequenced flanking regions. The process was repeated until we achieved the complete sequencing and cloning of the entire *pha* locus containing *phaC1*, *phaZ* and *phaC2* genes of *P. corrugata* 388 (Fig. 1a, GenBank Accession Number EF067339, 4,555 bp). A BLASTN search [2] using this sequence as a query showed that the best sequence match was found with the *pha* loci of *P. corrugata* strain CFBP5454 (GenBank Acc. No. AY910767) and *P. mediterranea* strain CFBP5447 (GenBank Acc. No. AY910768). These results are expected based on our earlier studies on the phylogenetic relationship of these organisms [5, 26]. A more in-depth sequence-comparative analysis was performed to understand the structural organization of the *pha* locus of *P. corrugata* 388. Table 1 shows that the PHA synthases of the two strains (388 and CFBP5454) of *P. corrugata* are nearly identical at both the nucleotide and amino-acid levels and the two PHA depolymerases are identical. Although the sizes of the two gene loci are the same (4,555 bp), there is in fact a one-nucleotide insertion and deletion in the *phaC1–phaZ* and *phaZ–phaC2* intergenic regions, respectively, of the *pha* locus of *P. corrugata* 388 in comparison to that of the CFBP5454 strain. Aside from these one-nucleotide differences, the two intergenic regions of these *P. corrugata* strains are identical. These results not only reaffirm our earlier contention that the Δ*phaC1* and Δ*phaC2* gene fragments of various *P. corrugata* stains isolated from distant locations worldwide are highly conserved [26], but also expand it to include the entire *pha* locus that includes the intergenic regions and *phaZ*. The results in Table 1 also show that the three *pha* genes of *P. corrugata* 388 are highly homologous to those of *P. mediterranea* CFBP5447. The %-match values range from 93 to 95 at the nucleotide sequence level to 96–98 at the amino-acid sequence level. Furthermore, the nucleotide sequences of the *phaZ–phaC2* intergenic regions of *P. corrugata* 388 (110 bp) and *P. mediterranea* CFBP5447 (108 bp) are 91% matched. The *phaC1–phaZ* intergenic regions of the two phylogenically related strains, however, are vastly different. The length of this region in *P. corrugata* 388 is 224 bp, whereas that of *P. mediterranea* CFBP5447 is only 103 bp, resulting in a 30%-match of these sequences. More importantly, a strong hairpin structure ($\Delta G = -41.8$ kcal) that is predicted based on the dyad symmetric sequence found in the *phaC1–phaZ* intergenic regions of *P. corrugata* 388 (Fig. 1b) is in effect eliminated in the much shorter *phaC1–phaZ* intergenic sequence of *P. mediterranea* CFBP5447. This dyad symmetry likely is an intrinsic (versus rho-dependent) transcription terminator for the *phaC1* gene of *P. corrugata* 388. As is characteristic of this kind of terminator [17], the dyad symmetry of *P. corrugata* 388 in the *phaC1–phaZ* intergenic region is constituted of 24 bp with a high GC content, and precedes a stretch of dA residues (nucleotide number 1865–1874) by 11 bases. A dyad symmetry with a ΔG value of −80.7 kJ was identified in the *phaC1–phaZ* intergenic region of *P. aeruginosa* PAO1 [27]; it did not precede a stretch of dA residues and was not classified as a putative terminator. These sequence differences between the *pha* loci of *P. corrugata* 388 and *P. mediterranea* CFBP5447 may explain the slight variation in the PHA composition observed between these strains [26].

We next constructed mutants of *P. corrugata* 388 where the *phaC1–phaZ* intergenic region including the putative terminator sequence is disrupted to probe its possible role on PHA synthesis. A kanamycin-resistance gene was used to effect the sequence interruption and at the same time served as a selection marker. This gene was introduced into the targeted region using a homologous recombination approach that we had previously employed to successfully construct *phaZ* gene knockout of *P. resinovorans* [25]. Two mutants, designated as *P. corrugata* clones XI 32-1 and XI 32-4, were selected for further characterization. The putative genetic structures of these mutants are shown in Fig. 3. These were confirmed by PCR amplification followed by

Table 1 Sequence comparison of *pha* loci

Bacterium	Size of *pha* locus (bp)[a]	Nucleotide level		Amino-acid level	
		Gene	% Match[b]	Gene product	% Match[b]
P. corrugata 388	4,555	$phaC1_{Pc388}$	–	$PhaC1_{Pc388}$	–
		$phaZ_{Pc388}$	–	$PhaZ_{Pc388}$	–
		$phaC2_{Pc388}$	–	$PhaC2_{Pc388}$	–
P. corrugata CFBP5454	4,555	$phaC1_{Pc5454}$	99 (5/1680)	$PhaC1_{Pc5454}$	99 (1/559)
		$phaZ_{Pc5454}$	100 (0/858)	$PhaZ_{Pc5454}$	100 (0/285)
		$phaC2_{Pc5454}$	99 (4/1680)	$PhaC2_{Pc5454}$	99 (2/560)
P. mediterranea CFBP5447	4,432	$phaC1_{Pm5447}$	95 (79/1680)	$PhaC1_{pm5447}$	98 (9/559)
		$phaZ_{Pm5447}$	94 (45/858)	$PhaZ_{Pm5447}$	98 (4/285)
		$phaC2_{Pm5447}$	93 (112/1683)	$PhaC2_{Pm5447}$	96 (22/560)

[a] The size of *phaC1–Z–phaC2* locus includes the intergenic regions

[b] Numbers in brackets show the number of mismatched bases or amino-acid residues over the length of the sequence. Sequence comparison was performed using Clone Manager Professional Suite program

sequence determination of the junction regions of the recombination sites. As depicted in Fig. 3, PCR amplification of the genomic DNA of clone XI 32-1 with the two primer pairs, SQ11-50-C1F1/SQ11-50-C1R1-4 and SQ11-50-ZF1-4/SQ11-50-C2, should result in 0.7- and 1.3-kb DNA fragments, respectively. Figure 4 shows that these PCR-amplified fragments of 0.7 kb (lane 1) and 1.3 kb (lane 3) were indeed obtained with clone XI 32-1 using the appropriate primer pairs. Similarly, PCR amplification of the clone XI 32-4 genomic DNA with primer pairs SQ11-50-C1F1/SQ11-50-C1R1-3 and SQ11-50-ZF1-3/SQ11-50-C2 should yield 0.8- and 1.2-kb amplicons, respectively (Fig. 3). These DNA pieces were indeed obtained when XI 32-4 genome was amplified with the respective primer pairs (lanes 5 and 7 of Fig. 4). Sequence determination of these PCR amplicons further confirmed the preservation of the nucleotide sequence at the recombination sites (data not shown).

The production of PHA by mutant clones XI 32-1 and XI 32-4 lacking the terminator sequence in the *phaC1–phaZ* intergenic region was compared to that by a recombinant strain of *P. corrugata* 388 containing pCN51 plasmid with a kanamycin-resistance marker. This recombinant was used to discount any effect the addition of kanamycin might have on the PHA biosynthesis of *P. corrugata* 388. Oleic acid and glucose representing related and non-related substrates, respectively, for mcl-PHA synthesis were used as carbon sources. The results in Table 2 show that the major repeat units of mcl-PHA produced on oleic acid feedstock are 3-hydroxyoctanoate (C8, 54–58 mol%), 3-hydroxydecanoate (C10, 20–22 mol%) and 3-hydroxytetradecenoate (C14:1, 12–15 mol%) regardless of the bacterial strain used. With glucose as a feedstock, the most predominant repeat unit of the biopolymer is 3-hydroxydecanoate (C10, 35–54 mol%) with all three producing organisms tested.

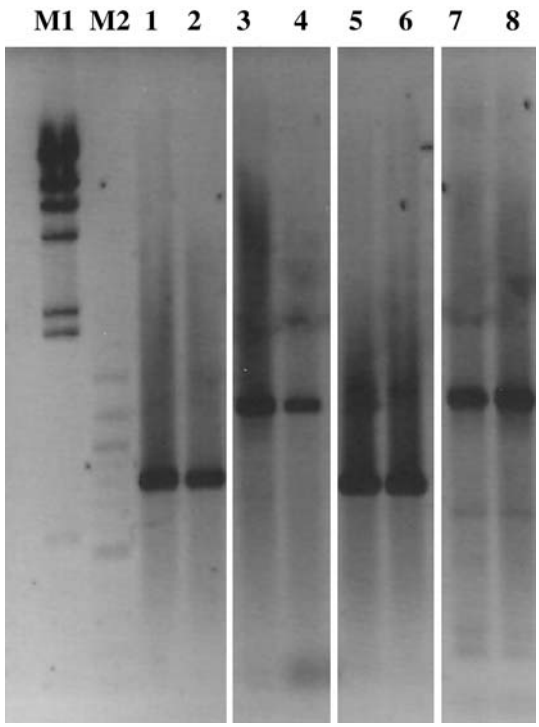

Fig. 4 Agarose-gel electrophoretic analysis of PCR products. *M1* λ DNA-*Hin*dIII digest (sizes from top to bottom 23, 9.4, 6.6, 4.4, 2.3, 2 and 0.56 kb), *M2* 100 bp DNA ladder (New England Biolabs, Beverly, MA, USA; sizes of visible bands from top to bottom 1.5, 1.2, 1.0 and 0.5 kb), *1* clone XI 32-1 with PCR primer-pair *A/B-1*, *3* XI 32-1 with *C-1/D*, *5* XI 32-4 with *A/B-2*, *7* XI 32-4 with *C-2/D*. Note that clones in *lanes 2*, *4*, *6* and *8* were not characterized further in this study

The same pattern of substrate-dependent differential incorporation of 3-hydroxyalkanoates had been previously reported [10, 15]. Those authors showed that recombinant bacteria expressing individual *Pseudomonas phaC1* or *phaC2* gene produced PHA with C8 and C10 as the predominant monomer when grown on fatty acids (i.e., octanoate

Table 2 PHA production and β-hydroxyalkanoate repeat-units of mcl-PHA from *P. corrugata* grown on various feedstocks

Feedstock	Strain	Cell density (g CDW/L culture)	PHA productivity		Composition (mol%)[a, b]					
			% CDW	Volumetric Yield (g/L culture)	C6	C8	C10	C12:0	C12:1	C14:1
Oleic acid (2% v/v)[c]	388 [pCN51]	3.1 ± 0.3	24 ± 2	0.8 ± 0.1	5 ± 2	54 ± 1	20 ± 3	5 ± 1	–	15 ± 0
	Clone XI 32-1	2.9 ± 0.3	23 ± 4	0.7 ± 0.1	4 ± 0	57 ± 2	21 ± 1	5 ± 1	–	13 ± 1
	Clone XI 32-4	2.7 ± 0.1	19 ± 3	0.5 ± 0.1	3 ± 1	58 ± 2	22 ± 2	5 ± 1	–	12 ± 1
Glucose (2% w/v)[c]	388 [pCN51]	1.3 ± 0.2	2 ± 0	>0.10 ± 0.0	2 ± 1	28 ± 1	35 ± 0	9 ± 1	14 ± 3	9 ± 1
	Clone XI 32-1	2.5 ± 0.1	7 ± 1	0.16 ± 0.02	1 ± 1	9 ± 1	54 ± 4	8 ± 0	26 ± 6	–
	Clone XI 32-4	2.0 ± 0.2	5 ± 0	0.10 ± 0.01	1 ± 1	15 ± 1	45 ± 3	7 ± 1	31 ± 1	–

CDW Cell-dry-weight

[a] Values are the averages of at least two compositional determinations from the duplicate cultures. – Trace amount or not detected. *C6* 3-hydroxyhexanoate, *C8* 3-hydroxyoctanoate, *C10* 3-hydroxydecanoate, *C12:0* 3-hydroxydodecanoate, *C12:1* 3-hydroxydodecenoate, *C14:1* 3-hydroxytetradecenoate

[b] When grown in glucose medium, 3-hydroxybutanoate (C4) was detected in the PHA of *P. corrugata* 388 [pCN51] (4 ± 0 mol%) and Clone XI 32-1 (2 ± 1 mol%), and 3-hydroxytetradecanoate (C14:0) was found in the polymers of *P. corrugata* Clone XI 32-1 (1 ± 0 mol%) and Clone XI 32-4 (2 ± 1 mol%)

[c] Bacteria were grown in E* medium supplemented with the indicated carbon source. Duplicate cultures were set up for each experimental run. Cells were grown for 72 h (oleic acid) or 48 h (glucose) in bench-top fermentors operated at 30°C, 300 rpm and 2 L/min air-flow. (See Materials and methods)

and decanoate) and gluconate, respectively. However, the next most abundant repeat units of the polymer from clones XI 32-1 and XI-32-4 grown on glucose were 3-hydroxydodecenoate (C12:1, 26–31 mol%) and 3-hydroxyoctanoate (C8, 9–15 mol%). On the contrary, the second and third most abundant repeat units of polymer from the recombinant *P. corrugata* 388 [pCN51] were C8 (28 mol%) and C12:1 (14 mol%), respectively. Our results thus show that from the perspective of the repeat-unit composition, the removal of the terminator sequence in the *phaC1–phaZ* intergenic region results in the production of mcl-PHA having a high content of the unsaturated C12:1 monomer when glucose is used as substrate. Since the unsaturated bond provides a site for further chemical or enzymatic modification of the polymer to alter its properties, the mcl-PHA produced by terminator knockout strains are thus useful for the preparation of PHA derivatives. Aside from this observation on PHA composition, the data in Table 2 also showed that in general, the level of PHA production (both in terms of % cell-dry-weight and volumetric yield) is lower with the non-related substrate (i.e., glucose) than it is with oleic acid, a related substrate. A previous study with *P. corrugata* 388 also showed a similar pattern of mcl-PHA product yields as a function of carbon source [24]. Hoffman et al. [11] established that the lack of transacylase PhaG activity is responsible for the inability of *P. oleovorans* ATCC 29347 to synthesize mcl-PHA from gluconate (an unrelated carbon source). The low mcl-PHA yields observed with *P. corrugata* 388 grown on glucose may also reflect a low PhaG activity in this organism.

The effect of the removal of the *phaC1–phaZ* intergenic region on the expression of *phaC1* and *phaC2* genes was investigated. The results showed that when clones XI 32-1 and XI-32-4 were grown on oleic acid for 48 h, the relative abundance of the *phaC1* and *phaC2* transcripts increased by 6.6–7.0 (*phaC1*) and 4.7–5.4 (*phaC2*) folds in comparison to the amounts of transcripts of the inoculants at time zero (Fig. 5a). In contrast, the wild-type cells (harboring only the KmR-pCN51 plasmid) exhibited a slight induction of *phaC1* (1.2 folds) and *phaC2* (1.4 folds) transcripts (Fig. 5a), in agreement with the observation of Conte et al. [6] for *P. corrugata* strain 388. These results suggest that the removed *phaC1–phaZ* region played a role in regulating the interaction of a fatty acid-substrate (or its metabolites) with the transcription of *phaC1* and *phaC2* genes. A different observation was made when glucose was used as a sole carbon source. The results in Fig. 5b showed that the *phaC1* transcripts in all three bacterial clones grown on glucose increased by about the same extents, i.e., 5.6 folds for *P. corrugata* 388 [pCN51], 6.3 folds for XI 32-1, and 8.2 folds for clone XI 32-4. The results also showed that within the SD values, the temporal-expression patterns of *phaC2* in these strains grown on glucose did not increase at the 48-h time point. Unlike in cells grown on oleic acid as a carbon source, it seems that the *phaC1–phaZ* intergenic region does not play a role in the transcription regulation of *phaC1* and *phaC2* when glucose was the carbon source. Overall, the results of the transcription study showed for the first time that the *phaC1–phaZ* intergenic region is involved in the regulation of carbon source-dependent expression of *phaC1* and *phaC2* genes. The exact mechanism of action, however, awaits further elucidation.

We had also investigated how the absence of the *phaC1–phaZ* intergenic region with its terminator signal affected

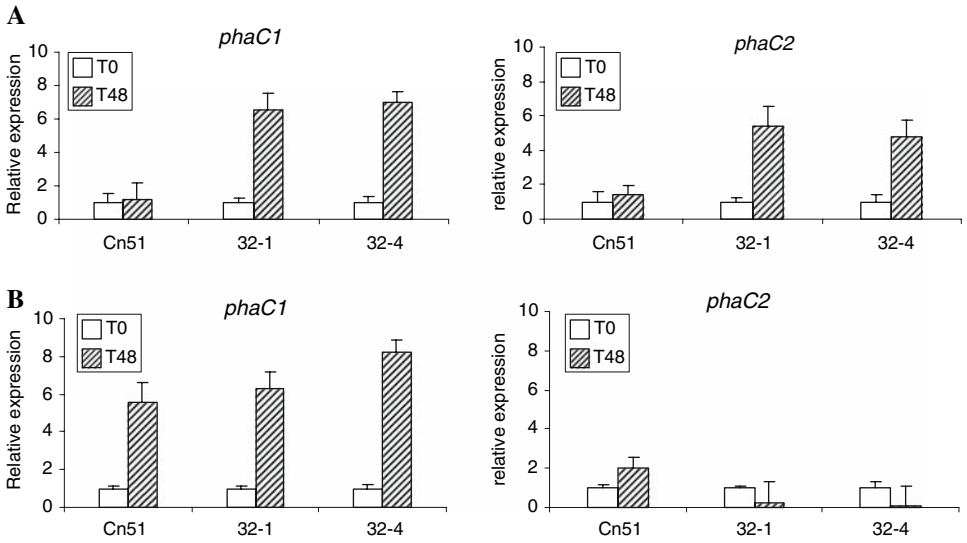

Fig. 5 Transcriptional analysis of *phaC1* and *phaC2* by RT-PCR. Expression of *phaC1* and *phaC2* was investigated by real-time PCR on total RNA samples prepared from *P. corrugata* cultures grown for 48 h at 30 °C in E* medium plus either 0.5% (v/v) oleic acid (**a**) or 2% (w/v) glucose (**b**) as a unique carbon source. Results are reported as the fold difference relative to data obtained for each strain grown in LB broth pre-inoculum growth conditions. The data were normalized to the 16S rRNA values. Mean values are calculated from duplicate sample from two separate experiments. Error bars represent ± one standard deviation from the mean. *Cn51 P. corrugata* 388 [pCN51], *32-1 P. corrugata* clone XI 32-1, *32-4 P. corrugata* clone XI 32-4, *T0* Pre-inoculum cells (at time zero), *T48* Cells harvested at 48 h of growth

the morphology of the PHA granules. For this purpose, cells at the time of harvest were processed accordingly for examination with a transmission electron microscope (see Materials and methods). Figure 6 shows the electron micrographs of cells grown on oleic acid at the time of harvest. The control-sample cells, *P. corrugata* 388 [pCN51], were shown to contain up to six large, electron-transparent PHA granules with the typical round or oblong morphology (Fig. 6a). Such PHA granules were not observed in cells of clones XI 32-1 and XI 32-4. In contrast, these cells contain

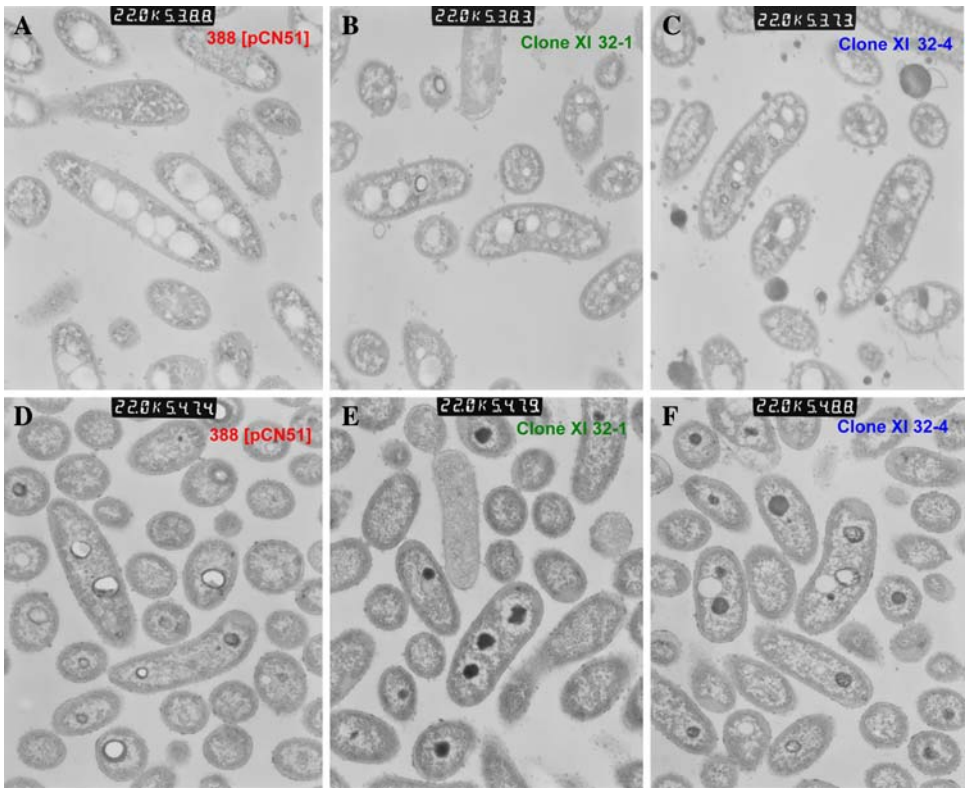

Fig. 6 Electron micrographs of *P. corrugata*. **a–c** Cells grown on oleic acid, **d–f** cells cultured in glucose, **a** and **d** *P. corrugata* [pCN51], **b** and **e** *P. corrugata* clone XI 32-1, *C and F P. corrugata* clone XI 32-4

a few PHA granules of medium size and numerous small granules that locate at the periphery of the cells (Fig. 6b, c). This is the first report of the observation of this kind of PHA-granule organization; it seems to suggest the involvement of *phaZ* and *phaC2*—and even the *phaD* gene located immediately downstream from *phaC2* (not shown in Fig. 1)—on the growth of the PHA granules to the large, oblong-shaped mature structure. Figure 6 also shows the electron micrographs of cells grown on glucose. In agreement with the smaller amounts of mcl-PHA isolatable from these glucose-grown cells in comparison to those obtained with cells grown on oleic acid (Table 2), much lower numbers of PHA granules were observed in cells shown in Fig. 6d–f. Interestingly, the cells of clones XI 32-1 and XI 32-4 grown on glucose contained a number of electron-dense PHA granules not observed in the oleic acid-grown cells. Even though this might be related to the high content (26–31 mol%, Table 2) of the unsaturated 3-hydroxydodecenoic acid repeat-units in the PHAs of these clones, the exact reason for this observation may only be affirmed by a selective compositional analysis of these granules.

In conclusion, we have completed the sequencing and cloning of the entire *pha* locus containing the *phaC1–phaZ–phaC2* genetic region of *P. corrugata* 388. We have further constructed *P. corrugata* deletion mutants, clones XI 32-1 and XI 32-4, which lack the *phaC1–phaZ* intergenic region with its terminator sequence to probe the function of this stretch of genomic DNA on PHA synthesis. The transcription of *phaC1* and *phaC2* genes was affected by the removal of this region when cells were grown on oleic acid, but was unaffected when glucose was the sole carbon source. Furthermore, these mutants when grown on glucose, a non-related substrate for mcl-PHA biosynthesis, produced PHA containing a high level of the unsaturated 3-hydroxydodecenoate as one of its repeat-unit monomers. In growth medium containing a mcl-PHA-related substrate, i.e., oleic acid, clones XI 32-1 and XI 32-4 synthesized small PHA granules that line the periphery of the cells. This is the first report of the effects of *pha* genetic organization on the repeat-unit composition and PHA granular morphology in a *Pseudomonas* species.

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