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ORIGINAL PAPER

The influence of nitrogen limitation on mcl-PHA synthesis by two newly isolated strains of *Pseudomonas* sp.

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Abstract The nucleotide composition of key enzymes involved in medium-chain-length polyhydroxyalkanoates (mcl-PHA) synthesis was analyzed in two newly isolated strains of *Pseudomonas*. The isolated strains were tested for their abilities to synthesize polyhydroxyalkanoates using three different substrates as a carbon source: sodium octanoate, oleic acid, and sodium gluconate. Both analyzed strains were able to accumulate mcl-PHA in a range from 2.07 to 21.40%, which depended on the substrate used. Potential nitrogen-dependent regulation of mcl-PHA synthesis was analyzed by cell cultivation in nitrogen-limiting and non-limiting conditions. The analyzed strains demonstrated an incremental increase of mcl-PHAs in response to nitrogen starvation when oleic acid and sodium gluconate were applied as the carbon source. The transcriptional analysis showed that the induction of gene coding for PHA synthases was correlated with an increment in mcl-PHAs content. Both analyzed strains revealed differences in terms of the studied gene's expression, showing a dependence on the carbon source used.

Keywords mcl-PHA · Polyesters · *Pseudomonas* sp. · Real-time PCR · Reverse transcription

Introduction

Polyhydroxyalkanoates (PHAs) are biodegradable polyesters that are accumulated by numerous bacterial species in the form of intracellular granules and that serve as reserves of energy [30]. PHAs have attracted extensive commercial interest because of their inherent biocompatibility and biodegradability [33]. These bacterial polyesters have been generally divided into two groups according to their structures: one group is represented by short-chain-length PHA (scl-PHAs) consisting of 3–5 carbon atoms, whereas the other group is medium-chain-length PHAs (mcl-PHAs) consisting of 6–14 carbon atoms [15]. In particular, mcl-PHAs show great promise as thermoelastomers for biomedical applications, such as wound management, drug delivery and tissue engineering [28]. However, to date, process development for the production of mcl-PHAs has been much less extensive than for scl-PHAs [29].

Although PHAs synthesis and accumulation is quite common in the bacterial world, mcl-PHAs production is typical mainly for the *Pseudomonas* species. The organization of genes involved in mcl-PHAs synthesis in pseudomonads is already known. The PHA biosynthesis gene cluster of *Pseudomonas* species comprises four genes encoding two PHA synthases (*phaC1* and *phaC2*), the intracellular depolymerase PhaZ and the proposed structural protein PhaD. Another gene cluster, containing the genes encoding the PHA granule-associated proteins *phaF* and *phaI*, proposed to be involved in PHA biosynthesis regulation, is located downstream of the PHA synthesis gene cluster and transcribed in the opposite direction [4]. An important role is played by the *phaG* gene that encodes the transacylase, which is essential for PHA synthesis from non-related carbon sources. *PhaG* is not co-localized with the previously mentioned genes [14].

It is believed that PHAs are produced by bacteria when the environmental conditions are not optimal for growing due to the limitation of a required nutrient and an excess

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of a carbon source. Nitrogen or phosphate limitation stimulates rapid PHA synthesis in most of the well-studied scl-PHAs synthesizing bacteria, such as *Wautersia eutropha* [28]. In the case of this species, it has been clearly demonstrated that the rate of PHA synthesis is greatly increased when nutrients such as nitrogen are in short supply [29]. Due to an assumption that mcl-PHAs production conditions must be similar to those of scl-PHAs accumulating bacteria, almost all experiments dealing with mcl-PHAs production have employed nitrogen or phosphate limitation [28, 29]. The role of nitrogen availability in the regulation of mcl-PHAs synthesis in *Pseudomonas* was studied by many authors, but the obtained results are debatable. According to Ramsay et al. [23, 24], mcl-PHAs synthesis by *Pseudomonas resinovorans* is greatly stimulated by nitrogen-limitation, but it was not observed in *P. oleovorans*. In various *P. putida* strains, the limitation of nitrogen positively influenced PHAs content [28]. Hoffmann and Rehm [13] showed the impact of substrate type on nitrogen regulation of mcl-PHAs synthesis in *P. putida*. In this species, nitrogen limitation was necessary when gluconate, as a carbon source, was applied, whereas *P. putida* cells accumulate mcl-PHAs in nitrogen excess when octanoate was used. Most probably, the impact of nitrogen limitation in *Pseudomonas* species depends on the microbial strain as well as on the form of substrate that is used as a carbon source.

The aim of this work was to characterize the nucleotide composition of *phaC1/phaZ/phaC2* genes of two newly isolated strains of *Pseudomonas* sp. These strains' physiological ability to produce mcl-PHAs production was tested by cultivation in medium supplemented with three forms of substrates as carbon sources. Potential nitrogen-dependent regulation of mcl-PHAs synthesis was analyzed by cultivating the cells in nitrogen-limiting and non-limiting conditions. The influence of nitrogen limitation on *phaC1/phaC2* genes' expression was also analyzed.

Materials and methods

Microorganisms

Pseudomonas sp. Gl01 and Gl06 strains were originally isolated from mixed microbial communities utilized for PHAs synthesis. The identification of strains was performed by 16S rRNA gene analysis, and DNA sequences are deposited in GenBank under accessions numbers: EU693921 and EU693923 for the Gl01 and Gl06 strain, respectively. Both strains were recognized as potential mcl-PHAs producers using PCR-based detection of *phaC1/phaC2* genes (data not published).

PHAs synthesis

Cultivation experiments were conducted as triplicates, and mean values and standard deviation are provided. Cells were preinoculated in Luria-Bertani medium, and then 5 ml of inoculum was transferred to 95 ml of E medium, containing per liter: 3.5 g of $Na_2HPO_4{\cdot}12H_20$, 0.7 g of KH_2PO_4, 0.8 g of $MgSO_4{\cdot}7H_2O$, and 5 g of $(NH_4)_2SO_4$. Nitrogen limiting conditions (N−) were created by lowering the amount of $(NH_4)_2SO_4$ to 1 g/l. Medium contains 1% of trace element solution that consists of 0.3 g of H_3BO_3, 0.2 g of $CoCl_2{\cdot}6H_2O$, 0.1 g of $ZnSO_4{\cdot}7H_2O$, 0.03 g of $MnCl_2{\cdot}4H_2O$, 0.03 g of $NaMoO_4{\cdot}2H_2O$, 0.028 g of $NiSO_4{\cdot}7H_2O$, 0.01 g of $CuSO_4{\cdot}5H_2O$ per liter. Medium was supplemented with sodium gluconate (20 g/l), oleic acid (10 g/l), and sodium octanoate (10 g/l) as carbon sources. Flasks were incubated in an orbital shaker (220 rpm) at 30°C. The pH was kept in a range from 6.5 to 7.0 using 1 N NaOH. After 48 h the cells from each culture were centrifuged and lyophilized for PHAs extraction; 5 ml of each culture was conserved in RNAlater (SIGMA) for RNA stabilization. Differences in biomass amount and PHAs content in bacterial cells were tested for significance using Student's *t* test employing Statistica software (StatSoft Inc., USA).

Analysis of nitrogen in the growth medium and culture

For measuring the residual concentration of nitrogen (as ammonium ion) in the culture supernatant, the culture broth was spun down for 15 min (11,200*g*). The ammonium ion concentration of the growth medium and the culture supernatant was measured according to the titrimetric method with the preliminary distillation step described in Standard Methods [2] with modification, which relied on applying 0.01 N HCl instead of 0.02 N H_2SO_4.

Extraction and analysis of PHAs

PHAs were extracted according to the dispersion method described by Hahn et al. [10]. Freeze-dried cells were recovered from the pellets and ground, and the resulting powder was extracted by vigorous shaking with a mixture of chloroform and 6% sodium hypochlorite (1:1, v/v) for 30 min at 250 rpm. After centrifugation for 15 min (11,200*g*), the chloroform phase was filtered through Whatman no. 1 filter paper, and the chloroform was evaporated from the filtrate to give the crude polymer. The crude polymer was dissolved in a small volume of chloroform and reprecipitated by drop-wise addition to cold methanol. The polymer was dried for 24 h under vacuum at room temperature. The PHA content was calculated in terms of

percentage of PHAs weight over cell-dry-weight (CDW). Samples for gas chromatography analysis were prepared accordingly to Braunegg et al. [3]. One milligram of extracted polymer was transferred into a 10-ml glass tube. Two milliliters of acidified methanol (3% H_2SO_4, v/v) and an equal volume of chloroform were added. The tube was placed in the oven for 20 h at 100°C temperature in order to perform esterification. Afterwards 1 ml of distillated water was added, samples were vigorously shaken, and after phase separation, the upper phase was discarded and the organic phase was dried using sodium sulfate. Obtained methyl esters were determined by gas chromatography according to the method described by Furrer et al. [9]. One microlitre of the ester solution was analyzed on a GC (GC Varian CP-3800) equipped with a capillary column Varian VF-5 ms (30 m × 0.25 mm) with a film thickness of 0.25 µm (Varian, Lake Forest, USA) at a split ratio of 1:10 and an initial temperature of 80° C. The temperature was raised with a rate of 10°C min^{-1} to 240°C. For quantification purposes by FID, known amounts of pure 3-hydroxyhexanoic, -octanoic, -nonanoic, -decanoic, -undecanoic and -dodecanoic acid were derivatised and measured in the same way as analyzed samples, and measured to calculate their response factors. 3-Hydroxy acids (purity 98%) were obtained from Larodan Fine Chemicals (Sweden).

DNA extraction, amplification and sequencing

Genomic DNA was isolated as follows: 1 ml of sample was pelleted by centrifugation (16,100*g*), suspended in 200 µl of proteinase K buffer (100 mM Tris–HCl; 10 mM EDTA; pH 8.0) and incubated at 55°C in the presence of sodium dodecyl sulphate (0.5%), proteinase K (200 µg/ml) and lysozyme (2 mg/ml). DNA was purified using phenol/chloroform/isoamyl alcohol (25/24/1, v/v/v) solution and precipitated with two volumes of ethanol (96%) and 0.1 volume of sodium acetate (pH 5.2). The pellet was washed with 70% ethanol, dried and re-suspended in 100 µl of TRIS/EDTA (10 mM Tris–HCl,1 mM EDTA, pH 7.5). With the aim of sequencing PHA biosynthesis operon containing *phaC1*, *phaZ* and *phaC2* genes chromosomal-walking method was applied. *Orf1*, *orf2* and *phaD* PCR primers, adjacent to each end of the *phaC1* and *phaC2* genes, were used (Table 1). The rest of PCR primers were synthesized on the base of already sequenced fragments and DNA sequences available in GenBank (NCBI, http://www.ncbi.nlm.nih.gov).

PCR was performed in Eppendorf® Mastercycler Gradient (Eppendorf, Germany). The mixtures used for PCR amplification contained 50 ng of extracted total DNA, 0.5 µM of each primer, 100 µM of deoxynucleoside triphosphate (Promega, Winsconsin, USA), 1 U of *Taq* DNA polymerase (Invitrogen, Life Technologies), 5 µl reaction buffer (500 mM KCl, pH 8.5; Triton X-100), 1.5 mM $MgCl_2$ and sterile water to a final volume of 50 µl. The temperature program for DNA amplification was as follows: 94°C for 5 min; 30 cycles of denaturation at 94°C for 30 s, annealing at 50–55°C for 45 s, extension at 72°C for 1 min and single final elongation at 72°C for 10 min. The PCR amplicons of PHA were checked by resolving on 1.5% agarose gels stained with ethidium bromide. Sequencing reactions were carried out with an ABI 373 Automated DNA Sequencer (PE Applied Biosystems, Foster City, CA, USA).

The sequences of PHA operon (*phaC1*/*phaZ*/*phaC2*) were compared with those from the GenBank database using the NCBI blast program [1]. DNA sequences were aligned using the ClustalW program [31]. Distance trees were estimated according to the neighbor-joining method of Saitou and Nei [25] with the Mega 2.1 software [18]. To determine the degree of statistical support for branches in the phylogeny, 1,000 bootstrap replicates of data were analyzed. The open reading frames were predicted using the on-line ORF Finder tool (NCBI, http://www.ncbi.nlm.nih.gov). DNA sequences were deposited in Genbank under accession numbers: FJ214728 and FJ214729.

Total RNA extraction and purification

All reagents and processes were made in/with sterile, disposable, nuclease-free laboratory ware. For RNA extraction, the cells had been stored in RNAlater (SIGMA) to maintain the quality of the extracted RNA. RNA was extracted from approximately 0.02 g of semi-dry weight of the cell pellet using a commercial RNA isolation kit (Total RNA, A&A Biotechnology, Poland) according to the manufacturer's instructions. To eliminate contaminating genomic DNAs, 3 U of RQ1 RNase-Free DNase (Promega, USA) was added to each RNA extract before incubation at 37°C for 30 min. The quantity of purified RNA was measured using Qubit fluorometer (Invitrogen, Carlsbad, NM) with Quant-iT™ RiboGreen® RNA Reagent and Kit application.

Reverse transcription real-time PCR

The reverse transcription (RT) reaction was carried out using the RevertAid First Strand cDNA Synthesis Kit (MBI Fermentas) in total volume of 40 µl. Total RNA in an amount of 1.0 µg and 2 µl of random hexamer primer (0.2 µg $µl^{-1}$) was added to distilled water. After that, the mixture was incubated at 70°C for 10 min to denature secondary structures of rRNA, the tube was placed on ice, and the following mixture was added: 8 µl of 5× reaction buffer [250 mM Tris/HCl (pH 8.3 at 25°C), 250 mM KCl, $MgCl_2$, 50 mM dithiothreitol (DTT)], 2 µl of ribonuclease inhibitor (20 U/µl) and 2 µl of 10 mM dNTP mix. The mixture was incubated at 25°C for 5 min to allow the

Table 1 PCR primers used in this study

PCR primer	Nucleotide sequence	Amplicon/strain	Purpose	Reference
GlC1	5′ aag gtc aac gcc ctg acc ctg ctg gt 3′	*phaC1*	Real-time PCR	This study
179R	5′ ggt gtt gtc gtt gtt cca gta gag gat gtc 3′	Gl01 and Gl06		[26]
GlC2R	5′ gt tgatctg(at)ggcggcacga(ct)cagca 3′	*phaC2*	Real-time PCR	This study
380L	5′ tca agg aac tgt tca act ccg g 3′	Gl01 and Gl06		
341F	5′ cct acg gga ggc agc ag 3′	*16S rRNA*	Real-time PCR	[21]
515R	5′ aat ccg cgg ctg gca 3′	Gl01 and Gl06		
orf1	5′ agc tgg caa gtt ccc tgc ag 3′	*phaC1/phaZ/phaC*	DNA sequencing	[6]
phaD	5′ gtc gcg ggt ttt cat cca g 3′	Gl01 and Gl06		
orf2	5′ cat gac agc ggc ctg ttc acc tgg 3′	*phaC1/phaZ/phaC*	DNA sequencing	This study
phaD	5′ gtc gcg ggt ttt cat cca g 3′	Gl01 and Gl06		[6]

annealing of the random hexamer primers. After adding 2.0 µl of RevertAid M-MuLV, reverse transcriptase (200 U/µl) reaction was carried out at 25°C for 10 min and at 42°C for additional 60 min.

The details of the PCR primers used for real-time amplification of *phaC1, phaC2* and *16S rRNA* genes are given in Table 1. The specificity of each primers pair was controlled by melt curves analysis. Reactions were performed using POWER SYBR green master mix (Applied Biosystems) in 25 µl final volume. Primers were used at a final concentration of 50 nM per reaction. Templates were either 3.0 µl of cDNA per reaction or water in NTC (no template control) in this same volume. Thermal cycling conditions were designated as follows: initial denaturation at 95°C for 3 min, followed by 40 cycles of 95°C for 15 s and 60°C for 30 s. Fluorescence measurement was recorded during each annealing step. An additional step starting from 90 to 60°C (0.05°C/s) was performed to establish a melting curve of each primer pairs. This allowed for the verification the specificity of PCR reaction. Real-time PCR was performed using GeneAmp 5700 Sequence Detection System (Applied Biosystems).

Gene expression was presented using a modification of the $2^{-\Delta\Delta Ct}$ method, described by Livak and Schmittgen [20]. Reaction was normalized by determining the amounts of RNA added to cDNA reaction. To examine the intrasample variation, the ±SD was determined from three sample replicates. As the endogenous control expression of 16S rRNA was applied. The expression of each gene was presented as $2^{-\Delta Ct}$, where $\Delta C_t = (C_{t\ N-} - C_{t\ N+})$.

Results

Genetic characterization and phylogenetic analysis

In this study, two newly isolated *Pseudomonas* strains were genetically characterized by analysis of their biosynthesis operon consisting of *phaC1/phaZ/phaC2* genes. The genome DNA walking approach was successfully employed to obtain the nucleotide sequence of both strains. Several semi-nested gene-specific primers were constructed on the basis of newly sequenced flanking regions and homologous DNA sequences deposited in GenBank (NCBI). As the results, two regions composed of 4,469 (*Pseudomonas* sp. Gl01) and 5,029 nucleotides (*Pseudomonas* sp. Gl06) were obtained. The sequenced region of both strains contains whole genes coding for PhaC1, PhaZ and PhaC2. Analysis of nucleotide sequences provided the details concerning the location and length of genes creating this locus. *PhaC1* and *phaC2* genes coding for PHA synthases were of the same length as homologous genes of other pseudomonads, 1,680 and 1,683 bp, respectively. The length of gene coding for PhaZ was different in studied strains, and *phaZ* gene consists of 852 (Gl01) and 858 (Gl06) bp. The size of the *pha* operon counted from the first nucleotide position of *phaC1* gene to the last nucleotide position of *phaC2* gene were 4,326 (Gl01strain) and 4,591 bp (Gl06 strain). The observed length deviation released mainly from the differences in lengths of intergenic fragments, which were significantly longer in the Gl06 strain—142 bp in *phaC1/phaZ* region and 228 bp in *phaZ/phaC2* region; in the Gl01 strain, the lengths of these regions were 62 and 49 bp, respectively. The analysis of intergenic regions nucleotide compositions revealed unexpectedly high content of cytosine (38%) in the *phaC1/phaZ* intergenic region of the Gl06 strain. Additional sequence analysis revealed the presence of putative ribosome-binding sequences (RBSs) located upstream of start codons of *phaC1*, *phaZ* and *phaC2* genes in both strains. In the Gl01 strain, putative promoter exhibiting homology to σ^{54}-dependent promoter of *E. coli* was founded upstream of *phaC1* gene. In the case of Gl06 strain, homologous promoters were found upstream of all three genes. Features of putative RBS and promoters are given in Table 2.

Table 2 Details of putative promoters and ribosome binding sites (RBSs) discovered in *phaC1/phaZ/phaC2* operon of *Pseudomonas* sp. Gl01 and Gl06 strains

Strain	Gene	Structure of putative σ^{54}-dependent promoter	Location of putative promoters[a]	Structure of putative RBSs
Gl01	*phaC1*	**CTGG**TC-N_7-TA**GC**A	69	GGAG
	phaZ	Not found	–	AGAG
	phaC2	Not found	–	GGAG
Gl06	*phaC1*	**CTGG**CA-N_6-CT**GC**A	129	GGAG
	phaZ	**CTGG**TA-N_7-AG**GC**G	47	GGGG
	phaC2	**CTGG**AA-N_8-CT**GC**G	130	GGAG

[a] Location of putative promoters is given in base pairs measured from start codon of genes

The obtained DNA sequences of *phaC1/phaZ/phaC2* genes were aligned with each other and with homologous sequences derived from GenBank, and a phylogenetic analysis was carried out. The phylogenetic relationship shown in the form of a neighbour-joining tree is presented in Fig. 1. *Pseudomonas* sp. Gl01 was clustered within *P. putida* group with the highest identity to *P. putida* F1 (99.2%). *Pseudomonas* sp. Gl06 was positioned within a cluster created by *P. pseudoalcaligenes*, *P. nitroreducens* and *P. mendocina*, with the highest identity to *Pseudomonas* sp. USM4-55 (90.5%) and to *P. mendocina* (90.1%). The similarity between the analyzed Gl01 and Gl06 strains was relatively low with identity at a level of 75.6%, which suggests that they belonged to a different species.

Production and characterization of PHAs

In order to recognize whether nitrogen presence influences the PHAs accumulation, one-stage cultivation was conducted in two variants. In the first one, the applied medium contains 0.212 g of nitrogen per liter, whereas in the second variant, the nitrogen was provided at a concentration of 1.06 g per liter. After 48 h of cultivation, the nitrogen in the growth medium was completely depleted in the first variant, whereas in the second variant it was reduced to an average value of 0.51 g/l (Table 3). Thus, it was stated that nitrogen-limiting (N−) and nitrogen non-limiting (N+) conditions were implemented.

The details of PHAs production by both studied strains are given in Table 3. After 48 h of growth, the total CDWs ranged from 1.0 to 3.1 g/l depending on the bacterial strain and the applied carbon sources. The highest CDW values were reached when both strains were cultivated in a medium supplemented with oleic acid (from 2.43 to 3.1 g/l). Generally, both strains achieved the lowest cell concentration when sodium octanoate was applied as the carbon source. Nitrogen content did not significantly influence CDW values ($p < 0.05$); there was also a lack of significant differences ($p < 0.05$) between biomass weights of analyzed strains.

Under all applied experimental conditions, both strains of *Pseudomonas* accumulated PHAs. The highest amount of PHAs was accumulated by *Pseudomonas* sp. Gl01 when it was grown on oleic acid under nitrogen limiting conditions (21.4% of CDW). Contrarily, the lowest PHAs concentration (2.07% of CDW) was noticed when sodium gluconate was applied. This substrate was also worst for PHA production by the Gl06 strain; in this case the concentration of PHAs was at the level of 2.30% of CDW. Similarly to Gl01 strain, oleic acid was the most efficient carbon source for the PHAs synthesis by Gl06 strain (16.20% of CDW). The concentrations of PHAs produced by both strains cultivated in a medium supplemented with sodium octanoate were similar (from 8.81 to 11.67% of CDW). The influence of nitrogen starvation on PHAs concentration was noticed only when oleic acid (*Pseudomonas* sp. Gl01) and sodium gluconate (*Pseudomonas* sp. Gl06) were used as carbon sources. Generally, the percentages of PHA accumulation in both strains were slightly different, but this difference was not statistically significant ($p < 0.05$).

The results show that the major repeat units of mcl-PHA produced by both strains on sodium octanoate are 3-hydroxyoctanoate and 3-hydroxyhexanoate; additionally trace amounts of 3-hydroxydecanoate were denoted. With oleic acid as a feedstock, the most predominant repeat units of the biopolymer were 3-hydroxyoctanoate and 3-hydroxydecanoate; moreover 3-hydroxyhexanoate, 3-hydroxynonanoate, 3-hydroxyundecanoate and 3-hydroxydodecanoate were found in lower amounts. The composition of PHAs synthesized by both strains from sodium gluconate was found to consist mainly of 3-hydroxyoctanoate and 3-hydroxydecanoate. A low fraction of 3-hydroxydodecanoate was noted when cells were cultured in nitrogen-limited conditions. Additionally, 3-hydroxynonanoate was produced by *Pseudomonas* sp. Gl01 cultured in low nitrogen conditions. What is interesting, in the case of sodium gluconate, is that the difference in proportions of 3-hydroxyoctanoate and 3-hydroxydecanoate were observed in nitrogen-limiting and non-limiting conditions. In the case of

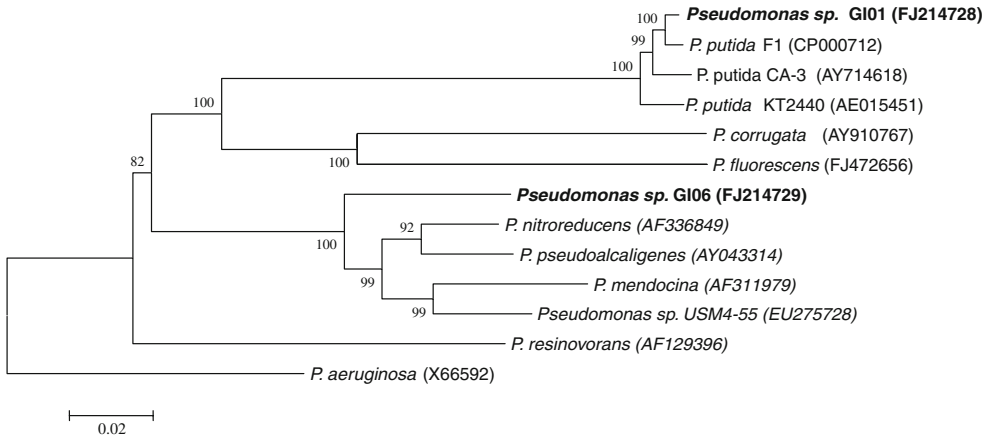

Fig. 1 Phylogenetic relationships of analyzed *Pseudomonas* sp. Gl01 and Gl06 strains based on *pha* operon (*phaC1*/*phaZ*/*phaC2*) DNA sequences. The tree was constructed using the neighbor-joining algorithm. Numbers adjacent to branch points are bootstrap percentages ($n = 1{,}000$ replicates). Analysis was performed by including nucleic acid gene sequences deposited in GenBank (accession numbers are indicated in *parentheses*). *Bar* 0.02 estimated substitution per sequence position

both strains, the amount of 3-hydroxydecanoate was significantly higher than 3-hydroxyoctanoate in nitrogen-limiting conditions, whereas in medium with nitrogen excess, the amounts of 3-hydroxydecanoate were slightly lower than 3-hydroxyoctanoate. This observation could suggest that monomer composition depends on nitrogen concentration in the medium. Moreover, in the Gl06 strain, there were more different monomers when cells were growing in medium supplemented with sodium gluconate in nitrogen limitation. There, the presence of 3-hydroxydodecanoate was noticed. Additionally, changes in monomer proportion were observed in this case (Table 3).

Relative gene expression

The effect of nitrogen limitation on the expression of *phaC1* and *phaC2* genes was investigated using reverse transcriptase real-time PCR. The applied PCR primers were developed to specifically bind to DNA sequences of target genes, and this specificity was controlled by melt curve analysis. The fold change in the target genes in nitrogen-limiting conditions relative to the expression of these same genes in nitrogen non-limiting conditions was calculated for each sample. The mathematical analysis was performed separately for each gene because the effectiveness of their amplification was different.

The results in Fig. 2 show that the *phaC1* and *phaC2* transcripts changed in different ways as a result of growing bacterial cells in unfavorable conditions. The transcripts of the Gl01 strain exhibit significant upregulation when cells were growing in medium supplemented with oleic acid. In this case, the genes expression increased 8.2 (*phaC1*) and 10.7 (*phaC2*) fold in comparison to their expression in nitrogen excess.

A different observation was made when the Gl06 strain was analyzed. Visible change of the *phaC1* gene transcript number was noticed when cells were cultured with sodium gluconate (4.7 folds); at the same time, expression of the *phaC2* gene was not altered.

The changes of *phaC1*/*phaC2* genes activity in the remaining variants were relatively low in some cases showing a decrease in the activity of the studied genes under nitrogen-limiting conditions (Fig. 2). The results of relative gene expression were correlated with values of PHAs content changes (Table 3). The increased PHA content in the Gl01 strain growing on oleic acid was correlated with the increments of both genes expression. The high induction of the *phaC1* gene of Gl06 strain cultured in a medium supplemented with sodium gluconate in nitrogen-limiting conditions was reflected in an increase of PHAs content.

Discussion

In this study, two newly isolated strains of pseudomonads were tested in the direction of mcl-PHA production. These strains were isolated from mixed culture selected towards PHA production (not published); thus, we assumed that they posses adequate physiological potential for PHAs synthesis. Here, the nucleotide composition of the whole *phaC1*/*phaZ*/*phaC1* operon is provided and analyzed. The phylogenetic analysis, based on DNA sequences of these genes, revealed that one of these strains (Gl01) exhibited the highest similarity to *P. putida* F1. The second one, Gl06, was clustered with *P. pseudoalcaligenes*, *P. nitroreducens* and *P. mendocina*, with identity at an average level of 90%. The performed phylogenetic study was not

Table 3 Accumulation and composition of PHA produced by *Pseudomonas* sp. Gl01 and Gl06 when grown in mineral medium with different carbon sources for 48 h at 30° C

Strain	Conditions/ N content (g/l)	Carbon source	CDW (g/l)	PHAs content (wt%)	PHAs monomeric composition (mol%)				
					3HHx	3HO	3HN	3HD	3HUD	3HDD
Gl01	N−/0.00	Sodium octanoate	1.00 ± 0.17	9.77 ± 0.78	14.1 ± 1.26	84.5 ± 0.94	nd	1.4 ± 0.06	nd	nd
	N+/0.63	Sodium octanoate	2.03 ± 0.64	11.67 ± 3.96	13.37 ± 1.19	84.08 ± 0.38	nd	2.55 ± 0.87	nd	nd
	N−/0.00	Oleic acid	2.56 ± 0.30	21.40 ± 0.80	8.35 ± 0.71	42.77 ± 4.11	1.99 ± 0.22	35.42 ± 1.61	1.65 ± 0.56	9.82 ± 1.26
	N+/0.58	Oleic acid	2.43 ± 0.15	13.17 ± 1.95	9.72 ± 0.55	45.69 ± 1.8	nd	35.87 ± 0.77	nd	8.72 ± 0.89
	N−/0.00	Sodium gluconate	2.46 ± 0.30	2.20 ± 0.35	nd	17.95 ± 5.41	4.28 ± 0.34	71.74 ± 3.33	nd	6.03 ± 1.73
	N+/0.39	Sodium gluconate	2.63 ± 0.28	2.07 ± 0.81	nd	56.45 ± 13.1	nd	43.55 ± 13.1	nd	nd
Gl06	N−/0.00	Sodium octanoate	1.50 ± 0.26	10.97 ± 2.27	13.77 ± 0.78	84.21 ± 1.68	nd	2.02 ± 1.0	nd	nd
	N+/0.59	Sodium octanoate	1.93 ± 0.23	8.81 ± 1.59	14.43 ± 0.84	83.88 ± 0.53	nd	1.69 ± 0.79	nd	nd
	N−/0.00	Oleic acid	3.10 ± 0.10	16.20 ± 1.56	7.1 ± 1.41	32.25 ± 6.3	2.55 ± 0.53	46.39 ± 6.13	nd	11.71 ± 1.86
	N+/0.54	Oleic acid	2.93 ± 0.90	16.17 ± 1.72	7.55 ± 1.19	40.99 ± 4.54	2.1 ± 0.38	37.44 ± 5.06	2.19 ± 0.77	9.73 ± 0.94
	N−/0.00	Sodium gluconate	1.33 ± 0.15	3.83 ± 0.15	nd	23.18 ± 6.43	nd	67.72 ± 5.79	nd	9.1 ± 0.66
	N+/0.34	Sodium gluconate	2.56 ± 0.15	2.30 ± 0.40	nd	57.71 ± 5.79	nd	42.29 ± 5.79	nd	nd

Values are the averages of three independent cultures

N−, nitrogen limiting conditions; *N+*, nitrogen non limiting conditions; *nd*, not detected; *3HH*, 3-hydroxyhexanoate; *3HO*, 3-hydroxyoctanoate; *3HN*, 3-hydroxynonaoate; *3HD*, 3-hydroxydecanoate; *3HUD*, 3-hydroxyundecanoate; *3HDD*, 3-hydroxydodecanoate

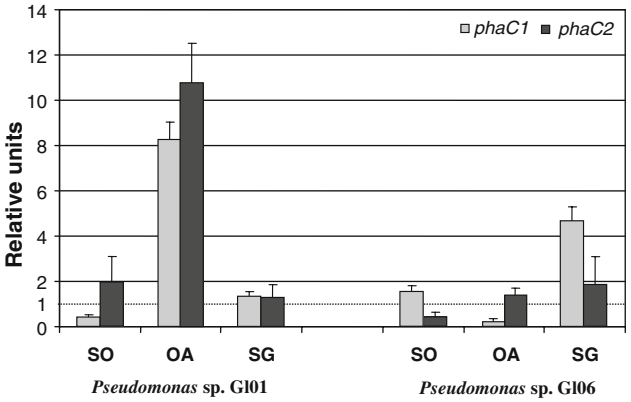

Fig. 2 Transcriptional analysis of *phaC1* and *phaC2* genes by real-time PCR of reverse transcribed total RNA. Results are reported as fold difference of the studied gene expression in nitrogen-limiting conditions relative to nitrogen non-limiting conditions. Mean values are calculated from triplicate samples; *error bars* represent standard deviation from the mean. The strains were grown on three substrates: sodium octanoate (SO), oleic acid (OA) and sodium gluconate (SG)

enough to exactly indicate the taxonomic position of this strain, but a high genetic similarity allowed us to expect that their physiological ability to mcl-PHA synthesis are comparable to the above-mentioned species. The length and nucleotide composition of the investigated genes do not have any unique features and are similar to the results described in previous papers [6, 32].

Although a nutrient limitation is commonly applied to the mcl-PHAs production process, some controversial results were reported. Limitation of nitrogen or phosphorus was shown to be stimulatory to the synthesis of PHA in *P. oleovorans* growing on *n*-alkanes, *P. resinovorans* and *P. putida* BM01 on octanoate [16] and *P. putida* KT2442 on oleic acid [19]. In contrast, it was demonstrated that nutrient limitation is unnecessary for PHA synthesis in *P. oleovorans* [8] and *P. putida* U [5] on octanoate. Most probably, the relation between PHA accumulation and growth conditions depends on the strain, carbon source and cultivation conditions, or a combination of these factors [28].

Processes involving complete nutrient depletion are usually two-stage fed-batch processes with PHA accumulation occurring mainly during the nutrient-depleted stage [29]. In this work, the nitrogen-limiting conditions were created by adding this nutrient in minimal concentrations, making them a growth-limiting factor. By comparing to cultivation cells in nitrogen excess conditions, we have been able to analyze the effect of nitrogen availability on PHA production by two taxonomically divergent species of *Pseudomonas*.

The analyzed strains were cultivated in flasks with sodium octanoate, oleic acid and sodium gluconate. Both strains showed comparable cell growth and a similar level

of PHA accumulation (Table 3). The highest content of PHAs was reached when a related substrate oleic acid or sodium octanoate was used. The PHA content was significantly lower when a non-related carbon source (sodium gluconate) was applied. The same pattern of substrate-dependent PHA accumulation has been previously reported by other authors. Solaiman et al. [27] growing *P. corrugata* on glucose and oleic acid noted that this species accumulated PHAs at a level of 2 and 24% of CDW, respectively. Results similar to ours were obtained in experiments described by Kim et al. [17] when various strains of *P. putida* were grown in medium supplemented with sodium gluconate and octanoate. In this work, the influence of nitrogen starvation was observed in the case of the Gl01 strain when oleic acid was used as the carbon source. The PHA content was 1.5 fold higher in nitrogen-limiting conditions. Moreover, the cells of the Gl06 strain showed nitrogen-dependent PHAs synthesis in medium supplemented with sodium gluconate in unfavorable conditions, the PHA concentration was 1.7 fold higher. Generally, differences in PHA content between the analyzed strains were not statistically significant.

The application of gas chromatography allowed us to obtain information about the monomer composition of mcl-PHAs accumulated under all experimental variants. The observed pattern of 3-hydroxyalkanoates incorporation was similar to those obtained by other authors when these same feedstocks were applied [7, 17, 27]. The possible influence of nitrogen availability on monomer composition when *Pseudomonas* cells are growing in medium supplemented with sodium gluconate could be interesting. Additional interesting information concerns the presence of small quantities of 3-hydroxynonanoate in cells of both strains when an even chain length substrate (oleic acid) was used. Although similar finding were described by other authors [22], the mechanism of odd chain length monomer synthesis from even chain length feedstock is still unclear.

Analysis of *phaC1* and *phaC2* gene transcription in two strains of *Pseudomonas* was performed in order to elucidate the induction of these genes' expression in response to nitrogen starvation. In the transcriptional analysis based on RT real-time PCR, two new pairs of PCR primers were successfully applied. The developed assay allowed us to estimate the relative level of *phaC1* and *phaC2* transcripts in both analyzed strains grown with three substrates as the carbon source. Using this approach, we demonstrated that nitrogen limitation affected the studied gene induction depending on the carbon source utilized for bacterial growth. A significantly higher expression of *phaC1* and *phaC2* genes in response to nitrogen starvation was observed when oleic acid was used during cultivation of the Gl01 strain. In Gl06 strain induction of only the *phaC1* gene was observed when this strain was fed with sodium gluconate. Thus, the co-transcription of *phaC1* and *phaC2* genes could be conjectured in the Gl01 strain, whereas independent transcription of these genes seems to be characteristic for the Gl06 strain. This could be affirmed by the fact that putative promoter in Gl01 strain was found only upstream of the *phaC1* gene. Moreover, the conclusion that PhaC1 and PhaC2 synthases work independently in the Gl06 strain could be supported by the observation concerning the differences in monomer composition, and their proportions resulted from application of sodium gluconate. A different contribution of each PHA synthase on this polyester synthesis in taxonomically similar species *P. mendocina* was previously proved [11]. The authors of this work revealed that PhaC1 is a major PHA synthase in this species, and PhaC2 synthase has a minor role in PHAs production with an unrelated carbon source. It should also be pointed out that there was a strong correlation between the induction of the *phaC1* gene and an increase in PHAs synthesis by the Gl06 strain grown in nitrogen starvation on sodium gluconate as a carbon source. This same relation was found when the Gl01 strain was grown on oleic acid.

The results obtained in this work seem to be very interesting in light of previously described works performed on *P. corrugata* [7, 27]. Conte et al. [7] showed that *phaC1* and *phaC2* genes in *P. corrugata* are not co-transcribed, and these genes are differentially regulated in a carbon source-dependent manner. In cultures grown with oleic acid, only expression of the *phaC1* gene was upregulated, whereas during cultivation in medium supplemented with glucose and sodium octanoate, both genes revealed expression at a similar level. A strong correlation between *phaC1*/*phaC2* genes and PHAs production was described, demonstrating that RT real-time PCR is a sensitive and reliable tool in studying PHAs biosynthesis at the molecular level. The results of the transcriptional analysis of *P. corrugata* described by Conte et al. [7] was confirmed in the work of Solaiman et al. [27] in which the function of the *phaC1-phaZ* intergenic region was investigated. The result of this work demonstrated that the phaC1-phaZ intergenic region is involved in *phaC1* and *phaC2* gene expression in a carbon source-dependent way. It played a regulatory function when cells of *P. corrugata* were grown on oleic acid, whereas when glucose was applied as the carbon source, it did not function as a transcription terminator. The results of the study of Solaiman et al. [27] showed for the first time the influence of the transcription terminator on PHAs biosynthesis. Analysis of the 224-bp-long DNA sequence of the *phaC1-phaZ* intergenic region revealed that it contains a strong hairpin structure suggesting that transcription of the *phaC1* gene is terminated in a Rho-independent manner. The second way of gene expression termination in prokaryotes is Rho-dependent termination, which contrary to Rho-independent

transcription needs a special protein (Rho) to stop the transcription. Rho-dependent terminators lack the obvious structural features that are associated with intrinsic terminators. Although several Rho-dependent terminators have been identified, their sequences do not conform to an obvious consensus. In general, they consist of a bipartite structure that extends for up to 150 bp of DNA and have a high proportion of C relative to G residues [12]. According to our analysis, the lengths of the *phaC1/phaZ* intergenic regions were 62 bp in the Gl01 strain and 142 bp in the Gl06 strain; additionally in the case of the Gl06 strain, this region contains a relatively high concentration of cytosine (38%). The discovered features of this region in conjunction with the results related to gene expression as well as monomer composition suggest that *Pseudomonas* sp. Gl06 could have Rho-dependent termination of the *phaC1* gene. In consequence, *phaC1* and *phaC2* genes in the Gl06 strain can be expressed independently, contrary to the Gl01 strain in which the *phaC1* gene is most probably co-transcribed with the *phaC2* gene. The intriguing carbon source-dependent expression of *phaC1* and *phaC2* genes is still unclear and needs further investigation.

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