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**Colonization and degradation of polyhydroxyalkanoates
by lipase producing bacteria**

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Draft

Abstract

Biodegradation of short chain length polyhydroxyalkanoates (scl-PHAs) and medium chain length polyhydroxyalkanoates (mcl-PHAs) was studied using two bacteria, *Pseudomonas chlororaphis* and *Acinetobacter lwoffii*, which secrete an enzyme, or enzymes, with lipase activity. These bacteria produced clear zones of depolymerization on petri plates containing colloidal solutions of PHA polymers with different monomer compositions. Lipase activity in these bacteria was measured using p-nitrophenyl octanoate as a substrate. In liquid medium, scl-PHA (PHBV) and mcl-PHA (PHO) films were used as the sole carbon source for growth, and after 7 days, 5-18% loss in weight of PHA films was observed. Scanning electron microscopy (SEM) of these films revealed bacterial colonization of the polymers, with cracks and pitting in the film surfaces. Degradation of polymers released 3-hydroxyhexanoate, 3-hydroxyoctanoate, and 3-hydroxydecanoate monomers into the liquid medium, depending on the starting polymer. Genes encoding secretory lipases, with amino acid consensus sequences for lipase boxes and oxyanion holes, were identified in the genomes of *P. chlororaphis* and *A. lwoffii*. Although amino acid consensus sequences for lipase boxes and oxyanion holes are also present in PHA depolymerases identified in the genomes of other PHA degrading bacteria, the *P. chlororaphis* and *A. lwoffii* lipases had low homology to these depolymerases.

Key words: Polyhydroxyalkanoates (PHAs); PHA depolymerase; Esterase/Lipase; Lipase box; Oxyanion hole consensus sequence; PHA films; *Pseudomonas chlororaphis*; *Acinetobacter lwoffii*.

Introduction

Polyhydroxyalkanoate (PHAs) polymers have recently gained popularity because they are considered to be biodegradable and biocompatible (Preito 2016). Moreover, some PHAs have physical and thermal properties that are similar to petroleum plastics, such as polyethylene and polypropylene. PHAs are natural polyester polymers that are synthesized by bacteria as carbon and energy storage compounds when conditions are not conducive for growth, for example under conditions of carbon excess but limited nitrogen and/or phosphorus (Luengo et al. 2003; Madison and Huisman 1999). Under conditions of carbon-limitation in the presence of sufficient concentrations of other nutrients (nitrogen, phosphorus, etc.), PHA polymers are hydrolyzed into monomer subunits that are metabolized to generate ATP and growth via the β -oxidation pathway. PHAs have been classified as short chain length PHAs or medium chain length PHAs, on the basis of number of carbon atoms in side-chains of the monomers. Short chain length (scl-)PHAs consist of subunits with 3-5 carbon atoms, while medium chain length (mcl-)PHAs consist of subunits with 6-14 carbon atoms (Madison and Huisman 1999). Inside the cell, PHAs are stored as granules, referred to as carbonosomes, in which the PHA polymers are covered by a lipid layer in which different proteins are embedded (Preito et al. 2016; Sznajder and Jendrossek 2011). Intracellular native PHA polymer (nPHA) granules exhibit an amorphous, rubbery state. They consist of highly mobile chains of the carbon backbone, giving them a disordered conformation. Upon extraction from the cell, the surface layer of nPHA granules is rapidly lost, and the PHA chains tend to adopt an ordered helical conformation and develop a crystalline phase known as denatured PHA (dPHA) (Jendrossek et al. 2007).

The biodegradability of PHA polymers makes them an attractive alternate to conventional petroleum plastics (Rujnić-Sokele and Pilipović 2017; Volova et al. 2017). Although the biodegradation of scl-PHAs, such as polyhydroxybutyrate (PHB), has been well characterized, the

biodegradation of mcl-PHAs is not well understood, and it is not known if all PHA polymers are equally biodegradable.

The hydrolysis of PHA polymers into monomer subunits is mediated by intracellular or extracellular PHA depolymerase enzymes (Jendrossek and Handrick 2002). PHA polymers synthesized by Pseudomonad bacteria have an intracellular PHA depolymerase gene (*phaZ*) in the PHA synthesis cluster, and the gene product of *phaZ* (PhaZ, depolymerase) is present on the surface of PHA granules. PHA depolymerases have very specific activities. In bacteria that synthesize scl-PHAs, such as poly(3-hydroxybutyrate, PHB), the intracellular PHB depolymerase that hydrolyzes polymers within PHA granules (nPHB) is not able to degrade polymers that have been extracted from the cell (dPHB), and *vice versa* (Merrick et al. 1999). Moreover, depolymerases of scl-PHAs are not able to degrade mcl-PHA polymers. For example, depolymerase from *Commamonas* sp. and *Paucimonas lemoignei* were specific for scl-PHB and did not hydrolyse mcl-PHO (poly 3-hydroxyoctanoate) (Molitoris et al. 1996).

Some bacteria have extracellular depolymerases, or “e-depolymerases”, that can hydrolyze PHAs released by other bacteria in the environment (Jendrossek and Handrick, 2002). Production of e-depolymerases gives the bacteria an additional survival strategy under hostile environmental conditions, using polymers produced by other bacteria. e-PHA depolymerases that degrade scl-PHAs have been studied in detail from *Bacillus* sp. strain NRRL B-14911 (Ma et al. 2011), *Ralstonia pikettii* T1, *Acidovorax* sp. TP4 (Wang et al. 2002), *Rhodospirillum rubrum* (Merrick et al. 1999; Handrick et al. 2004), *Streptomyces ascomycinicus* (Garcia-Hindalga et al. 2012, 2013), and *Burkholderia cepacia* DP1 (Azami et al. 2017; Handrick et al. 2001). Among PHA degrading bacteria that express depolymerases, *P. lemoignei* was unique. It was found to encode seven PHA e-depolymerases, which were specific for PHB and its copolymers. Of the seven PHA depolymerases in *P. lemoignei*, PhaZ7_{PI} was found to be specific for amorphous dPHB (Schober et al. 2000).

In contrast to depolymerases of scl-PHAs, very few depolymerases specific for mcl-PHA polymers have been studied. An extracellular depolymerase from *P. fluorescens* GK13 was shown to degrade mcl-PHA polymers synthesized from octanoic acid (PHO), which consisted of approximately 92 mol% C8 subunits, with 6 mol% C6, and 2 mol% C10 subunits (Schrimmer et al 1994; Molitoris et al. 1996). New extracellular mcl-PHA depolymerases have been detected in the obligated predator *Bdellovibrio bacteriovorus* HD100 (Martínez et al. 2012), in the Actinobacteria *Streptomyces roseolus* SL3 (Gangoiti et al. 2012), *Streptomyces exfoliatus* K10 DSMZ 41693 (Martínez et al. 2015), and *Streptomyces venezuelae* SO1 (Santos et al. 2013).

PHA depolymerases are in fact lipases, which are a subclass of the esterase enzyme family. Lipases hydrolyze carboxyl-ester bonds in triglycerides, such as oils and fats resulting in end-products of glycerol and fatty acids (Li and Zhang 2005). Lipases and depolymerases have common α/β hydrolase-fold and catalytic amino acid triad consisting of serine/cysteine, histidine, and aspartate. Functionally, lipases and depolymerases differ with respect to substrate preference: lipases show a preference for lipids/fats, while depolymerases have a preference for PHA polymers. However, a number of lipases have been shown to have depolymerising activity with PHB and PHB copolymers. Some commercial lipases have been reported to depolymerize PHB and its copolymers, as detected by calorimetric methods (Stoytcheva et al. 2012).

A method of identifying extracellular depolymerase activity based on change in opacity of colloidal PHB films was developed by Chang and Sudesh (2013). Using this method, lipases from bacteria, yeast, and animal origin were screened for degradation of poly (3-hydroxybutyrate-co-4-hydroxybutyrate) [P(3HB-co-4HB)] and confirmed to degrade P(3HB-co-4HB) (Mok et al. 2015). A lipase and depolymerase (PHZ7_{PI}), encoded by *P. lemoignei*, had a conserved amino acid consensus sequence for an oxyanion hole motif, as well as the catalytic triad sequence found in other PHA depolymerases (Mohamed et al. 2017).

Although the PHA depolymerase activities of purified lipase enzymes, as well as from lipase-producing bacteria, have been demonstrated for scl-PHB and PHB copolymers, very few depolymerases specific for mcl-PHA polymers have been studied in detail. In the present study, we report extracellular lipase/depolymerase activities that resulted in the depolymerization of a mcl-PHA polymer (PHO) by *P.chlororaphis* PA23-63-1 and of P(3HB-co-3HV) copolymers by *Acinetobacter lwoffii*.

Materials and methods

Bacterial cultures, media and growth conditions

P. chlororaphis PA23 is a plant growth promoting bacterium isolated from the rhizosphere of soybeans, capable of suppressing growth of the fungal pathogen *Sclerotinia sclerotiorum* due to its ability to synthesize and secrete two anti-fungal compounds, phenazine and pyrrolnitrin (Fernando et al. 2007; Savchuk and Fernando, 2004). *P. chlororaphis* PA23-63-1 is a mutant strain of *P. chlororaphis* PA23 that is defective in phenazine and pyrrolnitrin production, and is also able to synthesize mcl-PHAs (Sharma et al. 2018). *P. chlororaphis* PA23-63-1 was kindly provided by Dr. Teresa De Kievit, in the Department of Microbiology, at the University of Manitoba. *A. lwoffii* was isolated in the Levin's laboratory in the Department of Biosystems Engineering, at the University of Manitoba, from wastewater. This species was later found growing as a contaminant on a waste PHA film stored in the Levin Laboratory. It was subsequently confirmed as a PHA degrader by the presence of a zone of clearance around a colony growing on colloidal PHA films.

All bacteria used in this study were grown and purified on LB plates incubated at 30 °C for 24 hours (h). PHA degradation was studied in Ramsay's minimal medium (RMM) and PHAs with different monomer composition were used as a sole carbon source (Sharma et al. 2012). For solid RMM, 15 g/L agar was added to the medium. PHA polymers used in present study were produced using *Pseudomonas putida* LS46, cultured in 7 L Applicon Bioreactors containing 3 L RMM with hexanoic, octanoic, nonanoic, or decanoic acid as the sole carbon sources in separate fermentations

(Blunt et al. 2017). The monomer compositions of these PHA polymers has been published (Sharma et al. 2012), and are shown in Table 1. PHAs from canola oil was produced using *Pseudomonas chlororaphis* PA23 as described earlier (Sharma et al. 2017). Copolymer of 3-hydroxybutyrate and 3-hydroxyvalerate was produced from biodiesel fatty acid supplemented with 0.5% valeric acid using *Cuprividus necator* H16 (Sharma et al. 2016).

Identification of bacterial isolates

Polymerase Chain Reaction (PCR) amplification of 16S rRNA genes followed by nucleotide sequencing was used to identify the PHA degrading bacteria. Genomic DNA was isolated from LB grown bacterial cultures using Wizard DNA purification kit (Promega). Genomic DNA was amplified using 16S rDNA F27 and 1492R primers using RedTag as PCR mixture. Bacterial cultures were identified by sequencing the amplified 16S rDNA sequences, followed by BLAST analyses against the NCBI 16s DNA database, as described earlier (Sharma et al. 2012).

Degradation of mcl-PHA films on petri plates

Aliquots of PHA polymers produced from hexanoic, octanoic, nonanoic, or decanoic acid (2.5 g each) were dissolved in 100 mL chloroform. RMM-agar plates were overlaid with 2 mL PHAs dissolved in chloroform. The chloroform was allowed to evaporate under the fume hood for 24 h to remove residual chloroform. These plates were spotted with 50 μ L of each bacterial culture ($OD_{600} = 1.0$), grown overnight in LB broth. All plates were incubated at 30 °C for 7 days.

Preparation of plates with colloidal suspensions of PHA polymers

Colloidal solutions of PHA polymers synthesized by *P. putida* LS46 from hexanoic (PHHx), octanoic (PHO), nonanoic (PNO), decanoic acids (PDO), and from long chain fatty acids derived from canola oil (PCO) were prepared as described by Ramsay et al. (1994). Briefly, PHAs were dissolved in 100 mL acetone (1 mg per mL) and the solutions were poured slowly, drop wise, with continuous stirring into 10 mL cold water. Acetone was evaporated in a vacuum dryer, resulting in 10 mL colloidal solutions, each containing 10 mg/mL PHA. RMM (25 mL) was mixed with 1 mL

of each colloidal polymer solution and poured into petri plates. The plates were allowed to solidify in a biohazard hood. The plates were spotted with 50 μ L of each bacterial culture and incubated at 30 °C. After 7 days, the width (mm) of the zones of degradation resulting from the degradation of PHA polymers around the bacterial colonies was measured.

Preparation of solvent cast PHA films

PHA produced from octanoic acid (PHO) was used to produce PHA films. Aliquots of PHO and PHBV (5 g) were each dissolved in 100 mL acetone and 100 mL chloroform, respectively. Solutions of 5% PHO or PHBV (2.5 mL each) were poured into aluminum cups (45 mm diameter), and the acetone/chloroform was evaporated at room temperature for 48 h. After keeping cups at -20 °C overnight, the films were removed from the cups.

Growth of bacterial cultures in liquid medium with PHO or PHBV films as the sole carbon source

Films were weighed, sterilized with 95% ethanol for 20 minutes (min) and washed 3 times in sterilized nanopure water. Each type of PHA film ($\approx$ 0.25 g, 43.57 mm diameter, Thickness PHO 0.184 mm, PHBV 0.214 mm) was put into 250 mL Erlenmeyer flasks containing 50 mL RMM. Flasks were inoculated with 1 mL of overnight cultures that had been grown in RMM with 100 mg PHO or PHBV for 72 h. The flasks containing the PHA films with bacteria were then incubated for 7 days at 30 °C on rotary shaker (150 rpm). Growth was monitored by measuring optical density at 600 nm (OD_{600}) periodically after 24 h, up to 168 h. The loss of weight of the PHAs films was estimated after 7 days, after drying the films for 48 h in a biohazard hood..

Electron microscopy of PHO/PHBV films after incubation with bacterial cultures

Colonization of PHA films by the bacteria was studied by scanning electron microscopy (SEM). Samples were mounted on aluminum SEM sample pin-mounts. The pin-mounts were coated with copper tape and a piece of PHO/PHBV film (5 x 5 mm) was placed on each mount. Pin mounts with films were placed on a pin-mount platform and were coated with gold (60%) and

palladium (40%) for 45 seconds (sec) $\approx$ 15 nm using a Denton Vacuum Desk II cold sputter unit. PHO films were studied under a dual-beam Quanta FEG 650 scanning electron microscope (FEI Company, United States). The samples were examined under low and high vacuum at 5 kV.

Estimation of lipase activity

Lipase activity in bacterial cultures grown in RMM were estimated by using p-nitrophenyl octanoate (PNPO), as described by Schirmer et al. (1993). For this assay, supernatants from 1 mL of each bacterial culture were centrifuged. Supernatants were transferred to fresh sterile tubes and saved. Cell pellets were washed with PBS buffer, pH 7.0, and then centrifuged again to recover the cells. In each assay tube, 1 mL supernatant, or cells suspended in 1 mL PBS buffer, were mixed with 3.9 mL PBS buffer. To start the reaction, 100 μ L substrate p-nitrophenyl octanoate was added. This was time 0 for reaction. The mixture was vortexed for 10 sec and incubated at 30 °C on a rotary shaker. After the appearance of a yellow color, the reaction was stopped by adding 1 mL of 1 M sodium carbonate, and the length of time to the appearance of the yellow colour was noted. Total protein in the samples was estimated using the Bradford method (Bradford 1976). One unit of PNPO esterase activity was defined as the hydrolysis of 1 μ mol of PNPO per min, at 30°C. Lipase activity was also estimated using three additional p-nitrophenyl-alkanoate substrates: p-nitrophenyl-acetate, p-nitrophenyl-butyrate, and p-nitrophenyl-decanoate.

Two additional substrates PES (poly (ethylene succinate)) and PCL (polycaprolactone) were studied for their degradation by lipases produced by *P. chlororaphis* and *A. lwoffii*. The enzyme activity with PES and PCL was determined at 30 °C by measuring the decrease in turbidity at 650 nm of the polymer suspensions with the enzyme for 20 min. One unit of enzyme activity was the amount of enzyme capable of decreasing A_{650 nm} by 1 absorbance unit per min.

Identification of PHA monomers as hydrolysis products

After incubation of PHAs films for 7 days, 1.5 mL of each culture was centrifuged at 10000 g for 10 min. Samples (1 mL) were placed in 10 mL culture tubes and dried in an oven at 60 °C for

2 days. The dried samples were methanolized with 1 mL of chloroform containing 1 mg of benzoic acid (internal standard), and 1 mL of methanol containing 15% concentrated sulphuric acid as described by Braunegg et al. (1978). Tubes were boiled in a water bath for 6 h, and then 0.5 mL of water was added to each tube. The chloroform (lower) layer was transferred to a 2 mL GC vial and analyzed by gas chromatography using an Agilent GC (Model 7890A) fitted with a DB-23 capillary column and flame ionization detector. The initial oven temperature was maintained at 60 °C for 5 min, which was increased to 250 °C at a ramping rate of 15 °C/min. The 3-hydroxy fatty acid methyl ester peaks were identified by their retention time using different 3-hydroxy fatty acids as standards.

Results

Bacteria degrading PHAs

P. chlororaphis PA23-63-1 is able to grow in RMM containing different vegetable oils as a sole carbon source and produce PHAs (Sharma et al. 2018). The identity of this strain was confirmed on the basis of genome sequencing (Loewen et al. 2014). The other bacterium isolated from wastewater as a PHA degrader was identified as a variant related to *A. lwoffii* strain AL-24, with 92% 16S rDNA sequence identity. Based on genome sequence analysis of *P. chlororaphis* PA23 (Accession No. CP008696), *P. chlororaphis* PA23-63-1 (which is derived from PA23) encodes an intracellular depolymerase (*phaZ*), which is part of *pha* gene cluster. In contrast, no intra- or extracellular PHA depolymerase has been reported from sequenced and annotated *A. lwoffii* genomes in closely related *A. lwoffii* strain CIP 70.31 (Cerqueira et al. 2013).

Degradation of colloidal PHA and PHA films in minimal medium plate

Plates containing colloidal PHHx, PHO, PHN, or PHD films were opaque, with a milky white color. Only plates containing colloidal PCO (PHA from canola oil fatty acids) were not opaque. Both *P. chlororaphis* and *A. lwoffii* produced zones of clearance around their bacterial colonies on all colloidal PHA film plates (Figure 1). The zones of clearance were wider on PHHx

(28 and 25 mm) and PHN (25 and 21 mm) plates, compared to the zones of clearance on PHO and PHD plates (Table 2). No visible zone of clearance was observed on PCO plates.

Growth of bacteria in liquid RMM with PHA films as a sole carbon source

P. chlororaphis and *A. lwoffii* were able to grow on PHO as sole carbon source. *P. chlororaphis* reached the stationary phase after 96 hours post-incubation (h pi) with a final cell density of $OD_{600} = 1.21$, while *A. lwoffii* reached stationary phase after 144 h pi and a final cell density of $OD_{600} = 1.61$ (Figure 2). Growth of *P. chlororaphis* and *A. lwoffii* on PHO films was associated with a loss of weight of 4.18% and 5.42%, respectively, after incubation for 7 days (Table 3). Degradation of PHBV films by *P. chlororaphis* and *A. lwoffii* was better than PHO films, resulting in a loss of weight of 16.92% and 18.43%, respectively, after incubation for 7 days (Table 3).

Esterase/lipase activity of PHO grown cultures

The lipase activity of *P. chlororaphis* and *A. lwoffii* cultured with PHO, PHD, and PCO as sole carbon sources was estimated using the p-nitrophenyl-octanoate assay. *P. chlororaphis* had higher lipase activity than *A. lwoffii* on polymers derived from PHO and PHD, but *A. lwoffii* showed higher lipase activity on polymers derived from PCO (Table 4). The specificity of the lipase activities of *P. chlororaphis* and *A. lwoffii* after growth on PHO or PHBV derived polymers was further studied using p-nitrophenyl-acetate, p-nitrophenyl-butyrate, and p-nitrophenyl-decanoate, in addition to p-nitrophenyl-octanoate. These assays allowed an assessment of lipase activity with substrates consisting of C2, C4, C8, or C12 fatty acid esters.

P. chlororaphis grown on polymers derived from PHO had highest lipase activity with p-nitrophenyl-butyrate followed by p-nitrophenyl-octanoate, while *A. lwoffii* grown on polymers derived from PHO had its highest activity on p-nitrophenyl-butyrate, followed by p-nitrophenyl-dodecanoate. *P. chlororaphis* grown on polymers derived from PHO showed minimal activity for p-nitrophenyl-dodecanoate, while *A. lwoffii* grown on polymers derived from PHO had minimal

activity for p-nitrophenyl-acetate (Table 5). The order of lipase activity substrate preference for *P. chlororaphis* grown on PHO-derived polymers was $C4 > C8 > C2 > C12$, while the lipase activity substrate preference for *A. lwoffii* grown on polymers derived from PHO was $C4 > C12 > C8 > C2$.

The order of substrate preferences for lipase activities of *P. chlororaphis* and *A. lwoffii* grown on PHBV-derived polymers were different from those of the two bacteria grown on PHO-derived polymers. The order of substrate preference for lipase activity of *P. chlororaphis* grown on PHBV polymers was $C8 > C4 > C12 > C2$, while the order of substrate preference for lipase activity of *A. lwoffii* grown on PHBV polymers was $C2 > C8 > C4 > C12$. Thus, the extracellular lipase activities of *P. chlororaphis* and *A. lwoffii* did not appear to show any specificity for scl- versus mcl-PHA polymers. However, the lipase activities of *P. chlororaphis* and *A. lwoffii* were specific to PHA polymers, as no p-nitrophenyl-PES or p-nitrophenyl-PCL activities were detected for either bacterium.

Scanning electron microscopy (SEM) of PHO and PHBV films after growth of *P. chlororaphis* and *A. lwoffii*

PHO films on solid medium

Scanning electron microscopy of PHO films inoculated with *P. chlororaphis* or *A. lwoffii* showed the colonization of the PHA polymer film by bacteria. Growth of both bacteria resulted in erosion and cracks in the PHO film (Figure 3). Both *P. chlororaphis* (Figure 3A) and *A. lwoffii* (Figure 3B) were able to use PHO polymers as a sole carbon source for their growth. Prior to bacterial growth and colonization, the PHO films were semitransparent. After bacterial growth and colonization, the PHO films were opaque.

PHO films in liquid medium

PHO films that were not treated with *P. chlororaphis* or *A. lwoffii* showed small undulations in their surfaces, and the surfaces were free from cracks or pits (Figure 4 A, B, C). Changes in the PHO film surfaces were clearly visible after incubation with bacteria. Incubation with *P.*

chlororaphis (Figure 5 A, B, C) or *A. lwoffii* (Figure 6 A, B, C) resulted in erosion of the surfaces, with the appearance of small cracks and pits. The size of the pits varied from 1-6 μm diameter, and deeper cavities were filled with the bacteria. The surface of the films had a rough appearance, and no area on the surface of the films was unmodified after bacteria growth.

PHBV films

The surface of PHBV films that were not inoculated with *P. chlororaphis* or *A. lwoffii* were smooth, and no cracks or pits were observed at either low (100 x) or high (5000 x) magnifications (Figure 7 A, B, C). After incubation of PHBV films with *P. chlororaphis* (Figure 8 A, B, C) or *A. lwoffii* (Figure 9 A, B, C), the PHBV film surfaces were covered with bacteria. The film surfaces were eroded with cracks and pits of different diameters (Figures 5C and 6C). In comparison to *P. chlororaphis*, *A. lwoffii* produced more and deeper pits, giving the films a porous appearance. *P. chlororaphis* cells (Figure 8 B, C) and *A. lwoffii* cells (Figure 9 B, C) were observed to be attached to the entire surface area of the PHBV films.

Identification of degradation products of PHA films

Hydrolysis products from the degradation of PHO, PHD, and PCO polymer films by *P. chlororaphis* or *A. lwoffii* in liquid cultures were detected by gas chromatography analysis. While 3-hydroxyoctanoate monomers were detected in the supernatants of *P. chlororaphis* cultures growing on PHO films, 3-hydroxyhexanoate, 3-hydroxyoctanoate, and 3-hydroxydecanoate monomers were detected in the supernatants of *A. lwoffii* growing on PHO films (Table 6). No monomers were detected in the supernatants of *P. chlororaphis* cultures growing on PHD films, but 3-hydroxyhexanoate, 3-hydroxyoctanoate, and 3-hydroxydecanoate monomers were detected in the supernatants of *A. lwoffii* growing on PHD films (Table 6). In cultures using PCO polymers, 3-hydroxyoctanoate and 3-hydroxydecanoate monomers were detected in *P. chlororaphis* cultures, but no monomers were detected in the supernatants of *A. lwoffii* cultures. Finally, neither 3-

hydroxybutyrate, nor 3-hydroxyvalerate monomers were detected in the supernatants of *P. chlororaphis* or *A. lwoffii* grown on PHBV films.

Amino acid sequence alignments of PHA depolymerases and lipases of *P. chlororaphis* and *A. lwoffii*

Genome analyses identified seven esterases/lipases encoded by the *P. chlororaphis* PA23 genome [Acc. No. CP008696] (Loewen et al. 2014). Out of seven esterases/lipases, three were secretory proteins with signal peptides (EY04_17885, EY04_21540, and EY04_32435). In addition to this, an ATP-binding cassette for export of lipase (EY04_16115) was also present (Sharma et al. 2018). Six esterases/lipases encoding genes with signal peptides were identified in the *A. lwoffii* CIP 70.31 genome. Lipases from *A. lwoffii* CIP 70.31 (F924_03808, F924_03809, and F924_03810) had only 16.86-19.92% amino acid sequence identity with the PHB depolymerase PhaZ7 from *P. lamoignei* (Schrimmer et al. 1994).

The amino acid consensus sequences for the pentapeptide lipase box (GX SXG) and the PXXXXHG oxyanion pocket present in T1 lipase of *Geobacillus zahilae*, were also identified in secretory lipases from and *A. lwoffii* CIP 70.31 (F924_03808, F924_03809, and F924_03810). The three lipases (i.e. F924_03808, F924_03809, and F924_03810) had conserved PMV FVHG, PILMVHG and PIVFAHS anion pocket respectively (Figure 6). In addition to this, catalytic aspartic acid and histidine were also present. Lipases from *A. lwoffii* showed 19.8-213% amino acid sequence identity to PHA depolymerase from *S. roseolus*. The pentapeptide lipase box present in *S. roseolus* was also present in lipases from *A. lwoffii*. The intracellular PHA depolymerase encoded by *P. chlororaphis* PA23 (and hence the mutant strain *P. chlororaphis* PA23-63-1) also had a pentapeptide lipase box, but no oxyanion hole motif. Only EY04_02420 lipase from *P. chlororaphis* PA23 had a lipase box. No conserved pentapeptide motif was present in other lipases of *P. chlororaphis*.

Discussion

Polyhydroxyalkanoates (PHAs) are biodegradable polymers produced by a number of bacteria. Biodegradability of PHAs is either chemical or enzymatic. P(3HB-co-4HB) films can be degraded in buffer with pH 12-13, at temperatures above 50 °C (Mok et al. 2016). Enzymatic degradation of PHB and PHBV films has been reported using intra- or extracellular PHA depolymerases (Schirmer et al. 1995). The vast majority of PHA films used in these studies were scl-PHAs, which are mostly crystalline, with some amorphous regions. Extracellular depolymerases can degrade crystalline PHA polymers, but intracellular depolymerase have not been reported to degrade crystalline polymers. Intracellular PHA depolymerase genes are part of the *pha* operon in *Pseudomonas* species. Recently some extracellular PHA depolymerases have been cloned from various microorganisms and have been characterized for PHA degradation (Jendrossek 2005; Jendrossek et al. 1993, 2002; Jendrossek and Handrick 2002; Gangoiti et al. 2010). Lipase-producing bacteria, as well as commercial lipase enzymes produced from different bacteria, have been shown to degrade amorphous PHAs only. In the present study, we found that *P. chlororaphis* and *A. lwoffii* could grow on mcl-PHA films as a sole carbon source, even though neither of these bacteria has been reported to encode and express e-PHA depolymerases.

P. chlororaphis PA23 can synthesize mcl-PHA polymers utilizing products from canola and other vegetable oils (Sharma et al. 2017). The genome sequence of *P. chlororaphis* PA23 is available at the Integrated Microbial Genome (IMG) website (www.img.jgi.doe.gov). *P. chlororaphis* PA23 encodes seven genes annotated as esterase/lipase. Three of these lipases of *P. chlororaphis* PA23 encode a signal peptide for their excretion outside the cell (EY04_02420, EY04-17885, and EY04_21540). An intracellular PHA depolymerase (EY04_01535) was also present in the genome of this strain (Genome Acc. No. CP008696). The genome sequence of *A. lwoffii* strain 263AY1 is not available, but annotated genomes of other closely related strains of *A. lwoffii* are available at the IMG website, such as *A. lwoffii* ATCC 9957 = CIP 70.31 (Cerqueira et

al. 2013). The genomes of two strains, *A. lwoffii* CIP7031 and *A. lwoffii* NIPH, encode 3-4 secretory lipases. However, no genes encoding intracellular or extracellular PHA polymerases were identified in the genomes of these bacteria.

Lipases of bacterial, fungal, and animal origin have been known to degrade poly (3-hydroxybutyrate-co-4-hydroxybutyrate) [P(3HB-co-4HB)] (Mok et al. 2016). Lipases are known to have a consensus α/β hydrolase fold and a consensus pentapeptide Gly-X-S-X-Gly lipase box. Generally, PHA depolymerases differ from lipases in that they have a lipase box and oxyanion hole, but do not display lipase activity (Jaeger et al. 1995). However, four classes of PHA depolymerases have been identified, and one of these does not have a lipase box (Knoll et al. 2009). Thus, the distinction between lipases and PHA depolymerases is not entirely clear.

Generally, lipases and depolymerases have low amino acid homology. Comparison of T1 lipase from *Geobacillus zahilae* and PhaZ7_{PI}, the extracellular depolymerase from *P. lemoignei*, showed only 25% amino acid homology, but both enzymes had the same active site aspartic acid and histidine residues (Mohamad et al. 2017). The PhaZ7_{PI} amino acid sequence contained the motifs PXXXXHG and AHSMG, which are related to the oxyanion pocket and the lipase box pentapeptide (GX SXG) of many lipases and other serine hydrolases, respectively (Arpigny and Jaeger 1999; Jaeger et al. 1994; 1999). The PHA depolymerases of *S. exfoliatus*, *S. venezuelae* and *S. roseolus* are different than PHA depolymerases from *Pseudomonas* sp. and *Bdellovibrio bacteriovorus* (Martinez et al. 2015). The lipases from *S. exfoliatus*, *S. venezuelae*, and *S. roseolus* showed 19.88-21.34% amino acid sequence homology to extracellular mcl-PHA depolymerase from *Streptomyces roseolus* and a similar GX SXG pentapeptide lipase box was present in both. The secretory lipases of *P. chlororaphis* and *A. lwoffii* could function as extracellular PHA depolymerases.

Indeed, lipases have been used earlier to study degradation of scl-PHA or co-polymers of PHB and PHV (Chang and Sudesh 2013; Mok et al. 2016; Mohammad et al. 2017). PHA

degradation occurs at the polymer film surface, and both transparent PHO films and semi-transparent PHBV films become opaque after incubation with lipase producing bacteria. This was the basis of development of a densitometer method for PHB co-polymer degradation (Chang and Sudesh 2013). Degradation of P(3HB-co-4HB) films by triglycerol lipase enzyme using a sensitive and rapid densitometry method for evaluation of hydrolytic activity of lipase was also studied. Lipases from fungal, bacterial and animal origin hydrolyzed P (3HB-co-92 mol% 4HB) as detected by opacity of the hydrolysis spots (Chang and Sudesh 2013). We also observed changes in the opacity of PHA polymers after the growth of lipase producing bacteria.

In our study, lipase producing bacteria did not show any specificity for scl- versus mcl-PHA polymers, and grew on both scl- and mcl-PHA films. This is the first report of extracellular lipase activities that depolymerize both scl- and mcl-PHAs. This could be due to presence of multiple lipases in the cell, which can act on different substrates. Earlier lipases were reported to have specificity for hydrolysing PHAs substrates. The lipases from prokaryotes are reported to have narrow specificity, while lipases from eukaryotes have broader specificity. PHA depolymerase from *Alcaligenes faecalis* T1, *Commamonas testosteroni*, *Pseudomonas lamoignei* A and B could not degrade poly (3-hydroxyvalerate) or poly (3-hydroxyhexanoate) films (Mukai et al. 1993).

The different rate of degradation of PHO and PHBV films pointed to some specificity in the action of lipases in degradation of PHA films. Degradation of PHO and PHBV films by lipase producing bacteria was $\approx$ 5-18% in comparison to 90% degradation of PHB by PHB-depolymerase producing bacteria like *Ralstonia* sp. and *Cupriavidus* sp. (Martinez et al. 2018). However, the method of PHB film casting for this work was different, using acetic acid as a solvent rather than chloroform film casting. The fabrication methods have been reported to affect degree of crystallinity and hence degradation (Anbukarasu et al. 2015). Other extracellular PHB depolymerase producers, like *P. lemoignei*, *P. stutzeri*, and *C. testosteroni* showed less than 10% loss in weight (Martinez et al. 2018). In this respect, lipase producing *P. chlororaphis* and *A. lwoffii*

had comparable efficiency of PHBV degradation to PHB depolymerase producing bacteria.

Conclusions

P. chlororaphis PA23 is known to synthesized medium chain length PHA polymers. Analyses of the *P. chlororaphis* PA23 genome revealed that it encodes an intracellular depolymerase, which is known to act on native PHAs, but does not encode an extracellular depolymerase. *A. lwoffii* is not a PHA synthesizing bacterium, and analyses of its genome showed that it does not encode either extracellular or intracellular PHA depolymerases.. However, genomes of both of these bacteria were found to encode 3-5 extracellular lipases. Moreover, both lipase and PHA depolymerase have catalytic triad serine-aspartate-histidine as well as a conserved lipase box.

Lipases are classified as ester hydrolases, and are know to cleave ester bonds. Both lipase and most PHA depolymerases have catalytic triad serine-aspartate-histidine residues, as well as a conserved lipase box. PHA depolymerases differ from lipases in that they have a lipase box and oxyanion hole, but do not display lipase activity. Our data demonstrated that the culture media in which *P. chlororaphis* PA23 and *A. lwoffii* were grown contained a factor (or factors) that cleaved both the ester bonds of p-nitrophenyl-fatty acid substrates, and the ester bonds of PHA polymers of various subunit composition. Commercial lipases purified from *Candida antarctica*, *Candida rugosa*, *Mucor javanicus*, *Pseudomonas cepacia*, *Pseudomonas fluorescens*, *Rhizopus arrhizus*, *Rhizopus niveus*, *Rhizopus oryzae* and porcine pancreas have been reported to degrade PHAs (Chang and Sudesh 2013; Mok et al. 2016). Therefore, we concluded that the factor(s) in the media in which *P. chlororaphis* PA23 and *A. lwoffii* were grown were lipases.

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Figure Legends

Figure 1. Degradation of colloidal PHA polymers by *P. chlororaphis* and *A. lwoffii*. The two lipase-producing bacteria generated zones of clearance by hydrolyzing colloidal PHA polymers. A) PHHx, polyhydroxyhexanoate; B) PHO polyhydroxyalkanoates; C) PHN, polyhydroxynonaote; D) PHD, polyhydroxydecanoate.

Figure 2. Growth of *P. chlororaphis* and *A. lwoffii* in Ramsay's minimal medium with polyhydroxyoctanoate (PHO) or co-polymer of 3-hydroxybutyrate and 3-hydroxyvalerate [poly(3HB-co-3HV)] as the sole carbon sources. Growth of the bacteria was estimated by measuring optical density at 600 nm.

Figure 3. Colonization of polyhydroxyoctanoate (PHO) solvent cast films on solid medium by *P. chlororaphis* PA23-63-1 and *A. lwoffii*. PHO films were observed under scanning electron microscope. A) PHO films inoculated with *P. chlororaphis* PA23-63-1 with 7433 X magnification, B) PHO films inoculated with *A. lwoffii* with 2000 X magnification under the scanning electron microscope.

Figure 4. Scanning electron microscopy of uninoculated (Control) PHO films. A) Uninoculated (Control) film at 100 X magnification; B) Uninoculated (Control) film at 500 X magnification; C) Uninoculated (Control) film at 5000 X magnification, under the scanning electron microscope.

Figure 5. PHO film incubated with *P. chlororaphis* PA23-63-1 for 7 days in liquid mineral medium. A) PHO film incubated with *P. chlororaphis* PA23-63-1 at 100 X magnification; B) PHO film incubated with *P. chlororaphis* PA23-63-1 at 500 X magnification; C) PHO film incubated with *P. chlororaphis* PA23-63-1 at 5000 X magnification, under the scanning electron microscope.

Figure 6. PHO film incubated with *A. lwoffii* for 7 days in liquid mineral medium. A) PHO film incubated with *A. lwoffii* at 100 X magnification; B) PHO film incubated with *A. lwoffii* at 500 X magnification; C) PHO film incubated with *A. lwoffii* at 5000 X magnification, under the scanning electron microscope.

Figure 7. Scanning electron microscopy of uninoculated (Control) PHBV films. A) Uninoculated (Control) film at 100 X magnification; B) Uninoculated (Control) film at 500 X magnification; C) Uninoculated (Control) film at 5000 X magnification, under the scanning electron microscope.

Figure 8. PHBV film incubated with *P. chlororaphis* PA23-63-1 for 7 days in liquid mineral medium. A) PHBV film incubated with *P. chlororaphis* PA23-63-1 at 100 X magnification; B) PHBV film incubated with *P. chlororaphis* PA23-63-1 at 500 X magnification; C) PHBV film incubated with *P. chlororaphis* PA23-63-1 at 5000 X magnification, under the scanning electron microscope.

Figure 9. PHBV film incubated with *A. lwoffii* for 7 days in liquid mineral medium. A) PHBV film incubated with *A. lwoffii* at 100 X magnification; B) PHBV film incubated with *A. lwoffii* at 500 X magnification; C) PHBV film incubated with *A. lwoffii* at 5000 X magnification, under the scanning electron microscope.

Figure 10. Partial amino acid alignment of PHB depolymerase PhaZ_{7pl} of *P. lamoignei* with three lipases of *A. lwoffii* CIP 70.31 (F924_03808, F924_03809, F924_03810). Red boxes indicate the amino acid consensus sequences for the oxyanion pocket and lipase box in depolymerase and lipase proteins. Amino acids of the catalytic triad, S¹²², D²⁴⁷ and H²⁸⁹, are also shown by red boxes. H⁴⁹ is a part of oxyanion hole in F924_03409.

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Table 1. Composition of PHA polymers used in the present study

PHA	Produced by	Substrate	*Monomer composition of PHA (mol%)								
			3HB	3HV	3HHx	3HHp	3HO	3HN	3HD	3HDD	3HTD
PHHx	<i>P. putida</i> LS46	Hexanoic acid	nd	nd	82.34	nd	16.02	nd	1.64	nd	nd
PHO	<i>P. putida</i> LS46	Octanoic acid	nd	nd	6.52	nd	88.50	nd	3.81	31.0	nd
PHN	<i>P. putida</i> LS46	Nonanoic acid	nd	nd	nd	18.71	nd	81.29	nd	nd	nd
PHD	<i>P. putida</i> LS46	Decanoic acid	nd	nd	4.56	nd	47.5	nd	46.61	1.32	nd
PCO	<i>P. chlororaphis</i> PA23	Canola oil	nd	nd	9.28	nd	47.40	nd	35.80	5.57	1.78
PHBV	<i>C. nector</i> H16	Glucose/Valerate	76.93	23.07	nd	nd	nd	nd	nd	nd	nd

HHx, 3-hydroxyhexanoate; HHp, 3-hydroxyheptanoate; HO, 3-hydroxyoctanoate; HD, 3-hydroxydecanoate; 3HDD, 3-hydroxydodecanoate; 3HTD, 3-hydroxytetradecanoate; 3HB, 3-hydroxybutyrate; 3HV, 3-hydroxyvalerate; nd, not detected.

Table 2. Growth of bacterial isolates on RMM-agar plates with 10 mg colloidal PHAs

Strain	*Zone of clearance (mm)				
	#PHHx	PHO	PHD	PHN	PCO
<i>P. chlororaphis</i> PA23-63-1	28.0 ± 2.1	11 ± 0.8	12.3 ± 2.4	25.6 ± 1.8	0
<i>A. lwoffii</i> 263AY1	25.6 ± 1.1	11 ± 0.9	11.6 ± 1.5	21.0 ± 2.4	0

*Zone of clearance was measured after 7 days of incubation; PHAs were produced from hexanoic acid (PHHx), octanoic acid (PHO), decanoic acid (PHD), nonanoic acid (PHN), and canola oil long chain fatty acids (PCO): Data are presented as the means of three replications with standard deviations.

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Table 3. Loss of weight in PHO and PHBV films after growth of *P. chlororaphis* and *A. lwoffii*.

Strain	% Change in PHO film weight	% Change in PHBV film weight
<i>P. chlororaphis</i>	4.5 ± 0.33	16.92 ± 1.28
<i>A. lwoffii</i>	5.10 ± 0.62	18.43 ± 2.94

Table 4. Lipase activities of *P. chlororaphis* and *A. lwoffii* grown in RMM with different PHA polymers as the sole carbon source for 48 hours

Strain	*Specific activity (U/mg protein)*		
	PHO	PHD	PCO
<i>P. chlororaphis</i>	722 ± 10.0	155 ± 5.0	88 ± 11.0
<i>A. lwoffii</i>	542 ± 3.0	127 ± 8.0	156 ± 5.0

*p-nitrophenyl-octanoate as substrate, mean of three replications

Table 5. Lipase activity in supernatants of *P. chlororaphis* and *A. lwoffii* cultures grown in RMM with PHO or PHBV polymers as the sole carbon, and assayed with different p-nitrophenyl-alkanoate substrates.

Substrate	Specific enzyme activity (U/min/mg protein)			
	PHO		PHBV	
	<i>P. chlororaphis</i>	<i>A. lwoffii</i>	<i>P. chlororaphis</i>	<i>A. lwoffii</i>
*pnp-acetate	684.6 ± 0.9	409.3 ± 0.2	1671 ± 0.3	2088 ± 0.4
pnp-butyrate	4579 ± 0.3	2342 ± 0.4	2495 ± 0.5	1029 ± 0.3
pnp-octanoate	997.7 ± 0.1	1376 ± 0.1	2871 ± 0.9	1467 ± 1.2
pnp-dodecanoate	500 ± 1.4	1647 ± 1.2	1210 ± 1.0	837.9 ± 0.9
PES	0	0	0	0
PCL	0	0	0	0

*pnp, p-nitrophenyl; PES polyethylene succinate; PCL, polycaprolactone

Table 6. Identification of hydrolysis products (monomers) of different PHAs after growth of *P. chlororaphis* and *A. lwoffii* cultures for 7 days.

Strain	Substrate	Monomer detected in supernatants			
		3-HHx	3-HO	3-HD	3-HDD
<i>P. chlororaphis</i>	PHO	-	+	-	-
<i>A. lwoffii</i>	PHO	+	+	-	+
<i>P. chlororaphis</i>	PHD	-	-	-	-
<i>A. lwoffii</i>	PHD	+	+	+	-
<i>P. chlororaphis</i>	PCO	-	+	+	-
<i>A. lwoffii</i>	PCO	-	-	-	-

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A

	10/30/2018 3:59:02 PM	det ETD	HV 5.00 kV	mag 100 x	WD 9.9 mm	HPW 2.07 mm	pressure 6.87e-4 Pa	spot 3.0	500 µm
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MIM University of Manitoba

B

	10/30/2018 4:00:17 PM	det ETD	HV 5.00 kV	mag 500 x	WD 9.9 mm	HPW 414 µm	pressure 6.43e-4 Pa	spot 3.0	100 µm
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MIM University of Manitoba

C

	10/30/2018 4:03:04 PM	det ETD	HV 5.00 kV	mag 5 000 x	WD 9.9 mm	HPW 41.4 µm	pressure 6.01e-4 Pa	spot 3.0	10 µm
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MIM University of Manitoba

10/30/2018 3:32:38 PM	det ETD	HV 5.00 kV	mag 100 x	WD 9.2 mm	HPW 2.07 mm	pressure 3.48e-3 Pa	spot 3.0	500 μm	MIM University of Manitoba
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10/30/2018 3:36:12 PM	det ETD	HV 5.00 kV	mag 500 x	WD 9.0 mm	HPW 414 μm	pressure 2.17e-3 Pa	spot 3.0	100 μm	MIM University of Manitoba
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10/30/2018 3:39:47 PM	det ETD	HV 5.00 kV	mag 5 000 x	WD 9.1 mm	HPW 41.4 μm	pressure 1.65e-3 Pa	spot 3.0	10 μm	MIM University of Manitoba
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A

B

C

PhaZ7p1	MISKLMGAQRFLPAVAATVTSLVWGLAGTLVAPGEAALCTGTSNGFVCKGTQTYAGGF	60
F924_03409	-----MKKII-----YHLNLLLLSVIWTGPVQAAALQQYK-----	30
F924_03408	-MH-----MISKLMGAQRFLPAVAATVTSLVWGLAGTLVAPGEAALCTGTSNGFVCKGTQTYAGGF	30
F924_03410	-----MKFGILL-----IGLLLA-GTCLSTQASTQASQV-----	28
	Oxyanion pocket*	..:
PhaZ7p1	APGVGYGGGGGSGCTATKTPVIFIHGNDNAISFDMPPGNVSGYGTPARSVYAEKARGY	120
F924_03409	-----SNFVLSSYARTKYPMVFVHGFLGFNRIGTESIGLD-----YWYQIPQDLSS	77
F924_03408	-----IEKRSQDYAKTKYPILMVHGWLGSRIQGTDSIGLD-----YWYQILPDMAR	82
F924_03410	-----TAKASSQYAKTQYPIVFAHSM-AGFIRIGTDQFGLD-----YWYQILPDLAR	74
	.. : *: *::: *. . :. :..	
	Lipase box	
PhaZ7p1	NDCEIFGVTY--LSSSEQGSAYQNYHSSTKYAIKFTIDKVKAYTGKSVQDVAHSMGVS	178
F924_03409	HGAWVFSAEALSAVASN-----ELRGEQLLMQIDEVLALTGHKKNVNLIGHSGGGP	126
F924_03408	NGSTVFAAQLSPANIT-----AHRGEQLVQQVEDVLAITGKKKLNLIIGHSHGGP	131
F924_03410	NGGNVNAVTRVSPFNSS-----EIRGEQLLEQVNEILAITGAKKNVNLIGHSHGGP	123
	:. :.. :. : : : :. : * * * : : : : * * *	
PhaZ7p1	MSLATLQYNNWTS--VRKFINLAGGIRGLYSCTYTGAYANAAAPTCSGQNYNSYTFGFF	236
F924_03409	TI---QYIEGITHPKAASLTATAGAMQGTVPFVNAG-----QTPLEPFI	168
F924_03408	TV---LYVAATQPQYIASITGVAGTYHGSKVADDIQ-----NNSLTRTAF	173
F924_03410	TI---RYVAGAGNGIVASLTSGVAPHKGSVPVGDIL-----QAE-----	159
	* : :. : *	
PhaZ7p1	PEGWYGVVWSNPWTGSGSTNS----MRDMPAKRTAVSFY-----TLSAGFKDQVG	283
F924_03409	Q---AIGTIL-----GHSMLNV-----TGNQYPSVMIEALQAMNPEAIQAFNMKYG	211
F924_03408	N---ILDYIIGPLIALGQF-----KP-ELEIDFDSMKSLTQTGNTFNATVA	218
F924_03410	-----GTPIQGPLVGGINLVSKAITWAQGLDPNSFPHDSLAGGSSLTLAGSAQFNQKYP	213
	* : . *	
PhaZ7p1	CAT-----ASFWAGCDSAAKFSTTSNVKAQINVGAGSNATQADYDWAAGMPYNAGGGDT	338
F924_03409	S-----SAIPKDCQSQGS-----KLTPNGIYHYSWMGN-----RQA	242
F924_03408	QQVVKDGVLASTENCNKNLK-----QKDSKGIHYSWTGV-----AQA	256
F924_03410	L-----GMPTTACGEG-----KEKGDGIRHYSFTGI-----GVV	242
	* . *: . :.	
PhaZ7p1	TNGV-----GHFRKTNTGAIIRMLLTCTGL-----	366
F924_03409	TNSLDVIESTVVTLGSSFLKGEANDGA-----LPLCSGRYGQIIRQDYAHNLFDEVNQF	296
F924_03408	TNALDID-TILMQQLGPLSYGSKDNDGM-----VSRCSAFMGKVIHDQYKLNHTDLANMM	309
F924_03410	TNPLDPD-SALTVTALLIDGGKENDGL-----VSRCSAFGKGTIRDNYNWNLFDEVNQF	295
	** : : * * : * :	
PhaZ7p1	-----DEAAEYTTGPIKAT-----	380
F924_03409	FGILGPFAQDPVALYRQHANRLKLQGL	323
F924_03408	FGLGKMFAPDPVALYRQHANRLKLEGL	336
F924_03410	LGLKNIFAPDPVDVYRQHANRLKLQGL	322
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