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Journal:	<i>Canadian Journal of Microbiology</i>
Manuscript ID	cjm-2017-0412.R1
Manuscript Type:	Article
Date Submitted by the Author:	20-Sep-2017
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Is the invited manuscript for consideration in a Special Issue? :	N/A
Keyword:	<i>Pseudomonas chlororaphis</i> , phenazine-1-carboxylic acid, Polyhydroxyalkanoate (PHA) synthesis, medium chain length PHAs, vegetable oils

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Synthesis of Polyhydroxyalkanoates (PHAs) from vegetable oils and free fatty acids by wild-type and mutant strains of *Pseudomonas chlororaphis*.

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Abstract

Pseudomonas chlororaphis strain PA23 was isolated from soybean roots as a plant growth-promoting rhizobacterium (PGPR) and secretes a wide-range of compounds, including the antibiotics phenazine-1-carboxymide (PCA), pyrrolnitrin, and 2-hydroxyphenazine. We have determined that *P. chlororaphis* PA23 can synthesize medium chain length polyhydroxyalkanoate (PHA) polymers utilizing free fatty acids, such as octanoic acid and nonanoic acid, as well as vegetable oils as sole carbon sources. Genome analysis identified a *pha* operon containing 7 genes in *P. chlororaphis* PA23 which were highly conserved. A non-pigmented strain that does not synthesize PCA, *P. chlororaphis* PA23-63, was also studied for PHA production. *P. chlororaphis* PA23-63 produced 2.42 - 5.14 g/L cell biomass and accumulated PHAs from 11.7 - 32.5% of cdw when cultured with octanoic acid, nonanoic acid, fresh canola oil, waste canola fryer oil, or biodiesel-derived waste free fatty acids under batch culture conditions. The subunit composition of the PHAs produced from fresh canola oil, waste canola fryer oil, or biodiesel-derived free fatty acids did not differ significantly. Addition of octanoic acid and nonanoic acid to canola oil cultures increased PHA production, but addition of glucose did not. PHA production in the *phz*⁻ mutant, *P. chlororaphis* PA23-63, was greater than the parent strain.

Key words: *Pseudomonas chlororaphis*; phenazine-1-carboxylic acid (PCA); Polyhydroxyalkanoate (PHA) synthesis; Vegetable oils

Introduction

Polyhydroxyalkanoates (PHAs) have attracted extensive interest as environmentally friendly biodegradable plastics due to their promising material properties. PHAs are synthesized as microbial carbon and energy storage compounds under nutrient limited conditions. Depending on the carbon source and the strain, PHAs have diverse properties for industrial applications (Sudesh et al. 2011). PHAs are classified as short chain length (scl-) and medium chain length (mcl-) PHAs on basis of their monomeric composition. Scl-PHA polymers consist of 3-hydroxyalkanoate subunits containing 3 to 5 carbon atoms, and *Cupriavidus necator* (also known as *Ralstonia eutropha* H16) has been widely studied for the production of scl-PHAs. Some scl-PHAs have physical, mechanical, and thermal properties are similar to petroleum-based polymers, such as polyethylene (Prieto et al. 2016; Koller et al. 2017). In contrast, mcl-PHA polymers contain 3-hydroxyalkanoate subunits consisting of mainly of 6 to 14 carbon atoms (Chen et al., 2010a). mcl-PHAs are amorphous and have characteristics that resemble those of elastomers due to their low glass transition temperatures, making them interesting as biological rubber-like materials.

Pseudomonas species are known to produce mcl-PHAs from renewable carbon feed stocks like carbohydrates, lipids, alcohols, organic acids. Depending on the carbon source and the strain, the material obtained for industrial applications can range from thermoplasts to elastomers, latexes, and even high-performance, functional polymers (Chen, 2010b). *Pseudomonas putida*, *P. aeruginosa*, *P. oleovorans*, and other *Pseudomonas* species can use purified fatty acids or hydrolyzed vegetable oils, which increase the cost of PHA production (Ashy et al. 2001; Cromwick et al. 1996). Moreover, free fatty acids can be toxic at low concentrations and are not used widely in fed batch experiments. *Cupriavidus necator* (= *R. eutropha* H16) can use wide-range of industrial wastes for scl-PHAs, including animal fats and vegetable oils, but these industrial wastes are not used for mcl-PHAs production (Koller et al. 2005, 2010; Lee, 1996; Ng et al. 2011; Titz et al. 2012; Cromwick et al. 1996; Muhr et al. 2013; Solaiman et al. 2006; Sharma et al. 2012, 2014). Some *Pseudomonas* species, such as *P. aeruginosa*, *P. corrugata*, *P. sacchrophila*, *P. chlororaphis*, and

Comamonas testosterini have been reported to utilize vegetable oils directly as carbon sources to produce mcl-PHAs (Abid et al. 2016; Ashby et al. 2001; Fernández et al. 2005; Impallomeni et al. 2011; Marsudi, et al. 2008; Solaiman et al. 1999; Solaiman et al. 2001; Solaiman et al. 2002; Yun et al. 2003; Tan et al. 1997; Thakor et al. 2005).

In our laboratory we are using *P. putida* strain LS46 for large-scale production of mcl-PHAs from glucose, biodiesel-derived glycerol, medium chain length free fatty acids, long-chain free fatty acids derived from acid hydrolysis of canola oil and other vegetable oils, as well as biodiesel-derived free fatty acids (Sharma et al. 2012; Fu et al. 2014, 2015). *P. putida* LS46, like other *P. putida* strains is unable to use vegetable oil as substrate (Walsh et al. 2015).

P. chlororaphis DSM50083, synthesizes phenazine-1-carboxymide (PCA) and has been shown to synthesize mcl-PHAs from saturated biodiesel fractions (SFAE) derived from animal waste lipids (Muhr et al. 2013). Another PCA producer, *P. chlororaphis* strain PA23 was isolated from soybean root as a plant growth-promoting rhizobacterium (PGPR) and has been shown to protect canola from stem rot disease caused by the fungal pathogen *Sclerotinia sclerotiorum* (Savchuk and Fernando, 2004; Fernando et al. 2007). In addition to PCA, *P. chlororaphis* PA23 secretes a wide-range of compounds, including the antibiotics pyrrolnitrin (PRN) and 2-hydroxyphenazine (2-OH-PHZ), together with chitinase, protease, lipase, and siderophores (Salin et al. 2010; Zhang et al. 2006).

Synthesis of PCA conveys this strain with a bright orange color. Phenazines are nitrogen containing heterocyclic secondary metabolites, which act as antibiotics, electron shuttle compounds, and can also affect cellular responses (Laursen and Nielsen, 2004). Phenazine production may also compete with mcl-PHA production by carbon partitioning, using nitrogen and/or electrons required for growth. Therefore, we compared the mcl-PHAs production by phenazine deficient mutant, *P. chlororaphis* PA23-63, with the wild type strain. Our preliminary studies demonstrate that *P. chlororaphis* PA23 and *P. chlororaphis* PA23-63 can synthesize and accumulate mcl-PHA polymers using vegetable oils directly as carbon sources.

Materials and methods

Bacterial strain

Pseudomonas chlororaphis PA23, the wild-type strain was isolated from soybean rhizosphere (Savchuk and Fernando, 2004). *Pseudomonas chlororaphis* PA23-63 was a mutant defective in phenazine production. Both strains were procured from Dr. Teresa De Kievit, Department of Microbiology, University of Manitoba, Winnipeg, Canada.

Culture media and growth conditions

Luria Bertani (LB) medium with 2% agar was used for maintaining *P. chlororaphis* PA23 cultures. LB agar plates were streaked with *P. chlororaphis* and incubated at 30 °C for 24 hours (h). Single colonies were then picked and used to inoculate LB medium containing tetracycline (15 µg/mL). Ramsay minimal medium (RMM) containing different carbon substrates was used for mcl-PHA polymer production studies (Sharma et al. 2012).

PHA detection in *P. chlororaphis* PA23

P. chlororaphis PA23 was grown in RMM with canola oil or octanoic acid as sole carbon source. Cells (0.2 mL) were centrifuged and stained with 0.1% Nile blue dye. After for 15 minutes (min) cells were centrifuged at 10,000xg for 2 min, the cell pellets were resuspended in sterilized distilled water, centrifuged again, and the cell pellets were resuspended in 0.2 mL water. Wet mounted cells were observed under fluorescent microscope (Nikon) using green filter (636nm).

Identification of PHA synthesis genes

Genome of *P. chlororaphis* PA23 has been sequenced and is available on the NCBI website (www.ncbi.nlm.nih.gov) under the accession number CP008696. PHA synthesis genes were identified by BLAST analysis using the sequences of *pha* genes from *P. putida* strain LS46 to query the *P. chlororaphis* PA23 genome (Sharma et al. 2013; 2014). Amino acid sequences of the PhaC1 and PhaC2 proteins from other *Pseudomonas* species were downloaded from the NCBI website and aligned using Clustal (Thompson et al. 1994). A neighbour joining tree was constructed using Molecular Evolutionary Genetic Analysis version 7 (Kumar et al. 2016).

Growth of *P. chlororaphis* strains and analyses of PHA synthesis

Single colonies of *P. chlororaphis* PA23 or *P. chlororaphis* PA2363 were inoculated from LB agar streak plates and used to inoculate cultures with 10 mL LB broth. The inoculum cultures were grown at 30 °C for 18 hours (h) on a rotary shaker (150 rpm). Ramsay's Minimal Media (RMM, 100 mL) with different carbon sources were inoculated with 10% v/v of the inoculum culture. The different carbon sources used were: glucose (2% w/v); octanoic acid (20 mM); nonanoic acid (20 mM); biodiesel-derived free fatty acids (1% v/v); canola oil (1% v/v); and waste canola fryer oil (1% v/v). Other vegetable oils including soybean oil, coconut oil, and olive oil were also tested. Biodiesel-derived (waste) free fatty acids (REG-FFA) extracted from crude biodiesel-derived glycerol (REG-80) were obtained from the Renewable Energy Group LLC (Danville, IL, USA).

The test cultures were incubated at 30 °C on a rotary shaker (150 rpm) up to 96 h. Cultures were harvested by centrifugation at $4,190 \times g$ for 30 min. All tests were conducted with three independently replicated cultures (i.e., three biological replicates). The pellets were washed twice in phosphate saline buffer (PSB) and dried at 60 °C for 48 h to estimate cell dry mass. PHA subunit composition was determined by gas chromatography (Agilent) analysis as described by Braunegg et al. (1978).

Samples of dried cell biomass (5-10 mg) placed in 15 mL screw capped tubes and analysed for percent cell dry weight (% cdw) accumulation of PHA and subunit composition. To this biomass, 1 mL chloroform containing 1 mg benzoic acid (internal standard) and 1 mL methanol containing 15% concentrated sulphuric acid was added. Tubes were boiled in a water bath for 6 h, and then 0.5 mL water was added to each tube. The chloroform (lower) layer was transferred to a 2 mL GC vial and analysed by gas chromatography using an Agilent GC (Model 7890A) fitted with DB-23 capillary column and FID detector. The initial oven temperature was maintained at 60 °C for 5 min which was increased to 250 at ramping rate of 15 °C /min. The peaks were identified by their retention time and concentration of subunits was estimated by comparing with known concentration

of different 3-hydroxy fatty acid standards.

P. chlororaphis PA23-63 was also grown as a batch culture in a 13 L Applikon Bioreactor containing 10 L RMM supplemented with 1% v/v Canola oil. Conditions for the bioreactor culture were 30 °C, pH 7.0, with a dissolved oxygen (dO₂) concentration of 40%. The culture was harvested after 48 h and cell biomass was recovered by centrifugation at 4190 × *g* for 20 min. The pellet was washed twice in PSB and dried at 60 °C for 48 h to estimate cell dry mass. PHAs were extracted from the dry cell mass with chloroform as solvent in Soxhlet apparatus for 6 h. PHAs were concentrated by evaporating the chloroform and then precipitated using cold methanol. The supernatant was decanted and methanol was then evaporated. The precipitation and evaporation steps were repeated twice, and purified PHAs were stored at room temperature for further analysis.

Residual oil and ammonium nitrogen estimation

After pelleting the cells, supernatants were used for estimating residual oil and ammonium nitrogen in the medium. For analyses of residual oil, supernatants were vortexed for 3 to 5 min., and 1 mL samples were transferred from each treatment into 15 mL screw capped tubes. Samples were dried at 60 °C for 48 h and methylated as described earlier (Spiekermann et al. 1998). Methylated samples (fatty acid methyl-esters = FAMES) were analyzed by gas chromatography, as described above.. Uninoculated medium was used as a control and the percent decrease in fatty acid concentrations was calculated for different time points. Ammonium nitrogen was measured using the Quikchem method 10-107-06-1-I for determination of ammonium in wastewater by flow injection analysis (Lachat Instrument, Colorado, USA), and the measured concentrations were rounded-off and reported to 2 significant digits.

PHAs production by *P. chlororaphis* PA23-63 with mixed substrates

Production of mcl-PHAs by *P. chlororaphis* PA23-63 was compared to PHA production by *P. chlororaphis* PA23 in RMM containing canola oil alone, or with the addition of glucose (2% w/v), octanoic acid (20 mM), or nonanoic acid (20 mM). All cultures were inoculated with 10% of an overnight culture of *P. chlororaphis* PA23 or *P. chlororaphis* PA23-63, as described earlier. After

24 h, cultures were centrifuged to pellet the cells, followed by determination of the % cdw.

PHA synthesis by *P. chlororaphis* PA2363 in media with different C:N ratios

Ramsay's minimal medium was prepared containing different carbon and nitrogen ratios. For this canola oil concentration was used as 2% or 4% in combination with 0.5 and 1 g ammonium sulphate per litre of medium. With 2% oil and 0.5 and 1g ammonium sulphate C:N were 45:1 and 22.5:1 respectively. With 4% oil and 0.5 and 1g ammonium sulphate these ratios were 90: and 45:1 respectively. The media were inoculated with *P.chlororaphis* PA2363, cell dry weight and PHAs was estimated after 48 h as described earlier.

NMR and FTIR Spectra of PHAs Polymer

¹H-NMR spectrum of mcl-PHA polymers synthesized by *P. chlororaphis* PA23-63 were acquired by dissolving 10 mg of each polymer in deuteriochloroform (CDCl₃) and analyzed on a Bruker AMX-300 spectrometer at 22 °C with a 7.4 milli-second (ms) pulse width (30° pulse angle), 1 second (s) pulse repetition, 10330 Hz spectral width, 65,536 data points, and analyzed as described by Huijbert et al. (1992). FT-IR analysis of polymer sample was carried out on MB-3000, ABB FTIR spectrophotometer in range 4000–600 cm⁻¹ as described by Sharma et al. (2017).

Results

Detection of PHAs in *P. chlororaphis* PA23

P. chlororaphis PA23 cells grown in octanoic acid or canola oil and stained with Nile Blue gave very bright fluorescence indicating the synthesis of PHAs (Figure 1). Octanoic grown cell formed long filaments containing 5 to 15 PHA granules (Figure 1A), while cells grown in canola oil formed clumps with oil drops and had 1 to 5 PHA granules (Figure 1B).

Comparison of the *pha* operons and lipase genes encoded by *P. chlororaphis* PA23 and other *Pseudomonas* species

The *pha* operon of *P. chlororaphis* PA23 had seven genes (EY04_01510, EY04_01515, EY04_01520, EY04_01525 EY04_01530, EY04_01535 and EY04_01540). The locus numbers, organization, and product size of the encoded polypeptides are shown in Figure 2. Like other

Pseudomonas species, *P. chlororaphis* PA23 had two Class II PHA synthase genes, *phaC1* and *phaC2*, which were separated by a PHA depolymerase gene (*phaZ*). Three other regulatory genes, *phaD*, *phaF*, and *phaI*, which were identified earlier in other *Pseudomonas* species, were also present. Another putative polyhydroxyalkanoic acid system protein (EY04_01510), which was identified as a hypothetical protein, was also present in *pha* operon of *P. chlororaphis* PA23.

The organization of *pha* genes in *P. chlororaphis* PA23 was identical to other *P. chlororaphis* strains, like *P. chlororaphis* PCL1606, *P. chlororaphis* strain LMG 21630, and *P. chlororaphis* strain ATCC 13985. The organization of *pha* operon was also similar to *P. putida*, *P. oleovorans*, and *P. aeruginosa*. The hypothetical protein encoded by the *P. chlororaphis* PA23 gene located at EY04_01510 was also present in *P. putida*, *P. oleovorans*, and *P. aeruginosa*. However, the gene product encoded by the *P. chlororaphis* PA23 gene had only a 57% amino acid sequence identity with to the equivalent protein encoded by *P. putida* LS46.

Genome analyses also identified 21 lipase genes in *P. chlororaphis* PA23, while the genome of *P. putida* LS46 encoded only 11 lipase genes (Table 1). Some of the lipase proteins in *P. chlororaphis* PA23 and *P. putida* LS46 encoded signal peptides, but not all. The triacylglycerol lipase (EY04_02420, COG1075), which is present in the genomes of both *P. chlororaphis* PA23 and *P. putida* LS46 lack a signal peptide. An ABC transporter for lipase/protease (EY04_16115, COG 4618) was present in *P. chlororaphis* PA23, but was absent in *P. putida* LS46 (Table 1)

Phylogenetic relationships among PHA synthase proteins

A Neighbour Joining tree of the PHA synthase proteins (PhaC1 and PhaC2, encoded by *phaC1* and *phaC2*, respectively) from different *Pseudomonas* species was constructed. The *phaC1* and *phaC2* genes of *P. chlororaphis* PA23 shared only 73% nucleotide identity, while the proteins they encode shared 58% amino acid sequence identity. The PhaC1 and PhaC2 of different *Pseudomonas* species formed separate clusters in the Neighbor Joining tree, and the PhaC1 and PhaC2 proteins of *P. brassicacaerum*, *P. chlororaphis*, *P. fluorescens*, *P. putida*, *P. aeruginosa*, and *P. syringae* formed different clades within their respective clusters. All PhaC1 and PhaC2

proteins from *P. chlororaphis* strains had high amino acid sequence identity and formed a monophyletic clade (Figure 3).

Growth and PHA synthesis by *P. chlororaphis* PA23

Synthesis of phenazine-1-carboxylic acid (PCA) by *P. chlororaphis* PA23 confers a deep orange colour to the cells, which was more intense in cultures grown with canola oil than in cultures grown with octanoic acid or nonanoic acid. *P. chlororaphis* PA23 was able to grow in minimal medium with glucose, hexanoic acid, octanoic acid, nonanoic acid, canola oil, biodiesel-derived free fatty acids, and waste canola fryer oil as a sole carbon source. In cultures containing canola oil, the cells tended to form a biofilm, which floated on the surface of the liquid medium and pelleted with the cells after centrifugation.

P. chlororaphis PA23 accumulated 1.46 - 4.88 g/L cell biomass in minimal medium with different carbon substrates (Table 2). Cultures containing biodiesel-derived free fatty acids, waste canola fryer oil, and fresh canola oil generated greater cell mass (g cdw) than cultures containing glucose or the medium chain length free fatty acids (hexanoic, octanoic, and nonanoic acids). Among all of the substrates, biodiesel-derived free fatty acids supported the highest cell mass production. Among cultures containing medium chain length free fatty acids, nonanoic acid supported higher cell mass production than hexanoic acid and octanoic acid. PHA production was higher in glucose cultures than hexanoic and nonanoic acid cultures, but was less than cultures containing octanoic acid.

PHA polymers synthesized by *P. chlororaphis* PA23 from fresh canola oil, waste fryer oil, or biodiesel-derived free fatty acids had comparable subunit compositions, with 3-hydroxyoctanoate (C8), followed by 3-hydroxydecanoate (C10), and 3-hydroxydodecanoate (C12) as the dominant subunits (Table 2). The subunit compositions of PHA polymers synthesized by *P. chlororaphis* PA23 using medium chain length free fatty acids were different from the subunit compositions of polymers synthesized by *P. putida* strains on the same substrates. *P. putida* strains cultured with octanoic acid synthesize mcl-PHA polymers containing approximately 90 mol% 3-

hydroxyoctanoate (C8 subunits), while *P. putida* strains cultured with nonanoic acid synthesize polymers containing approximately 70 mol % of 3-hydroxynonanoate (C9 subunits). In comparison, *P. chlororaphis* PA23 cultured with octanoic acid synthesize polymers containing approximately 80 mol% C8 subunits, and polymers containing approximately 60 mol% C9 subunits when cultured with nonanoic acid.

Growth and PHA synthesis by *P. chlororaphis* PA23-63 on different substrates

P. chlororaphis PA23-63 is a deletion derivative of *P. chlororaphis* PA23, which is defective in phenazine production (*phz*⁻). The *P. chlororaphis* PA23-63 mutant was derived by insertional mutagenesis using transposon Tn5, which was inserted in *phzE* gene of the *phz* operon (*phzABCDEFG*). *P. chlororaphis* PA23-63 cannot produce phenazine and does not produce the orange pigment (Figure 4).

Growth of *P. chlororaphis* PA23-63 was tested on canola oil, waste fryer oil, and biodiesel-derived free fatty acids (Figure 5). Growth on canola oil and biodiesel fatty acid was comparable and produced 4.3g/L cdw in 24 h (Figure 5A). In cultures containing canola oil, cell mass increased slightly in next 24 h and then decreased thereafter. In cultures containing biodiesel-derived free fatty acids, cell mass stabilized after 48 h. In cultures containing waste fryer oil, cell mass increased up to 72 h and then decreased. Of the three substrates tested, biodiesel-derived free fatty acids were consumed most rapidly, while canola oil and Waste Fryer oil were consumed at approximately the same rates (Figure 5B). Analysis of residual nitrogen concentrations indicated that ammonium nitrogen in cultures containing canola oil, waste fryer oil, and biodiesel fatty acid was depleted within 24 h (Figure 5C). PHA accumulation by *P. chlororaphis* PA23-63 cultures with canola oil, waste fryer oil, and biodiesel-derived free fatty acids increased between 24 and 48 hours, despite the depletion of nitrogen in the culture (Figure 5D). PHA accumulation was greatest in cultures with waste fryer oil and biodiesel-derived free fatty acids, and lowest in cultures with fresh canola oil.

Growth and PHA subunit composition was also evaluated for *P. chlororaphis* PA23-63 grown up to 96 h in batch culture experiments with a number of different vegetable oils (canola oil, soybean oil, olive oil, coconut oil, waste oil), biodiesel-derived free fatty acids, and octanoic acid. Among the different oils, canola oil was preferentially used by *P. chlororaphis* 23-63, as indicated by the greatest cdw production (Table 3). Coconut oil and olive oil supported the lowest g cdw production. PHAs production by *P. chlororaphis* PA23-63 in cultures containing canola oil, waste fryer oil, and biodiesel fatty acids did not differ significantly (Table 3). PHA accumulation in *P. chlororaphis* PA23-63 was lowest in cells grown with soybean oil, followed by olive oil. Subunit compositions of PHA polymers synthesized by *P. chlororaphis* PA23-63 varied with the type of vegetable oil used as substrate, but 3-hydroxyoctanoate was the dominant monomer, followed by 3-hydroxydecanoate in all the polymers analyzed (Figure 6).

PHA synthesis by *P. chlororaphis* PA23-63 cultures containing in mixed substrates

Growth of *P. chlororaphis* PA23-63 on vegetable oils is associated with initial lag phase up to 4-5 h, presumably due to the need to induce synthesis and secretion of lipase enzyme that cleaves the ester bond between glycerol and the long chain fatty acids of the triacylglyceride molecules. The addition of medium chain length free fatty acids or sugar to cultures containing vegetable oils can reduced this lag phase period. Therefore, glucose (2% w/v), octanoic acid (20mM), and nonanoic acid (20mM) were used with canola oil (1% v/v) as mixed substrates.

Among the three substrates, glucose produced greater cell mass (3.16 g/L cdw) than octanoic acid (2.44 g/L cdw), and nonanoic acid (2.2 g/L cdw) (Figure 7). PHAs production was higher in octanoic acid (35% of cell dry weight) than glucose or nonanoic acid. Growth on canola oil showed maximum cell dry weight of 5.82 g/L and 30% accumulation of PHAs (% cdw). Addition of octanoic or nonanoic acid to cultures containing canola oil increased cell mass production (Fig. 7). However, there was no increase in cell mass or PHAs production when glucose was added to canola oil medium. Addition of octanoic or nonanoic acid to canola medium increased the PHAs production by 10-13% cdw.

PHA polymers produced on mixed substrates had different subunit compositions than those produced on single substrates. PHAs produced glucose had 3-hydroxydecanoate as the major subunit (64%) in comparison to 38% in polymers synthesized by cells growth with canola oil, and 51% in cells cultured with mixed substrates (canola oil + glucose). Similarly, the mol% of 3-hydroxyoctanoate in polymers produced from glucose and canola oil was 12% and 42%, respectively, but was 23% in polymers synthesized by cells cultured with mixed substrates.

PHA polymers synthesized by cells cultures with octanoic acid contained 90% 3-hydroxyoctanoate, but in mixed substrates with canola oil the mol% of 3-hydroxyoctanoate decreased to 69%, and was only 42.4% in polymers synthesized by cells cultured with canola oil. PHA polymers synthesized by cells cultured with canola oil contained subunits with even numbers carbon chain lengths, while polymers synthesized by cells cultured with nonanoic acid contained subunits with odd numbered carbon chain lengths (40% 3-hydroxyheptanoate and 60% 3-hydroxynonanoate). However, PHA polymers synthesized by cells cultures with mixed substrates of canola oil and nonanoic acid had both even and odd carbon number 3-hydroxy fatty acids. The major subunit component in these polymers was 3-hydroxyoctanoate, followed by 3-hydroxydecanoate, with 3-hydroxynonanoate making up 20% of the subunit composition.

PHA synthesis by *P.chlororaphis* PA2363 in media with different C:N ratios

Cell dry weight varied significantly in C:N ratio of 22.5:1 and 45:1 when 2% oil was used but varied significantly in 45:1 and 90:1 C:N ratios when 4% oil was used (Fig 8). PHAs production was highest in C:N ration of 45:1 when 4% oil was used. PHAs produced in media with different C:N ratios showed similar monomer composition with 8.6-9.7% of 3-hydroxyhexanoate, 43-49% 3-hydroxyoctanoate, 28-30% 3-hydroxydecanoate, 8.6-9.7% 3-hydroxydodecanoate and 4-11% 3-hydroxytetradecanoate. C:N ratios did not affect the mol% of 3-hydroxyhexanoate or 3-hydroxydecanoate, but 3-hydroxyoctanoate and 3-hydroxytetradecanoate varied with different C:N ratios.

Analyses of PHA polymer structure

Purified PHAs synthesized by *P. chlororaphis* PA23-63 grown with canola oil were analysed by ^1H -NMR, ^{13}C -NMR, and FTIR (Fourier Transform Infrared) analysis. ^1H -NMR spectra identify the origin of protons from different functional groups, and protons in five functional groups in the PHA polymers were identified by specific peaks in the ^1H NMR spectra (Figure 9). Left to right in Figure 9, the five peaks corresponded to protons located in: i) a methylene group next to the hydroxyl group (peak b); ii) a methylene group attached to a carbonyl group (peak a); iii) the first methylene group of saturated side chains (peak c); iv) a free methylene group (peak d); and v) the terminal methyl group (peak e).

The ^1H NMR spectra indicated wavelength shifts of 5.2, 2.5, 1.6, 1.2, and 0.8 ppm, respectively. These are signature peaks of polyhydroxyalkanoates. Other peaks indicating unsaturated carbon double bonds ($\text{C}=\text{C}$) in PHA side chains were also identified. FTIR spectrum could identify the presence of different functional groups, such as aliphatic $\text{C}-\text{H}$ bonds, $=\text{C}-\text{H}$ bonds, $=\text{CH}$ bonds, and $=\text{C}-\text{O}$ bonds, as well as $=\text{O}$ bond stretching, and $=\text{C}-\text{H}$ bond deformation. It has been established that carbonyl group peaks identified by FTIR mcl-PHAs at 1737. The methylene $\text{C}-\text{H}$ peaks (located at 2928 cm^{-1}) are more prominent in MCL-PHAs (Figure 10).

Discussion

In our laboratory, we isolated *P. putida* LS46 and have demonstrated the production of mcl-PHAs using by-products of the biodiesel industry (Sharma et al. 2012; Sharma et al. 2014; Fu et al. 2014). *P. putida* LS46 can utilize biodiesel-derived free fatty acids to synthesize mcl-PHAs, but cannot use vegetable oils directly as a carbon source. Hydrolysis of vegetable oils to release the fatty acids adds significant cost to the production of PHAs. Therefore we searched for a PHA producing strain that can utilize vegetable oil directly. *Pseudomonas chlororaphis* strains were used earlier to produce PHAs from saponified palm kernel oil, saponified waste rapeseed oil, or substrates derived from animal fat waste (SFAE) (Annur et al. 2007; Gumel et al. 2012; Mozejko and Ciesielski 2011, 2013; Muhr et al. 2013; Sun et al. 2003;), but had not previously been shown to produce PHAs growing directly on vegetable oils.

The *P. chlororaphis* PA23 genome, like the genomes of other *Pseudomonas* species (*P. putida*, *P. aeruginosa*, *P. fluorescens*) encoded genes for class II PHA synthase, which are associated with mcl-PHA production (Sharma et al. 2014, Zou et al. 2017). Phylogenetic analyses revealed that the *phaC1* and *phaC2* genes of the *P. chlororaphis* strains formed distinct clusters that were separate from the clusters formed by the *phaC1* and *phaC2* genes of *P. fluorescens* and *P. putida*. None-the-less, these differences in *phaC1* genes did not affect the polymerase specificity and *P. chlororaphis* synthesized mcl-PHAs with monomer compositions similar to those synthesized by *P. putida* LS46 (Sharma et al. 2012). *P. chlororaphis* HS-1 was unable to utilize glucose or gluconate as sole carbon source for PHA production (Sun et al. 2003). In contrast, *P. chlororaphis* PA23 could use glucose as a sole carbon source for PHA production, indicating that it uses both β -oxidation and *de novo* synthesis of fatty acids to produce PHAs.

The *P. chlororaphis* PA23 genome encodes an ABC transporter for lipase/protease (EY04_16115, COG 4618) that is absent in the genome of *P. putida* LS46. It may be that expression of this lipase/protease transporter confers *P. chlororaphis* PA23 with the ability to grow directly on vegetable oils. Production of extracellular lipase enzymes is induced by fatty acids and vegetable oils. In *Pseudomonas alcaligenes* M-1, lipase synthesis was induced by a limited number of natural oils, such as soybean oil, and some unsaturated fatty acids, such as oleic acid. (Gerritse et al. 1998), It is also induced by a two component regulatory system (Krzeslak et al. 2008) and by surfactant like Tween 60 and Tween 80 (Lu et al. 2012).

Lipase is also induced by cell density-dependent regulation controlled by a quorum sensing mechanism (Rosenau and Jaeger, 2000). Data from our mixed substrate experiments data are consistent with this observation. Addition of octanoic acid or nonanoic acid (20 mM) to cultures containing canola oil resulted in both increased cell biomass and increased synthesis and accumulation of mcl-PHAs. Our previous results with proteomic analysis of PHA synthesis by *Cupriavidus necator* H16 have shown that when *C. necator* was grown on glycerol bottom or biodiesel derived fatty acids, enzymes of β -oxidation were induced (Sharma et al. 2016). Octanoic

and nonanoic acids are preferential sources of carbon for *P. chlororaphis* PA23-63. Cultures grew faster in these media and reached minimal threshold number to induce lipase.

FTIR analysis has been used to differentiate scl-PHA and mcl-PHA (Hong et al. 1999). Canola oil has 58.6% monounsaturated fatty acids and 20.6% polyunsaturated acids. Unsaturated fatty acids were identified in the mcl-PHA polymers synthesized by *P. chlororaphis* from Canola oil by gas chromatography (data not shown), and further confirmed by ¹H-NMR (Huijberts et al. 1992), which detected the mcl-PHA specific NMR signals.

P. chlororaphis PA23 secretes a wide-range of compounds, including the antibiotics phenazine-1-carboxymide (PCA), pyrrolnitrin (PRN), and 2-hydroxyphenazine (2-OH-PHZ), as well as chitinase, protease, lipase, and siderophores (Salin et al. 2010; Zhang et al. 2006). The phenazine minus (*phz*⁻) mutant strain, *P. chlororaphis* PA23-63, displayed greater cell mass production and mcl-PHA accumulation than the parental strain on a range of substrates. Both phenazine and PHA synthesis are controlled by common regulatory mechanisms. They are both synthesized and accumulate in stationary phase and are regulated by the Stationary Phase Sigma Factor RpoS (Movrati et al. 2013).

PHA is accumulated during stationary phase of cell grown under conditions of nutrient limitation (for example, nitrogen-limitation) in the presence of abundant carbon. Intracellular levels of the stress response regulator, RpoS, are very low in the exponential phase and increase in stationary phase (Ryan et al. 2013). RpoS is known to positively regulate PHA synthesis after the onset of the stationary phase or under conditions of nutrient limitation in the presence of abundant carbon. In *P. putida* KT2440, deletion of the *rpoS* gene was associated with an increase in the expression of enzymes involved in PHA depolymerization, and the *rpoS* mutant displayed lower survival and tolerance to hydrogen peroxide (H₂O₂) stress (Raiger-Lustman and Ruiz, 2008). In *P. chlororaphis* PCL1391, mutation of *rpoS* resulted in diminished expression of the *phaC2* gene (Girard et al. 2006). Thus, the stress response regulator, RpoS, appears to play a role in regulation of PHA synthesis.

Quorum-sensing regulators are also known to regulate both phenazine and PHA synthesis. Treatment of *P. aeruginosa* cells with the quorum sensing inhibitor, azithromycin, decreased the accumulation of PHAs in *P. aeruginosa* (Xu et al. 2011), and resulted in reduced expression of *gacA* and *rsmA*, which encode the GacA-rsm components of the GacS/GacA-rsm regulatory cascade (see above), which positively regulates *rsmY* and *rsmZ*, and which in turn, regulate quorum sensing (Perez-Martinez and Haas, 2011).

Global regulators like GacA/GacS also regulate the synthesis of both phenazine and PHAs. The two-component GacS/GacA system forms the sensory apparatus of the Gac/Rsm cascade, which involves interactions with small RNA regulatory molecules affecting a range of microbial metabolic pathways (Lapouge et al. 2008). Disruption of the *gacS*, sensor kinase gene in a *P. putida* CA-3 mutant was found to eliminate mcl-PHA synthesis. Disruption of the *gacA* gene in *P. aeruginosa* resulted in 2- to 17-fold lower levels of transcription of the *pha* operon genes (*phaC1*, *phaZ*, *phaC2*, *phaI*, and *phaF*) compared to the wild-type strain (Pham et al. 2004; Wang et al. 2013b).

Conclusion

P. chlororaphis is a versatile non-pathogenic microorganism with a number of applications in agriculture and other industries. In agriculture it has been developed as a biological control agent due to production of several antifungal compounds against soil-borne plant pathogenic fungi (Chan et al. 2012; Huang et al. 2011; Calderon et al. 2015). Industrially it can be used to produce valuable bioactive rhamnolipids and medium chain length polyhydroxyalkanoates. However, commercial viability of the PHAs depends on several factors, of which low cost substrate(s) for fermentation is the most important. Carbon substrates used for PHB synthesis account for 40 - 48% of the production costs (Choi & Lee, 1997). High production costs make mcl-PHAs economically unattractive compared to petroleum-based plastics. There is now a global effort to decrease production costs by using low cost carbon substrates. Vegetable oils and saponified oils are good and low cost substrates for mcl-PHA production. Optimization of process control using *P.*

chlororaphis may lead to cost effective production of mcl-PHAs from vegetable oils.

Acknowledgements

This work was funded by the a Natural Sciences and Engineering Research Council of Canada (NSERC) through an NSERC Discovery grant (RGPIN-5961-2016), and by Genome Canada, through the Genome Applications Partnership Program (GAPP) grant titled, “Fibre composite and biomatrix genomics (FiCoGen: Applications to the ground transportation industry”.

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Figure Captions

Figure 1. *P. chlororaphis* PA23 cultured with A) 20 mM octanoic acid and B) fresh canola oil for 24 h. Cells were stained with Nile blue and observed under fluorescent microscope. Red fluorescence shows polyhydroxyalkanoate production.

Figure 2. Organization of *pha* genes in *pha* operons of *P. chlororaphis* PA23 and *P. putida* LS46. Genes are not drawn to scale. Triangle shows the direction of the transcription. Homology to *P. putida* LS46 proteins was calculated on the basis of protein blast.

Figure 3. Phylogenetic tree of PhaC1 and PhaC2 proteins depicting its relationship among *Pseudomonas* species. Sequences were aligned by ClustalW and Neighbor joining trees were generated using MEGA5. Bootstrap values are mentioned at the node.

Figure 4. *P. chlororaphis* PA23 versus *P. chlororaphis* PA23-63. A) The Phenazine producing strain, *P. chlororaphis* PA23; B) The *phz*⁻ mutant strain, *P. chlororaphis* PA23-63.

Figure 5. A) Cell growth, as measured by cell dry weight (cdw) production; B) substrate consumption; C) residual ammonium nitrogen concentrations; and D) PHA synthesis, measured as % cdw accumulation in the cells in *P. chlororaphis* PA23-63 cultures containing canola oil, waste fryer oil, and biodiesel-derived free fatty acids.

Figure 6. Subunit compositions of PHA polymers synthesized by *P. chlororaphis* PA23-63 in cultures containing different vegetable oils versus octanoic acid.

Figure 7. A) Cell biomass and PHAs production by *P. chlororaphis* PA23-63 in cultures containing canola oil, or canola oil supplemented with glucose, octanoic acid, or nonanoic acid; B) Monomer composition of PHAs produced by *P. chlororaphis* PA23-63 in cultures containing canola oil, or canola oil supplemented with glucose, octanoic acid, or nonanoic acid.

Figure 8. A) Cell biomass and PHAs production by *P. chlororaphis* PA23-63 in minimal medium with different C:N ratios; B) Monomer composition of PHAs produced in minimal medium with different C:N ratio (B).

Figure 9. ^1H -NMR spectrum of PHAs synthesized by *P. chlororaphis* PA23-63 cultured with canola oil. The structure of PHA is drawn on the basis of our GC results. The letters in the spectra indicate the position of protons located in the structure. Left to right, these are: **b**, a methylene group next to the hydroxyl group; **a**, a methylene group attached to a carbonyl group; **c**, the first methylene group of saturated side chains; **d**, a free methylene group; and **e**, the terminal methyl group

Figure 10. FTIR spectra of PHA synthesized by *P. chlororaphis* PA23-63 cultured with canola oil. Arrows indicate mcl-PHA specific peaks in the FTIR spectrum correlated with $\text{CH}_2\text{-CH}_2$ and $\text{CH}_3\text{-CH}_2$ groups.

Table 1. Comparison of lipase genes of *P. chlororaphis* PA23 with *P. putida* LS46, and *P. putida* KT2440

Locus Tag	Gene Product Name	Genome Name	COG
PPUTLS46_026451	Acetyl esterase/lipase	<i>P. putida</i> LS46	COG0657, Acetyl esterase/lipase
PP_4218	lipase/esterase family protein	<i>P. putida</i> KT2440	COG0657, Acetyl esterase/lipase
PP_3812	lipase, putative	<i>P. putida</i> KT2440	COG0657, Acetyl esterase/lipase
EY04_08410	Acetyl esterase/lipase	<i>P. chlororaphis</i> PA23	COG0657, Acetyl esterase/lipase
EY04_21540	Acetyl esterase/lipase	<i>P. chlororaphis</i> PA23	COG0657, Acetyl esterase/lipase
EY04_16115	ATP-binding cassette, subfamily C, exporter for protease/lipase	<i>P. chlororaphis</i> PA23	COG4618, ABC-type protease/lipase transport system, ATPase and permease components
PPUTLS46_000355	Esterase/lipase	<i>P. putida</i> LS46	COG1647, Esterase/lipase
EY04_09635	Esterase/lipase	<i>P. chlororaphis</i> PA23	COG1647, Esterase/lipase

Table 2. Synthesis and subunit composition of mcl-PHAs produced by *P. chlororaphis* PA23 in RMM containing different substrates.

Substrate	CDW (g/L)	PHA Accumulation % cdw	Subunit Composition (mol %)						
			HHx	HHp	HO	HN	HD	HDD	HTD
Octanoic Acid	2.42 ± 0.61	32.46 ± 1.34	9.95	-	83.29	-	3.63	-	-
Nonanoic acid	3.40 ± 0.75	28.24 ± 0.98		39.44		60.55	-	-	-
Glucose	3.38 ± 0.45	30.56 ± 1.41	5.72	-	46.70	-	36.30	12.38	1.56
Hexanoic acid	1.46 ± 0.27	4.26 ± 1.09	66.22	-	17.41	-	8.43	7.92	-
Biodiesel fatty acid	4.03 ± 0.43	24.17 ± 2.56	5.94	-	48.58	-	33.18	9.59	2.68
Canola oil	4.37 ± 0.16	11.48 ± 3.43	6.88	-	51.94	-	28.90	11.38	1.3
Waste Fryer oil	4.88 ± 0.29	10.5 6± 1.68	5.32	-	45.6	-	31.67	10.78	6.63

HHx, 3-hydroxyhexaoate; HHp 3-hydroxyheptunoate; HO, 3-hydroxyoctanoate; HN 3-hydroxynonanoate; HD, 3-hydroxydecanoate; HDD, 3-hydroxydodecanoate; HTD, 3-hydroxytetradecanoate

Table 3. PHAs production by *P. chlororaphis* PA23-63 (*phz*⁻ mutant) on different substrates.

Substrate	CDW (g/L)	PHA Accumulation % cdw	Subunit Composition (mol %)				
			HHx	HO	HD	HDD	HTD
Canola oil	4.58 ± 0.16	24.87 ± 3.05	9.28	47.4	35.8	5.57	1.78
Soybean oil	3.36 ± 0.32	10.1 ± 3.19	11.56	49.05	28.58	4.25	6.32
Oilve oil	2.41 ± 0.51	14.92 ± 4.56	9.87	50.7	30.27	5.01	4.11
Coconut oil	2.71 ± 0.10	26.11 ± 3.55	10.3	61.69	23.38	2.28	2.31
Waste oil	4.11 ± 0.12	24.81 ± 2.42	10.79	54.58	28.25	4.03	2.32
*Biodiesel-FFAs	4.3 ± 0.03	26.14 ± 1.53	8.17	46.0	36.63	3.89	3.2
Octanoic acid	2.55 ± 0.15	37.98 ± 1.61	12.45	83.9	1.39	0.63	1.47

HHx, 3-hydroxyhexaoate; HO, 3-hydroxyoctanoate; HD, 3-hydroxydecanoate; HDD, 3-hydroxydodecanoate;

HTD, 3-hydroxytetradecanoate; * Biodiesel-derived free fatty acids

Figure 1.

Figure 2 (Please convert to Black and White)

Figure 3.

Figure 4.

Figure 5.

Figure 6.

Figure 7.

Figure 8.

The figure displays the ^1H NMR spectrum of poly(3-hydroxybutyrate) (PHB). The chemical structure is shown at the top, with protons labeled (a) through (k). The spectrum below shows the corresponding peaks, with assignments (a) through (k) indicating the specific proton environments. The x-axis represents the chemical shift in ppm, ranging from 9.5 to -1.5.

Chemical Structure and Proton Labels:

The structure is a repeating unit of PHB: $-(\text{O}-\text{CH}_2-\text{CH}_2-\text{C}(=\text{O}))_n-(\text{O}-\text{CH}(\text{OH})-\text{CH}_2-\text{C}(=\text{O}))_m-$. The protons are labeled as follows:

- (a)**: CH_3 of the $\text{CH}_3\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_3$ chain.
- (b)**: CH_2 of the $\text{CH}_3\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_3$ chain.
- (c)**: CH_2 of the $\text{CH}_3\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_3$ chain.
- (d)**: CH_2 of the $\text{CH}_3\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_3$ chain.
- (e)**: CH_2 of the $\text{CH}_3\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_3$ chain.
- (f)**: CH_2 of the $\text{CH}_3\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_3$ chain.
- (g)**: CH_2 of the $\text{CH}_3\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_3$ chain.
- (h)**: CH_2 of the $\text{CH}_3\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_3$ chain.
- (i)**: CH_2 of the $\text{CH}_3\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_3$ chain.
- (j)**: CH_2 of the $\text{CH}_3\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_3$ chain.
- (k)**: CH_2 of the $\text{CH}_3\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_3$ chain.

Peak Assignments:

- (a)**: CH_3 of the $\text{CH}_3\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_3$ chain.
- (b)**: CH_2 of the $\text{CH}_3\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_3$ chain.
- (c)**: CH_2 of the $\text{CH}_3\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_3$ chain.
- (d)**: CH_2 of the $\text{CH}_3\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_3$ chain.
- (e)**: CH_2 of the $\text{CH}_3\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_3$ chain.
- (f)**: CH_2 of the $\text{CH}_3\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_3$ chain.
- (g)**: CH_2 of the $\text{CH}_3\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_3$ chain.
- (h)**: CH_2 of the $\text{CH}_3\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_3$ chain.
- (i)**: CH_2 of the $\text{CH}_3\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_3$ chain.
- (j)**: CH_2 of the $\text{CH}_3\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_3$ chain.
- (k)**: CH_2 of the $\text{CH}_3\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_3$ chain.

Figure 10.

Figure 9.

Figure 10.