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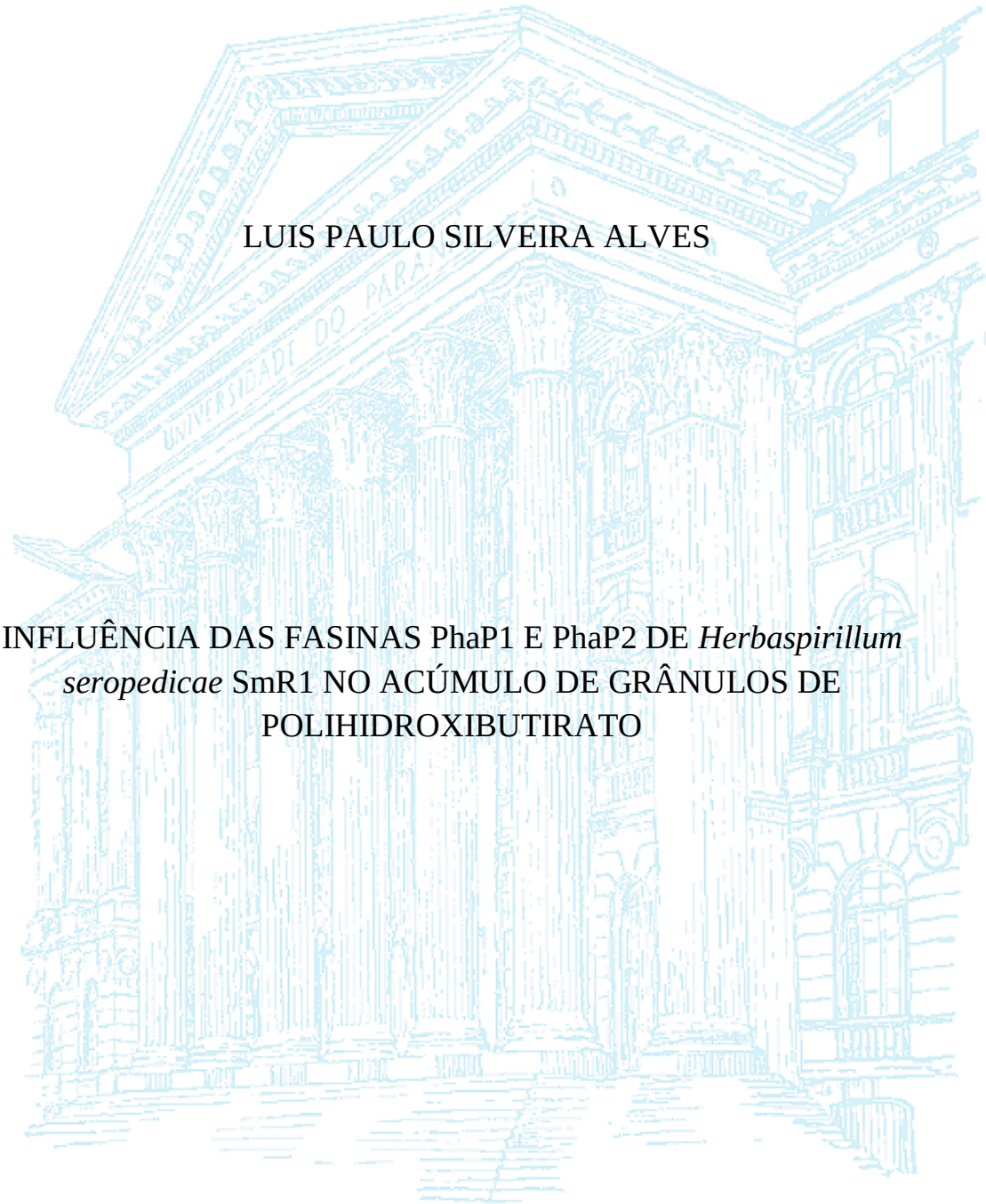

LUIS PAULO SILVEIRA ALVES

INFLUÊNCIA DAS FASINAS PhaP1 E PhaP2 DE *Herbaspirillum seropedicae* SmR1 NO ACÚMULO DE GRÂNULOS DE POLIHIDROXIBUTIRATO

CURITIBA

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Dissertação apresentada como requisito parcial para obtenção do título de Mestre em Ciências (Bioquímica) pelo Programa de Pós-graduação em Ciências (Bioquímica) do Departamento de Bioquímica e Biologia Molecular, Setor de Ciências Biológicas da Universidade Federal do Paraná.

Orientador: Prof. Dr. Marcelo Müller dos Santos
Co-orientadora: Profª Drª Leda Satie Chubatsu

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TERMO DE APROVAÇÃO

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Curitiba, 26 de março de 2014.

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D.O. = densidade óptica

PHB = polihidroxibutirato

DNA = ácido desoxirribonucléico

mL = mililitro

µL = microlitro

ONPG = o-nitrofenil-β-D-galactosídeo

PHA = polihidroxialcanoato

rpm = rotações por minuto

mmol/L = milimol por litro

mM = milimolar

°C = graus Celsius

LB = Luria – Bertani

nM = nanomolar

GC = cromatografia gasosa

mg = miligramas

h = hora

FSC = canal de dispersão para a frente (forward scatter channel)

SSC = Canal de dispersão lateral (side scatter channel)

FL3-H = intensidade (altura) no canal FL3 (intensity (height) in the FL3 channel)

IPTG = Isopropil β-D-1-tiogalactopiranosideo

RESUMO

Polihidroxibutirato (PHB) é um polímero produzido por várias bactérias em situações de excesso de carbono e falta de outro nutriente essencial para o seu crescimento. PHB funciona como uma reserva energética e é armazenado intracelularmente na forma de grânulos insolúveis. Na superfície de grânulos de PHB, algumas proteínas estão ligadas e são importantes para a estrutura e para o metabolismo do PHB. As fasinas são as proteínas associadas aos grânulos mais abundantes e, desempenham uma importante função no controle da forma e tamanho dos grânulos. Neste trabalho, nós apresentamos os resultados relacionados à caracterização de estirpes isogênicas de *Herbaspirillum seropedicae* mutadas em *phaP1, pahP2* ou em ambos os genes. Os resultados mostraram que apesar da alta similaridade entre PhaP1 e PhaP2, PhaP1 é a principal fasina de *H. seropedicae*, uma vez que a sua deleção reduz o acúmulo de PHB em torno de 50% em comparação com a estirpe selvagem e com *ΔphaP2*. Além disto, o padrão diferencial de transcrição de *phaP2* nos leva a propor que possivelmente PhaP2 pode executar outra função do que apenas ligar e formar os grânulos de PHA em *H. seropedicae*.

ABSTRACT

The polyhydroxybutyrate (PHB) is a polymer produced by several bacteria in situations of carbon excess and deficiency in other essential nutrient. PHB works as an energetic reservoir and it is stored intracellularly in the form of granules. On the surface of PHB granules, some proteins are attached being important for the structure of the granules and metabolism of PHB. The phasins are the most abundant granule-associated proteins and, they perform important structural role controlling the shape and size of granules. In this work, we present results concerning the characterization of isogenic strains of *Herbaspirillum seropedicae* defective in *phaP1*, *phaP2* or in both genes. The results showed that despite the high similarity among PhaP1 and PhaP2, PhaP1 is the major phasin in *H. seropedicae*, since its deletion reduces PHB accumulation around 50% in comparison to the wt and Δ*phaP2* strain. In addition, the differential pattern of transcription of *phaP2* prompts us to propose that possibly PhaP2 has evolve to perform other function than only bind and form PHA granules in *H. seropedicae*.

INTRODUÇÃO

Herbaspirillum seropedicae é uma bactéria da classe β-proteobacteria, aeróbica, prototrófica, endofítica, fixadora de nitrogênio e promotora de crescimento vegetal (PEDROSA *et al.*, 2011). Sabe-se da ocorrência de *H. seropedicae* no interior de plantas de interesse comercial, tais como milho, cana-de-açúcar, arroz e sorgo (PIMENTEL *et al.*, 1991; OLIVARES *et al.*, 1996; OLIVARES *et al.*, 1997; JAMES *et al.*, 1997; JAMES *et al.*, 2002). Logo, há um grande interesse no estudo de aspectos fisiológicos desta bactéria, bem como da sua associação com plantas de interesse econômico, visando sua aplicação na agricultura como um biofertilizante.

Herbaspirillum seropedicae também demonstra grande potencial biotecnológico quanto a produção de polihidroxialcanoatos (PHAs). Os PHAs são poliésteres formados pela condensação enzimática de 3-hidroxiacil-CoA e estocados intracelularmente na forma de grânulos insolúveis (ANDERSON & DAWES, 1990). O tipo mais comum de PHA produzido por bactérias é denominado poli-3-hidroxibutirato (PHB). Este é formado pela condensação enzimática de 3-hidroxibutiril-CoA (MADISON & HUISMAN, 1999).

Os polihidroxialcanoatos são biodegradáveis e consequentemente apresentam baixo impacto ambiental, não são tóxicos, possuem alto grau de polimerização, insolúveis em água, altamente cristalino – se extraído de seus recursos naturais - e opticamente ativos (STEINBÜCHEL *et al.*, 1991, 1996; HOCKING *et al.*, 1994). Alguns estudos indicam que os polihidroxibutiratos possuem uma boa processabilidade em equipamentos que processam plásticos sintéticos, como por exemplo, polietileno e polipropileno, tornando-os adequados para moldagem por injeção e extrusão. Portanto, isto demonstra as diversas aplicações indústrias que os

PHAs podem ter substituindo de forma eficaz e eficiente os plásticos sintéticos não biodegradáveis (HOCKING *et al*., 1994; MÜLLER *et al*., 1993).

No metabolismo de PHB, existem diversas proteínas envolvidas com funções específicas e extremamente relevantes. Há enzimas como a beta-tiolase (PhbA), a 3-hidroxibutril-CoA (PhbB) e a PHA sintase (PhaC). Além das proteínas envolvidas na síntese, também há proteínas estruturais com papel ativo no metabolismo deste polímero, sendo estas o regulador negativo PhaR (PhbF em *H. seropedicae*) e a proteína estrutural fasina (PhaP) (PÖTTER *et al*., 2006; KADOWAKI *et al*., 2011).

As fasinas cobrem a superfície dos grânulos de PHB formando uma interface entre o citoplasma hidrofílico e o núcleo hidrofóbico dos grânulos (STEINBÜCHEL *et al*., 1995). O isolamento dos grânulos é importante para evitar o fenômeno de coalescência, quando ocorre a união de todos os grânulos de PHB em um único grânulo. Portanto, as fasinas são importantes para controlar o número e o tamanho dos grânulos no interior da bactéria (PÖTTER *et al.*, 2004).

Para controlar de forma efetiva a expressão do gene *phaP*, *Herbaspirillum seropedicae* possui o repressor denominado PhbF (YORK *et al.*, 2001; MAEHARA *et al.*, 2001; PÖTTER *et al*., 2002; KADOWAKI *et al*., 2011). Este repressor age de maneira que, no momento em que é iniciada a produção de PHB, PhbF é sequestrada do DNA pelo polímero, liberando a expressão dos genes alvos, incluindo o gene *phaP*. Portanto, PhbF atua como um dispositivo que reconhece a formação de cadeias de PHB e libera a expressão de *phaP* somente no momento em que ocorra acúmulo do polímero. As fasinas produzidas se associam com as cadeias de PHB nascentes e moldam a estrutura dos grânulos.

Sendo assim, este trabalho de Mestrado visou avaliar a influência que as fasinas PhaP1 e PhaP2 tem na produção de PHB em *H. seropedicae*

SmR1, se ambas fasinas são reguladas por PhbF e, se a produção de PHB na ausência de fasinas pode acarretar em respostas de estresse na bactéria.

1. REVISÃO BIBLIOGRÁFICA

1.1 *Herbaspirillum seropedicae*

O gênero *Herbaspirillum* refere-se a uma bactéria que está associada a plantas herbáceas que não produzem tecido lenhoso, quanto à espécie *seropedicae* corresponde especificamente a uma homenagem à cidade de Seropédica no estado do Rio de Janeiro, Brasil, onde a espécie foi isolada pela primeira vez. (BALDANI *et al,* 1982).

Herbaspirillum seropedicae é uma bactéria gram-negativa, frequentemente vibróide, diazotrófica e geralmente helicoidal. Além disto, *H. seropedicae* pode possuir de 1 a 3 flagelos localizados em um único polo ou em ambos os polos da célula (BALDANI *et al,* 1984), e pode se associar a muitas plantas de relevante interesse para a agricultura (PIMENTEL *et al*., 1991; OLIVARES *et al*., 1996; OLIVARES *et al*., 1997; JAMES *et al*., 1997; JAMES *et al*., 2002). Assim sendo, devido a crescente demanda pelo aumento na produção vegetal, há uma necessidade mundial de se aperfeiçoar a utilização de nitrogênio como fertilizante, entretanto de forma economicamente viável e segura para o meio ambiente (LADHA & REDDY 1995; LADHA *et al*., 1997; JAMES *et al*., 2000). Dentro deste contexto, a utilização de bactérias endofíticas fixadoras de nitrogênio e promotoras de crescimento surge como uma alternativa viável para substituição de fertilizantes de origem exclusivamente química (LADHA *et al*., 1997).

1.2 POLIHIDROXIALCANOATOS

Herbaspirillum seropedicae desponta também como um importante produtor de polihidroxialcanoatos (PHAs) **(Fig. 1)**, os quais são compostos de monômeros unidos por ligações ésteres. Normalmente, PHAs são produzidos quando as bactérias encontram situações de excesso de carbono ou quando seu crescimento é diminuído ou restringido pela falta de algum nutriente, como fósforo, enxofre, nitrogênio ou oxigênio (STEINBÜCHEL, A., 1990; ANDERSON & DAWES, 1990). Do ponto de vista fisiológico, os PHAs podem ser utilizados como reserva de carbono e energética, já que durante a síntese há o consumo de equivalentes reduzidos na forma de NADPH (MADISON *et al.*, 1999). Em condições desfavoráveis, tais como baixa disponibilidade de carbono no meio, as bactérias podem mobilizar o PHA estocado para a produção de acetil-CoA e regeneração de NADPH (Ren *et al*., 2009).

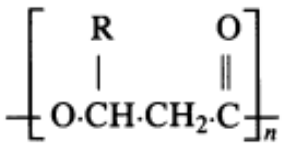

Figura 1- Estrutura química de um polihidroxialcanoato genérico (Anderson & Dawes, 1990). R representa um radical que normalmente varia de 1 a 12 átomos de carbono.

PHAs tem atraído o interesse da área industrial, uma vez que problemas gerados pelo despejo de resíduos plásticos vêm aumentando e prejudicando o meio ambiente. Portanto o desenvolvimento de materiais com propriedades biodegradáveis têm despertado o interesse das indústrias e do meio acadêmico. As propriedades físico-químicas de alguns PHAs são

similares a de plásticos de origem petroquímica como polietileno e polipropileno, indicando uma possível substituição destes materiais por uma fonte renovável (HOLMES 1988; LEE 1996a; STEINBÜCHEL 1991a; 1992).

Alguns estudos mencionam que como os polihidroxialcanoatos podem ser de cadeias curtas, médias e longas, hetero ou homopolímeros, isto pode ser a explicação para o motivo pelo qual os PHA tem uma ampla gama de diferentes famílias de polímeros (BYROM 1987; WILLIAMS & POVOS 1996; MADISON & HUISMAN, 1999), e isto pode ser explicado de acordo com a fonte de carbono que a bactéria utiliza para produzir o polímero. O principal PHA produzido por bactérias e, mais estudado até o momento, é o poli(3-hidroxibutirato) ou polihidroxibutirato ou PHB (CHOI e LEE, 1999; REDDY, GHAI e KHALIA, 2003).

1.3 POLIHIDROXIBUTIRATO

O PHB é produzido a partir da condensação de duas moléculas de acetil-CoA formando o intermediário acetoacetil-CoA pela ação da enzima beta-cetotiolase (PhbA). O intermediário acetoacetil-CoA é reduzido pela acetoacetil-CoA redutase levando a formação de 3-hidróxi-butiril-CoA pela ação da enzima (PhbB). Nessa reação é consumido 1 equivalente redutor na forma de NADPH. O intermediário 3-hidróxi-butiril-CoA é o substrato da enzima PHA sintase (PhaC) que polimeriza unidades de 3-hidróxi-butiril-CoA formando o polímero denominado PHB. O PHB é estocado intracelularmente na forma de grânulos que são cobertos por uma monocamada fosfolipídica e proteínas estruturais, sendo a principal delas a proteína fasina (JENDROSSEK, 2009). Também há enzimas que atuam na mobilização (catabolismo) dos grânulos de PHB, gerando como produtos acetil-CoA e NADPH (JENDROSSEK e HANDRICK, 2002). A PHB

depolimerase (PhaZ) hidrolisa o polímero liberando ácido 3-hidróxi-butírico (3HB). O 3HB é oxidado a acetoacetato gerando 1 equivalente redutor na forma de NADPH ou NADH pela ação da enzima 3HB desidrogenase. O acetoacetato reage com succinil-CoA por intermédio da enzima succinil-CoA : 3-cetoácido-CoA transferase formando acetoacetil-CoA e succinato. Por fim, acetoacetil-CoA é revertido a acetil-CoA consumindo uma molécula de coenzima A através da reação reversa da beta-cetotiolase (PhbA). O estado metabólico que favorece a produção de PHB consiste em: (i) alta taxa de metabolização da fonte de carbono, (ii) parte do acetil-CoA gerado pelo catabolismo não é aproveitado pelo ciclo do ácido cítrico e (iii) disponibilidade de excesso de equivalentes redutores, principalmente na forma de NADPH. Já o estado metabólico que favorece a mobilização de PHB ainda não é totalmente compreendido. Estudos sugerem que quando a bactéria é incubada de forma prolongada na ausência de carbono e em baixas concentrações de nitrogênio o PHB acumulado é rapidamente mobilizado (JENDROSSEK e HANDRICK, 2002).

Herbaspirillum seropedicae surge como um modelo importante para se estudar a produção de PHB já que esta bactéria é capaz de produzir o polímero metabolizando diferentes fontes de carbono, tais como glucose, galactose, manitol, xilose e succinato (CATALÁN *et al*., 2007). Em 2007, CATALÁN e colaboradores relataram que *H. seropedicae* Z69 produziu PHB a 36% do peso seco da cultura, utilizando glucose a 50 mmol/L como fonte de carbono e $(NH_4)_2SO_4$ a 20 mmol/L como fonte de nitrogênio. Também foi possível demonstrar em nossos laboratórios que *Herbaspirillum seropedicae* SmR1 é capaz de produzir PHB usando malato a 37 mM como única fonte de carbono e NH_4Cl a 20 mM como fonte de nitrogênio (KADOWAKI, 2012; TIRAPELLE, 2012).

1.4 BIOSSÍNTESE DO POLÍMERO

Os polímeros de polihidroxialcanoatos podem ser obtidos através de diferentes processos. Sabe-se que no final da década de 70, polihidroxibutirato e polihidroxivalerato foram obtidos através de processos biológicos de fermentação, e as estirpes utilizadas na época foram *Alcaligenes latus* e *Ralstonia eutropha*. Atualmente houve um grande progresso na analise molecular e bioquímico das vias de biossíntese dos polihidroxialcanoatos. Além disto, muitos polímeros de PHA podem ser obtidos por diferentes maneiras, como produção química, combinação de produção química e biotecnológica, produção biotecnológica e isolamento a partir de fontes naturais. (STEINBÜCHEL & FÜCHTENBUSCH, 1998).

Algumas bactérias normalmente biossintetizam PHA a partir de acetil-CoA através de uma via de três passos: 1) condensação de duas moléculas de acetil-CoA formando acetoacetil-CoA através da ação da β-cetotiolase (PhbA); 2) redução de acetoacetil-CoA a R-(-)-3-hidróxi-butiril-CoA pela enzima acetoacetil-CoA redutase NADPH dependente (PhbB) e 3) polimerização de R-(-)-3-hidróxi-butiril-CoA pela enzima PHB sintase (PhbC) (STEINBÜCHEL & FÜCHTENBUSCH, 1998).

Para a biossíntese dos polihidroxialcanoatos existem muitos genes envolvidos, e a maioria destes genes encontram-se dentro de um operon com um gene que codifica para a tiolase (phaA), redutase (phaB) e por fim para a sintase (phaC) (SCHUBERT *et al*., 1988; PEOPLES *et al*., 1989; SLATER *et al*., 1988; LIEBERGESELL *et al*., 1992), **(Fig. 2)**

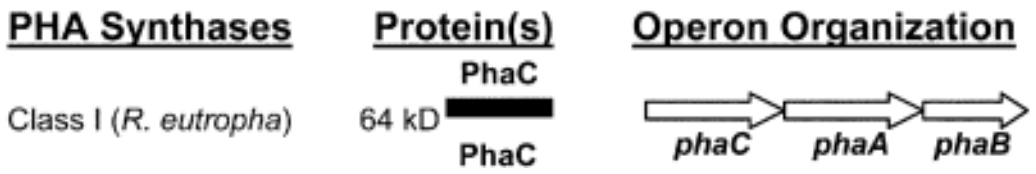

Figura 2. Organização do operon em *Ralstonia eutropha* (Stubbe e Tian, 2003).

No entanto, mais genes estão envolvidos no metabolismo de alguns polihidroxialcanoatos, com algumas depolimerases (*phaZ*) (SAEGUSA, 2001), proteínas como as fasinas (*phaP*) (WIECZOREK, 1995) e também uma proteína regulatória (*phaR*) (MAEHARA *et al*., 1999; MAEHARA *et al*., 2001; PÖTTER *et al*., 2002). Segundo as pesquisas, estes genes estão envolvidos principalmente no controle da homeostase do polímero (STUBBE & TIAN, 2003).

1.5 FASINAS

Logo após a síntese de cadeias de PHAs, estes são envoltos por uma camada de fosfolipídios e proteínas, sendo que a principal proteína associada é a fasina (PhaP). A estrutura formada pela associação de proteínas as cadeias de PHA recebe o nome de grânulo. As fasinas ainda não tem estrutura tridimensional conhecida, entretanto análises computacionais apontam que estas são proteínas com propriedades anfipáticas, que poderiam interagir simultaneamente com um solvente aquoso e com uma estrutura hidrofóbica (NEUMANN *et al*., 2008). Esta proteína pode afetar de forma positiva a síntese do polímero, e também – função principal das fasinas – o número e o tamanho dos grânulos (PÖTTER *et al*., 2004; STEINBÜCHEL *et al*., 1995; WIECZOREK *et al*.,1995;).

O principal papel das fasinas provavelmente seja formar uma camada de isolamento entre o citoplasma hidrofílico e o núcleo hidrofóbico dos grânulos de PHA (STEINBÜCHEL *et al*., 1995). Este isolamento dos grânulos é de extrema relevância, uma vez que as fasinas impedem a

coalescência entre os grânulos, levando a formação de um único e grande grânulo no interior da célula (PÖTTER *et al*., 2004). Além disso, Han e colaboradores (2001) propôs que as fasinas podem reduzir o estresse causado pela presença de cadeias intracelulares de PHA. Esta hipótese pôde ser sustentada, já que em *Escherichia coli*, uma bactéria que naturalmente não produz e não estoca PHAs na forma de grânulos, a transferência de genes de biossíntese de PHB na ausência de fasinas levou a superexpressão de genes relacionados a resposta anti-estresse como *dnaK*, *groEL* e *groES* (HAN *et al*., 2001). Tessmer e colaboradores (2007) encontraram uma proteína de choque térmico HspA associada a grânulos de PHB em *E. coli*, mostrando que de fato na ausência de fasinas outras proteínas são necessárias para aliviar o estresse causado pelo acúmulo de polímero. Na ausência de fasinas as cadeias de polímero poderiam interagir de forma inespecífica com proteínas celulares, levando a desnaturação de algumas proteínas importantes e desencadear resposta de estresse (STEINBÜCHEL *et al*., 1995; LIEBERGESELL *et al*., 1992; HOROWITZ e SANDERS, 1995; TESSMER *et al*, 2007).

A ocorrência de fasinas já foi relatada para vários microrganismos produtores de PHAS, sendo estes: *Ralstonia eutropha* (WIECZOREK *et al*., 1995; HANLEY *et al*., 1999), *Rhodococcus ruber* (PIEPER-FÜRST *et al*., 1994, 1995), *Acinetobacter sp*. (SCHEMBRI *et al*., 1995), *Chromatium vinosum* (LIEBERGESELL & STEINBÜCHEL, 1992), *Paracoccus denitrificans* (MAEHARA *et al*., 1999), *Bacillus megaterium* (MCCOOL & CANNON, 1999), *Aeromonas caviae* (FUKUI & DOI., 1997; KICHIZE *et al*., 2001), *A. hydrophila* (LU *et al*., 2004), *Pseudomonas putida* (VALENTIN *et al*., 1998), *P. oleovorans* (PRIETO *et al*., 1999) e *Pseudomonas* (MATSUMOTO *et al*., 2002) e *Herbaspirillum seropedicae* (TIRAPELLE *et al*., 2013).

Para a bactéria gram-negativa e organismo modelo, *Ralstonia eutropha*, foram realizados diversos estudos mostrando que a supressão de genes que codificam fasinas alterou a morfologia e a quantidade de grânulos no interior da célula, pois devido à ausência de fasinas na superfície dos grânulos ocorreu o fenômeno de coalescência (WIECZOREK *et al*., 1995; PIEPER-FURST *et al*., 1994; FULLER *et al*., 1992; PIEPER-FURST *et al*., 1995; KUCHTA *et al*., 2007). Além disso, foi comprovado que tanto a ausência de fasinas como a superexpressão destas proteínas leva a uma baixa produção de PHB (PÖTTER *et al*., 2005).

Em *R. eutropha*, a principal fasina encontrada associada aos grânulos de PHB é denominada PhaP1, embora sabe-se que este microrganismo possui outras fasinas cobrindo os grânulo de PHB (PÖTTER *et al*., 2004; PÖTTER *et al.*, 2005; KUCHTA *et al*., 2007). Os resultados apresentados por Wieczorek et al. (1995), mostram claramente que na ausência de PhaP1, as células de *R. eutropha* possuíam apenas um único e grande grânulo dentro da célula, sendo que na estirpe selvagem vários grânulos de tamanho médio foram observados.

A parte de *Ralstonia eutropha*, até o momento, pouco se sabe sobre a resposta de outras bactérias produtoras de PHB à deleção de genes que codifiquem fasinas. Wang *et al*. (2007) identificaram duas fasinas no genoma da bactéria *Sinorhizobium meliloti* 1021. Quando as duas fasinas foram mutadas não houve produção de PHB, entretanto a produção de glicogênio foi aumentada em cerca de 50%. A caracterização do mutante também mostrou que o duplo mutante *ΔphaP1phaP2* teve tempo de geração 4,1 h mais lento que a estirpe selvagem, sugerindo um papel importante das fasinas para o crescimento normal da bactéria. Além disso, a fixação de nitrogênio foi comparada entre o mutante *ΔphaP1phaP2* e uma estirpe ΔphbC, que tem os genes das fasinas mas não o gene que codifica a PHB sintase, portanto não sintetiza PHB. Os dados obtidos

mostraram que o mutante *ΔphaP1phaP2* teve atividade específica de redução de acetileno cerca de 80% da atividade da estirpe selvagem, porém a estirpe ΔphbC apresentou apenas 10% da atividade obtida com a estirpe selvagem. Esses resultados mostram que as fasinas podem além de estabilizar os grânulos de PHB ainda possuírem outras funções importantes para a fisiologia bacteriana que ainda não são conhecidas (WANG *et al*., 2007).

Para *Herbaspirillum seropedicae*, a qual produz polihidroxibutirato sob condições desbalanceadas de crescimento (excesso de carbono, ou falta de algum outro nutriente, como fósforo ou nitrogênio) (CATALAN *et al*., 2007; KADOWAKI *et al*., 2011), foram identificados trezes genes que estão envolvidos no metabolismo do PHB (KADOWAKI *et al*., 2011; PEDROSA *et al*., 2011), os quais podemos citar dois *phbC* (sintases), *phaZ* (depolimerases) e *phaP* (fasinas) e o gene que codifica para a proteína reguladora PhbF (KADOWAKI *et al*., 2011), assim estes genes codificaram algumas proteínas que irão, posteriormente, se ligar ao grânulo de PHB, auxiliando na formação do mesmo **(Fig. 3)**.

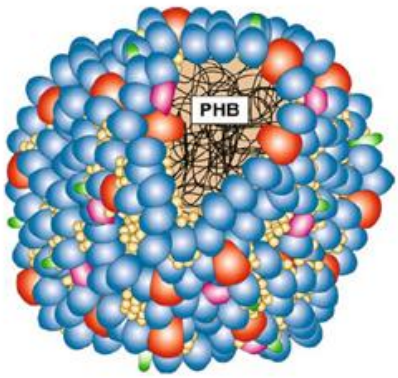

Figura 3. Modelo da estrutura do grânulo de pha. Fasinas: azul; pha sintases: vermelho; pha depolimerases: rosa; repressor transcricional das fasinas (phbf): verde; fosfolípidios: amarelo (pötter *et al*. 2005).

1.6 PROTEÍNA REGULATÓRIA DAS FASINAS (PhaR/PhbF)

Como observado por York *et al*., (2002) em *Ralstonia eutropha*, e em outras bactérias que produzem PHA (REHM & STEINBÜCHEL, 1999) como *Sinorhizobium meliloti* (TOMBOLINI *et al*., 1995; POVOLO & CASELLA, 2000), a proteína PhaR (PhbF em *Herbaspirillum seropedicae*) parece ter um papel fundamental que envolve a regulação da biossíntese das fasinas e assim dos grânulos de polihidroxibutirato, além de possuir uma autoregulação. Além disto, sabe-se da presença de PhaR em *Paracoccus denitrificans*, e verificou-se que esta proteína era capaz de regular negativamente a síntese das fasinas (MAEHARA *et al*., 1999, 2001).

Em *Ralstonia eutropha* alguns estudos sugerem que PhaR promove a síntese de polihidroxibutirato por regulação da expressão das fasinas, e também, age de uma forma que regula a expressão de outras proteínas envolvidas na síntese do polímero em questão (YORK *et al*., 2002)

Portanto, no momento em que é iniciada a produção de PHB, PhbF é sequestrada do DNA para o polímero, liberando a expressão dos genes alvos, incluindo o gene *phaP* que codifica para fasina. Logo, PhbF atua como um dispositivo que reconhece a formação de cadeias de PHB e libera a expressão de *phaP* para que assim as fasinas se liguem as cadeias de PHB nascentes e moldem a estrutura dos grânulos, além disto PhbF/PhaR pode reprimir sua própria expressão **(Fig. 4)** (YORK *et al*., 2001; PÖTTER *et al*., 2002; MAEHARA *et al*., 2001; KADOWAKI *et al*., 2011).

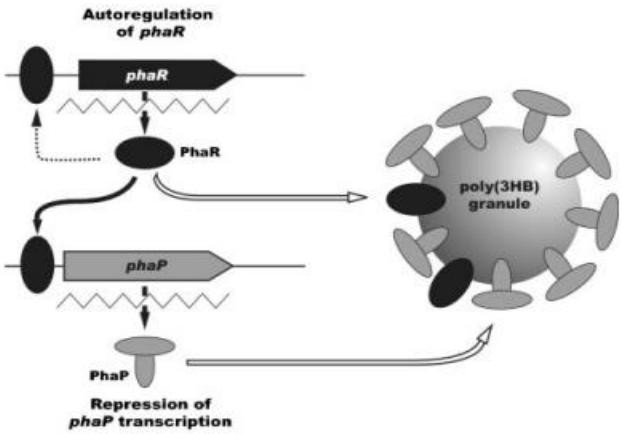

Figura 4. Modelo que sugere a regulação da expressão de p*haR* e *phaP* por phaR em *Ralstonia eutropha* H16 (Pötter *et al.*, 2002)

Em um estudo realizado por Tirapelle *et al*., (2013) sugere-se que a expressão da fasina PhaP1 de *H. seropedicae* SmR1 é regulada pela produção de PHB e pelo regulador PhbF. Quando a bactéria iniciou o acúmulo de grânulos de PHB, ocorreu um aumento na expressão da fusão transcricional do promotor (P) *phaP1-lacZ.* Entretanto, quando a expressão da fusão *PphaP1-lacZ* foi determinada no mutante *ΔphbC1*, o qual não produz PHB, não foi observada a expressão do gene repórter, mostrando que na ausência de PHB o repressor PhbF se mantém ativamente ligado ao DNA.

1.7 PHA SINTASE – Classe I (PhaC1/PhbC1)

A biossíntese do polímero de PHA ocorre em três passos, e sua etapa final e mais relevante somente acontece se houver a presença da enzima PHA sintase (PhaC), a qual polimeriza as porções acil do 3 hidroxibutiril-CoA formando o PHB e liberando concomitantemente a coenzima A. Portanto PHA sintase é a enzima chave na biossíntese dos polihidroxialcanoatos e obviamente dos grânulos de polihidroxibutirato (OEDING *et al*., 1973; HAYWOOD *et al.*, 1988; REHM *et al*., 2002).

Alguns estudos revelam que há uma associação entre a superfície dos grânulos de PHB e as PHA sintases de *Ralstonia eutropha* e *Allochromatium vinosum* (HAYWOOD *et al*., 1989; GERNGROSS *et al*., 1993; LIEBERGESELL *et al*., 1992).

Pötter *et al*., (2005) mencionam que PhaC é de extrema importância para a síntese do polímero, uma vez que as outras duas enzimas que participam do processo – PhaA e PhaB – podem ser substituídas por outras isoenzimas.

Atualmente mais de 60 genes da PHA sintases foram sequenciados de diferentes microrganismos, e de acordo com a homologia entre estes genes as PHA sintases foram separadas em diferentes classes: Classe I (PHA sintases que sintetizam PHAs de cadeia curta, como o PHB), Classe II (auxiliam na síntese de PHA de cadeias de tamanho médio), Classe III (sintetizam PHA de cadeias curtas, no entanto são compostos por subunidades diferentes) e Classe IV (compostos por subunidades diferentes e ocorrem somente em espécies que pertencem ao gênero *Bacillus* (McCOOL *et al*., 2001).

A Classe I e a Classe III das PHA sintases de *Ralstonia eutropha* e *Allochromatium vinosum* são consideradas como enzimas modelo para estudo da biossíntese de polihidroxialcanoatos de cadeia curta (PHB). Além disto, sabe-se que a possível ausência da PHA sintase pertencente à Classe I pode acarretar em uma diminuição no peso molecular (SIM *et al*., 1997; KRAAK *et al*., 1997) assim como na defasagem de acumulo de grânulo de PHB dentro do microrganismo.

The influence of phasins PhaP1 and PhaP2 on PHB accumulation in *Herbaspirillum seropedicae* SmR1

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Abstract

The polyhydroxybutyrate (PHB) is a polymer produced by several bacteria in situations of carbon excess and deficiency in other essential nutrient. PHB works as an energetic reservoir and it is stored intracellularly in the form of granules. On the surface of PHB granules, some proteins are attached being important for the structure of the granules and metabolism of PHB. The phasins are the most abundant granule-associated proteins and, they perform important structural role controlling the shape and size of granules. In this work, we present results concerning the characterization of isogenic strains of *Herbaspirillum seropedicae* defective in *phaP1*, *phaP2* or in both genes. The results showed that despite the high similarity among PhaP1 and PhaP2, PhaP1 is the major phasin in *H. seropedicae*, since its deletion reduces PHB accumulation around 50% in comparison to the wt and Δ*phaP2* strain. In addition, the differential pattern of transcription of *phaP2* prompts us to propose that possibly PhaP2 has evolve to perform other function than only bind and form PHA granules in *H. seropedicae*.

Introduction

Herbaspirillum seropedicae SmR1 is a diazotrophic β-Proteobacterium, which associates with graminae (1) and produces PHB (2, 3). In *H. seropedicae* SmR1, thirteen genes potentially involved in PHB metabolism were identified (3, 4), including four *phaC*, two *phaZ* and two *phaP* genes which code for PHA synthases, PHA depolymerases and phasins, respectively. In addition, the *phbF* gene which encodes a transcriptional repressor protein was identified as well (3). It has been suggested that PHB has an important role in nitrogen fixation and in plant-bacteria interaction (5-8), however the importance of PHB production for *H. seropedicae* was not totally determined, so far.

Polyhydroxybutyrate (PHB) is an aliphatic polyester member of the polyhydroxyalkanoates (PHA) family of polymers, biosynthesized by several bacteria as a means of carbon storage and source of reducing equivalents (9, 10). PHB is usually produced under conditions of carbon oversupply and low levels of other nutrients including nitrogen, phosphate and oxygen (11). The polymer synthesis is dependent on, at least, three enzymes: 3-ketothiolase, acetoacetyl-CoA reductase and PHA synthase, encoded by *phbA*, *phbB* and *phaC* genes, respectively (12). The biosynthesis of PHB chains occurs by condensation of acetyl-CoA forming acetoacetyl-CoA, then reduction of acetoacetyl-CoA to 3-hydroxyacyl-CoA

(3HB-CoA) and finally polymerisation of 3HB-CoA yields PHB (13). When carbon/energy is requested, the polymer is degraded by PHA depolymerase enzymes, encoded by *phaZ* genes (12). The PHB polymer is stored as intracellular insoluble granules coated with proteins reaching about 0.5 to 2% of the granule weight (14, 15). Phasins are small amphiphilic proteins attached on the surface of polyhydroxyalkanoates (PHA) inclusions in Bacteria and Archaea (14, 16, 17). These proteins are important to control the size and the number of PHB granules into the cells (18-22). Phasins seem to be present in all PHA producer bacteria, and despite this family of proteins are not highly conserved in terms of amino acid sequence, they perform the same functions of binding to PHA granules and promoting their formation and stabilization in different microbes (21, 23, 24). In *Ralstonia eutropha* H16, the well-studied model of PHB metabolism, there are a total of seven phasins (25-28). Nevertheless, it appears that PhaP1 is the major phasin and the only that has influence on PHB accumulation (25).

The genome sequencing of *H. seropedicae* SmR1 revealed the presence of two paralogous phasin genes with 75% of homology, coding for proteins of the exact same length (186 amino acids). To investigate if their similarity also reflected in a conservation of function, we determined the pattern of PHB accumulation and growth of three phasin mutants with precise deletion in the *phaP1*, *phaP2* or in both genes. Furthermore, we

determined the transcriptional pattern of *phaP1* and *phaP2*, using transcriptional fusions with *lacZ* reporter in different genetic backgrounds. The results are discussed correlating the phenotypes of mutants and the transcriptional pattern of each phasin, leading to a possible divergence of function between PhaP1 and PhaP2.

Materials and Methods

Reagents

All chemicals were Analytical or Molecular Biology grade and were purchased from Merck (Germany), Sigma (USA), J.T.Baker (Netherlands) or Invitrogen (USA). Restriction enzymes were from Fermentas (Lithuania) or Invitrogen (USA). Oligonucleotides were purchased from IDT (USA).

Bacterial Strains and Growth Conditions

Strains and plasmids used in this work are listed in Table 1. *Escherichia coli* strains Top10 (Invitrogen, USA) and S17.1 (29) were used for cloning and conjugation procedures. The *E. coli* strain ET8000 was used in transcriptional assays. They were grown at 37° C in LB, Terrific Broth, SOC or SOB medium at 160 rpm. *Herbaspirillum seropedicae* wild-type SmR1 (30) and mutants strains were grown in NFbHP medium with 37

mM DL-malate or 25 mM glucose and 20 mM NH_4Cl at 30 C and 120 rpm (31).

PHB Quantification

The amount of PHB was determined by methanolysis and GC-FID analysis as described previously (32). Methanolysis was carried out with 5–10 mg of lyophilized bacteria in 2 ml of chloroform and 2 ml of methanol containing 15% sulphuric acid in borosilicate glass tubes with screw caps. As an internal standard, it was added 0.5 mg of benzoic acid per reaction. The reactions were incubated at 100 C for 3.5 h in a dry heating block. After cooling, 1 ml of distilled water was added and the tubes were vortex for 60 s. The upper aqueous phase was removed and the lower organic phase containing the resulting 3-hydroxybutyric methyl ester (Me-3-HB) was dried with Na_2SO_4 and analyzed by GC in a 450 GC Varian chromatograph with a CP-Sil-5 CB column (10 m × 0.53 mm ID). Argon was used as carrier gas at 0.9 ml min^{-1}. The injector was set at 250 C and the detector at 275 C. The oven temperature programmed was: initial temperature 50 C for 2 min, then from 50 C up to 110 C at a rate of 20 C

Table 1. Bacterial strains and plasmids used in this work.

Strain or plasmid	Relevant characteristics	Reference/source
E. coli strains		
Top10	Cloning strain	Invitrogen
S17-1	Conjugation strain	(29)
ET8000	Wild-type strain	(33)
H. seropedicae strains		
SmR1	Wild-type, Nif^+, Sm^R	(30)
Δ*phaP1*	Chromosomal deletion of *phaP1*	(34)
Δ*phaP2*	Chromosomal deletion of *phaP2*	(34)
Δ*phap1.2*	Chromosomal deletion of *phaP1* and *phaP2*	(34)
Δ*phaC1*	Chromosomal deletion of *phaC1*	(34)
Δ*phbF*	Chromosomal deletion of *phbF*	This work
Plasmids		
pTZ18R	Cloning plasmid	(35)
pDK6	Expression vector *tac* promoter *lacI*^{q}, Km^R	(36)
pMMS31	Derivative of pDK6 encoding PhbF from *H. seropedicae* SmR1	(3)
pMP220	Promoter less *lacZ* containing vector used to construct transcriptional fusions; Tc^R	(37)
pEFT11	pMP220 harbouring the regulatory region of *phaP1* cloned upstream of *lacZ*	This work
pEFT12	pMP220 harbouring the regulatory region of *phaP2* cloned upstream of *lacZ*	This work
pK18*mobsacB*	Suicide vector; Km^R, *sacB*, mobilizable	(38)

	plasmid	
pCST31	Deletion product Δ*phbF* cloned into the pTZ18R	This work
pCST32	Deletion product Δ*phbF* cloned into the pK18*mobsacB*	This work

min^{-1} and finally up to 250 C at a rate of 20 C min^{-1}. The PHB amount in each sample was normalized by the weight of the lyophilized bacteria and expressed as % of PHB/cell dry weight (cdw).

Construction of Δ*phbF* mutant of *H. seropedicae* SmR1

The *H. seropedicae* Δ*phbF* mutant was constructed by deleting the *phbF* gene (Hsero_2997; genome GenBank accession number NC_014323.1). The unmarked *H. seropedicae phbF* deletion mutant was generated by overlapping PCR (39). Briefly, PCR with genomic DNA template from SmR1 strain was used to generate fragments to either side of the *phbF* locus for deletion. For amplification of the 5'-flanking region of *phbF*, primers Fw_*phbF*_UP (5'-GAGGATCCCGTGACCGTCAACACCGTCT-3') and Rev_*phbF*_UP (5'-agatctcttaggtcgtctctaTGCAGTAGTCATCTGAAGTCCAGTC-3') were used, resulting in the *phbF*_UP PCR product of 556 bp. For amplification of the 3'-flanking region of *phbF*, primers Fw_*phbF*_DOWN (5'-tagagacgacctaagagatctATGTTCGGCACCTTCCCC-3') and Rev_*phbF*_DOWN (5'-GTTCTGCAGTTGCCGCGATTCATGGTGG-3') were used, resulting in the *phbF*_DOWN PCR product of 510 bp. Primers Rev_*phbF*_UP and Fw_*phbF*_DOWN contained a 21 bp complementary sequence at their 5' ends (lower case). PCR products resulting from these two amplifications were gel purified and used as template for a PCR using

primers Fw_*phbF*_UP and Rev_*phbF*_DOWN. A fragment of 1087 bp lenght was purified and ligated into the pTZ18R plasmid digested with SmaI. After the sequence was confirmed, the Δ*phbF* fragment was then subcloned into the sites BamHI and PstI of the pK18mobsacB, generating the pCST32 plasmid. The pCST32 was transformed in *E. coli* S17-1 and conjugated to *H. seropedicae* SmR1 by biparental mating, selecting single-recombinants in NFb-malate agar with Sm 80µg mL^{-1}, Nal 5 µg mL^{-1} and Km 500 µg mL^{-1}. A single-recombinant colony was collected in 3 mL of NFb-malate and cultivated overnight without antibiotics. The culture was serially diluted and plated in NFb-malate agar containing 10% of sucrose. The colonies that grew on sucrose were screened by Km sensitivity. Finally, the sucrose resistant and Km sensitive colonies were screened by PCR using the primers FO_*phbF* (5'-GGTTTCGGTCGCATCATCAA-3') and RO_*phbF* (5'-CTTCATCGGACTTGACCAGG-3') designed to anneal 115 bp upstream and 123 bp downstream of the targeted deletion, respectively.

Construction of transcriptional fusions

The regulatory regions of *phaP1* (containing 333 bp including 28 bp of the *phaP1* coding sequence) and *phaP2* (containing 224 bp including 54 bp of the *phaP2* coding sequence) were amplified from the *H. seropedicae*

SmR1 genomic DNA and cloned into pMP220 (37), upstream the promoter less *lacZ* gene to yield the respective plasmids pEFT11 and pEFT12.

β-galactosidase activity assay

β-galactosidase activity was determined in *E. coli* ET8000 carrying transcriptional fusion plasmids (pEFT11 or pEFT12), in the presence of plasmid pMMS31 (expressing PhbF) or pDK6 (negative control), grown in LB medium as described above. For analysis in *H. seropedicae*, the plasmids pEFT11 or pEFT12 were conjugated by biparental mating with *E. coli* S17-1. The transconjugants were selected in NFb-malate agar with Tc 10µg mL^{-1}. The β-galactosidase activity assay was performed as described (40). The Abs_{410} of o-nitrophenol, the OD_{550} and OD_{600} was recorded using a microtiter plate reader BioTek (Germany) with 200 µL of reaction or culture.

Staining of bacterial cultures for microscopy analysis

To visualize the PHB granules into the cells of *H. seropedicae* SmR1 and *phaP* mutants, analysis of fluorescence microscopy were performed after staining the bacteria with the fluorescent probe Nile Red, which stains specifically neutral lipids (41, 42). The bacterial cultures (1ml) were harvested by centrifugation for 60 seconds at 12,000 *g*. Afterward, the supernatant was discarded, the pellet resuspended in ethanol 30% in PBS,

and then, 3µL of the Nile Red 1.6 mM in DMSO were added, incubating the cultures for 5 minutes in the dark. The samples were centrifuged again for 60 seconds at 12,000 *g*, resuspended in PBS and analysed by fluorescence microscopy.

Results

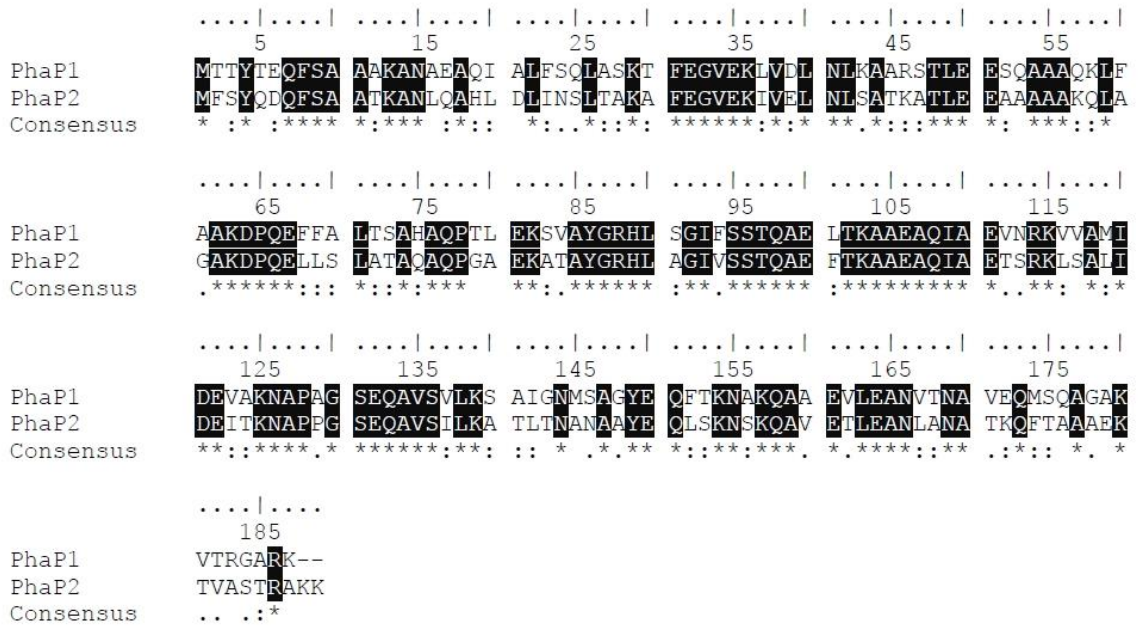
The genome of *Herbaspirillum seropedicae* SmR1 contains multiple genes coding phasins

The genome sequencing of *H. seropedicae* SmR1 revealed the presence of two genes coding for putative phasins (3, 4). The proteomic analysis of the proteins associated to the PHB granules in *H. seropedicae* revealed that the phasins PhaP1 (original annotation: Hsero_1639) and PhaP2 (Hsero_4759) were the most abundant proteins coating the granules (34). In fact, the genes *phaP1* and *phaP2* are paralogous and seem to be a case of gene duplication, since the amino acid sequence identity among PhaP1 and PhaP2 is 59% (Fig. 1A). Interestingly, during proteomic inspection of the granule-associated proteins, it was found a third phasin less homologous to PhaP1 and PhaP2, named PhaP3 (Fig. 1B) (34). To demonstrate the low homology of PhaP3 against the other phasins from *H. seropedicae,* we estimated the pairwise distance of the protein sequences of the three phasins from *H. seropedicae*. As a control for comparison, a group of five already described phasins from *Ralstonia eutropha* H16 were also

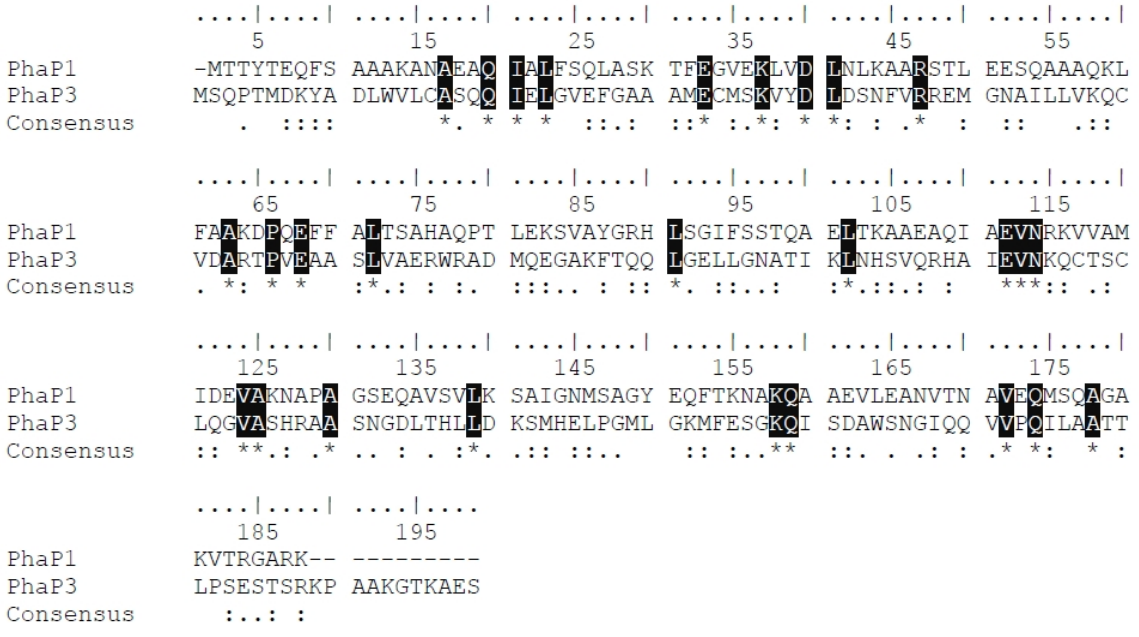
submitted to analysis (Fig. 1C). As expected, PhaP1 and PhaP2 from *H. seropedicae* presented the short evolutionary distance (0.373), as estimated by the p-distance method. It is worthy to note that PhaP3 is so distant from PhaP1 and PhaP2 as from the five other phasins from *R. eutropha* H16, indicating a divergence and sequence and maybe in function. Indeed, the PhaP3 was only detected on PHB granules when *phaP1* was deleted, nevertheless it was clearly less abundant than PhaP2, the main phasin in the absence of PhaP1 (34). From these results, it is likely that PhaP1 and PhaP2 should be the main phasins acting during the PHB biosynthesis and granules formation. To assess this hypothesis, we analysed the phenotypes of PHB accumulation and phasin expression in three different mutant strains of *H. seropedicae* SmR1.

Figure 1. Protein sequence alignment of phasins from *H. seropedicae* SmR1.

(A) ClustalW alignment of PhaP1 against PhaP2.



(B) ClustalW alignment of PhaP1 against PhaP3.



The protein sequence of PhaP1 (Hsero_1639) was aligned against PhaP2 (Hsero_4759) (A) and PhaP3 (Hsero_2402) (B) through ClustalW in the MEGA 6.0 sotware using the Gonnet protein weight matrix with gap opening penalty 10 and gap extension penalty 0.1. The black shaded letters indicate fully conserved residues.

(C) Estimation of evolutionary divergence between protein sequences of phasins from *H. seropedicae* and *R. eutropha*.

Species	PhaP1_Hse	PhaP2_Hse	PhaP3_Hse	PhaP1_Reu	PhaP2_Reu	PhaP3_Reu	PhaP4_Reu
PhaP1_Hse							
PhaP2_Hse	**0.373**						
PhaP3_Hse	0.894	0.908					
PhaP1_Reu	0.831	0.873	0.944				
PhaP2_Reu	0.880	0.908	0.930	0.930			
PhaP3_Reu	0.908	0.908	0.908	0.923	0.655		
PhaP4_Reu	0.901	0.908	0.930	0.951	0.387	0.634	
PhaP5_Reu	0.887	0.866	0.930	0.915	0.923	0.901	0.894

The numbers of amino acid differences per site between sequences are showed. The analysis involved 8 protein sequences of phasins. All positions containing gaps and missing data were eliminated. There were a total of 142 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (43).

The *phaP1* deletion reduces PHB accumulation in *H. seropedicae*

To verify the role of *phaP1* and *phaP2* on the PHB accumulation in *H. seropedicae*, three isogenic strains within the *phaP1*, *phaP2* and both genes deleted were generated. Their growth behaviour and PHB accumulation capacity were measured and compared to the wt strain. The growth curves of wt and mutant strains showed that the Δ*phaP1* and the double mutant Δ*phaP1.2* had their growth slightly negatively affected during the exponential phase, while the Δ*phaP2* mutant presented the same growth profile than wt strain (Figure 2A). The intracellular accumulation of PHB in *H. seropedicae* wt and mutant strains grew on malate as the carbon source was determined in four different growth phases: (i) early exponential, OD_{595} 0.6; (44) mid exponential, OD_{595} 0.8 (iii) early stationary, OD_{595} 1.2 and (iv) late stationary, OD_{595} 1.4. The wt strain accumulated PHB to the maximum of 13% (w/w) of cell dry weight (cdw) in 12 h of growth, when the culture reached the early stationary phase (Figure 2B), followed of a decrease to 11% of PHB in the late stationary phase. The Δ*phaP2* mutant accumulated PHB to 15% (w/w) of cdw when it reached the late stationary phase, showing that the deletion of *phaP2* did not negatively affected the PHB accumulation. However, in the Δ*phaP1* mutant the PHB accumulation was reduced more than 50% as compared to the quantity of PHB accumulated by wt strain, in all growth phases (Figure 2B). The deletion of both phasin genes, in the Δ*phaP1.2* mutant, presented

a more drastic effect on the PHB accumulation, since in any of the assayed points the polymer was detected (Figure 2B). To exclude that the effect of the mutations was not due to the use of malate as carbon source, the PHB accumulation was also determined in a medium with glucose as carbon source. In this condition, *H. seropedicae* SmR1 also accumulated PHB as previously reported for the strains Z67, Z69 and Z78 of *H. seropedicae* (2). The wt strain reached a maximum of 40% of PHB/cdw in the late-stationary phase. The production of PHB of Δ*phaP2* on glucose had a similar pattern than wt, however, reaching 56% of PHB/cdw. For the Δ*phaP1* mutant, the maximum PHB content accumulated on glucose as carbon source was only 18%, corresponding to 50% of the PHB content of the wt. In the double mutant Δ*phaP1.2*, no PHB accumulation was detected when cultivated on glucose. In conclusion, these results pointed that regardless of the carbon source employed and the PHB level accumulated into the cells, the absence of PhaP1 or both phasins exerted a negative effect on PHB accumulation. The growth profile of wt and Δ*phaP2* strains on glucose was very similar. Interestingly, the growth curve for Δ*phaP1* and Δ*phaP1.2* was totally different from those observed previously on malate (Figure 2C). The Δ*phaP1* exhibited a lag phase of 12h, 2-fold longer than wt and Δ*phaP2* strains. Since in exponential phase, the mutant grew only until the OD_{600} of 1.0, showing a decline of OD_{600} after this point. For the Δ*phaP1.2*, the effect in the growth was more drastic, since it

reached the OD_{600} of 0.6 in 16h of growth and, after this point it had a decline of OD_{600} to 0.4, maintaining the growth halted in the consecutive 4 hours. Thus, it is possible to infer that the low level of PHB (e.g. Δ*phaP1*), or the lacking of PHB granules (e.g. Δ*phaP1.2*) impaired the growth of *H. seropedicae* on glucose. Accordingly, the Δ*phaC1* mutant of *H. seropedicae*, the isogenic strain with the PHA synthase coding gene *phaC1* (Hsero_2999) deleted, which did not produce PHB, was also unable to grow on glucose (data not shown).

Figure 2.

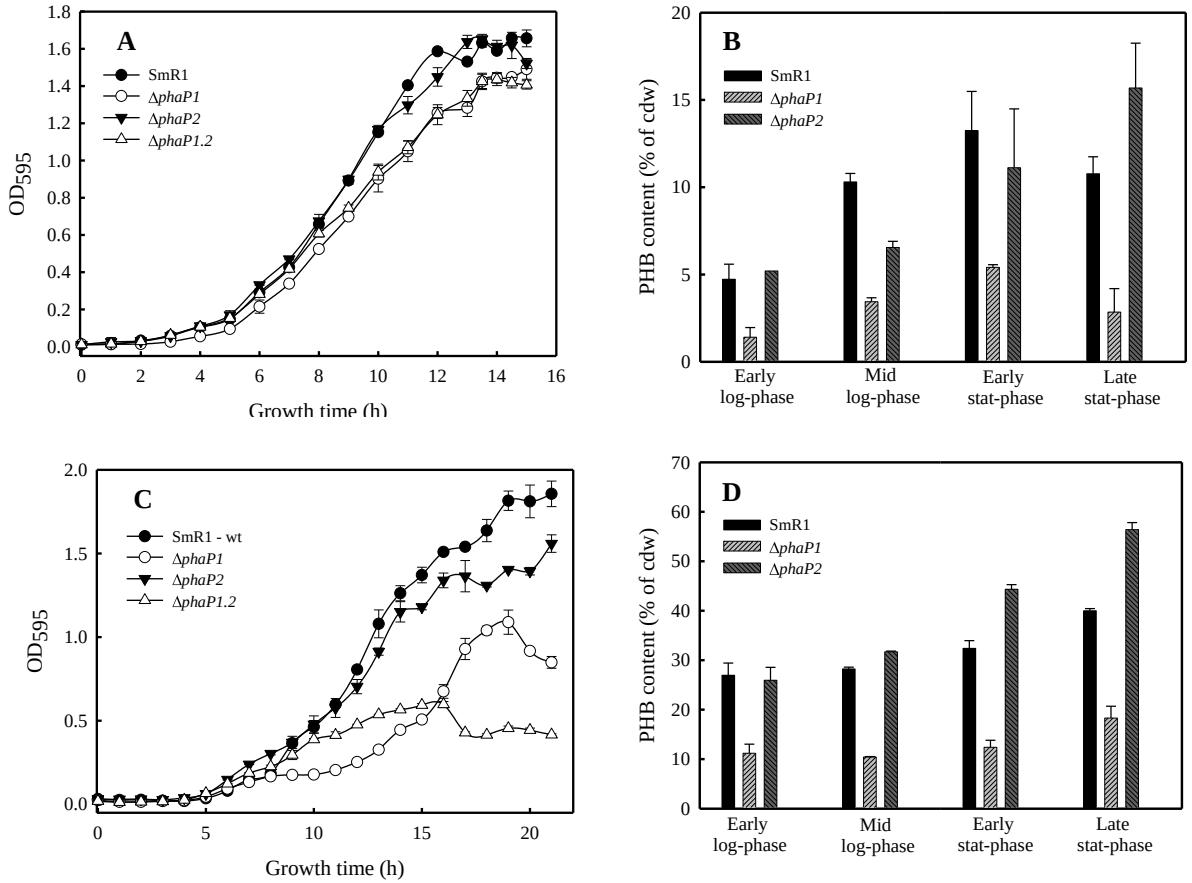


FIG. 2. Growth and PHB accumulation profiles of H. seropedicae SmR1 (wt) and mutants *ΔphaP1, ΔphaP2* and *ΔphaP1.2*. Strains were grown in NFb medium with 20 mM of ammonium chloride and 0.5% of DL-malate (A and B) or 0.25% of D-glucose (C and D) at 30 C and orbital agitation (120 rpm). The data of OD_{595} for growth measurement were obtained from three independent cultures. The PHB content was determined from four independent cultures. The PHB level in the Δ*phaP1.2* mutant was less than 1% in all growth phases.

The microscopic analysis revealed alterations in the number of granules per cell.

To verify the size and the number of granules into the bacterial cells, the cultures of wt and mutant strains were grown, fixed and stained with Nile Red to fluorescence microscopy analysis. In the Fig. 3 is presented a representative image from each strain after staining. The wt strain presented 92% of the cells containing at least two PHB granules, while 8% contained only one granule (n = 100 counted cells). On the other hand, the Δ*phaP1* mutant has only 18% of the cells containing two granules, while 82% had only one granule (n = 100 cells). The Δ*phaP2* mutant presented almost the same profile of granule distribution as compared to the wt, 91% of cells contained two granules against 9% of one-granule cells. As expected, in the double mutant Δ*phaP1.2* there were no cells with two PHB granules. Surprisingly, 9% of the counted cells (n = 100) presented one PHB granule. It is not clear if they were PHB granules *sensu stricto* coated by other proteins or if it was just a transient agglomerated mass of polymer within the cytoplasm, since at all times examined the level of PHB accumulated in this mutant was always below 1% of cdw. In alignment with the results of quantification of PHB, the number of granules in the Δ*phaP1* mutant was significant lower than in the wt and Δ*phaP2* mutant, demonstrating the importance of PhaP1 in formation and stabilization of PHB granules into *H. seropedicae* SmR1.

Figure 3.

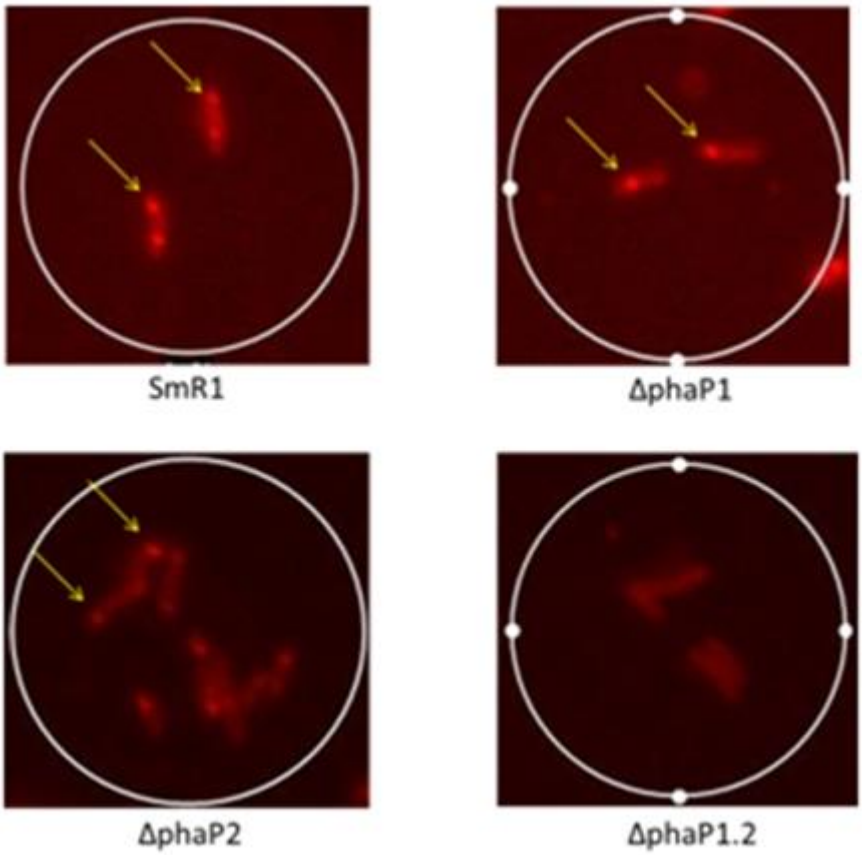


FIG. 3. Fluorescence microscopy of H. seropedicae SmR1 and phasin mutants. The strains were grown in NFb medium with 37 mM of DL-malic acid and 20 mM of NH_4Cl until reach the OD_{600} of 1.0. Cells were stained with Nile Red and visualized by excitation with 543 nm light. The arrows indicate the Nile Red stained granules of PHB.

The transcription of *phaP1* and *phaP2* is repressed by PhbF.

Previously, we demonstrated that the transcriptional repressor PhbF from *H. seropedicae* (homologous to PhaR in *R. eutropha* H16) bound to the regulatory region of *phaP1* and probably acts repressing its transcription, accordingly to the regulatory model proposed by other studies (3, 45-48). In order, to evaluate if *phaP1* and *phaP2* are indeed repressed by PhbF, we evaluated their transcription by beta-galactosidase activity measurement from transcriptional fusions with the reporter *lacZ*, encoding beta-galactosidase activity (P*phaP1-lacZ* and P*phaP2-lacZ*), in *E. coli* ET8000 expressing and not expressing the PhbF from *H. seropedicae*. As expected for *phaP1*, when *E. coli* expressed PhbF the fusion *phaP1-lacZ* was totally repressed, even without the addition of the inductor IPTG (Fig. 4). This demonstrated that only the expression leakage of *phbF* was already enough to repress the transcription of *phaP1*. On the other hand in the absence of PhbF the fusion was highly transcribed, expressing a total of 12,000 Miller units. When *E. coli* expressed PhbF, the P*phaP2-lacZ* fusion was also repressed, however no expression of PhbF resulted in high beta-galactosidase activity (12,000 Miller units). These results showed that both phasin genes are repressed by PhbF and probably, they are transcribed only when the bacteria reach a condition appropriated for PHB accumulation.

Figure 4

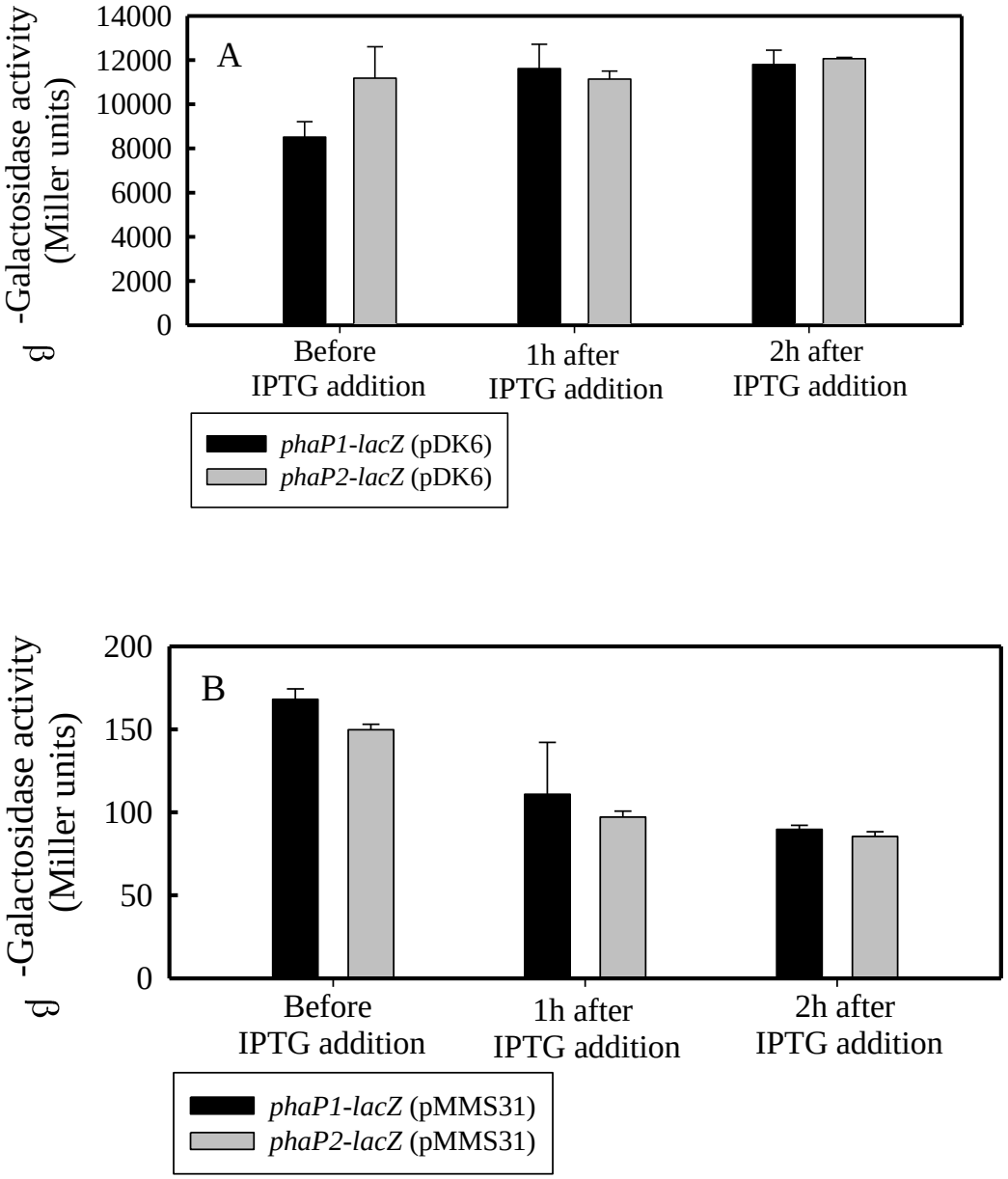


FIG. 4. Transcriptional analysis of *phaP1-lacZ* and *phaP2-lacZ* fusions in *E. coli* ET8000 not expressing (A) the PhbF or expressing (B) PhbF from *H. seropedicae* SmR1. The cultures were grown in LB medium until OD_{600} of 0.6. At this point, the IPTG was added to final concentration of 1 mM. The activity was monitored for two more hours after inducer addition.

The transcription of *phaP1* and *phaP2* is differentially regulated.

To verify the hypothesis that the onset of PHB accumulation is essential to phasin expression, we evaluated the transcriptional time-course profile of P*phaP1-lacZ* and P*phaP2-lacZ* in several background strains of *H. seropedicae* and in medium with DL-malate or glucose as carbon source. In the wt strain growing in DL-malate, we observed that the transcription of *phaP1* was dependent of the growth stage, increasing after OD_{600} of 0.5 when the onset for PHB accumulation is already achieved (Fig. 5A and 2B). The same profile was observed for the Δ*phaP1* mutant, however in this case the transcription of P*phaP1-lacZ* was 42% less than in the wt strain. In the Δ*phaP2* mutant, the transcription of P*phaP1-lacZ* was constitutive with a average value of 2,000 Miller units (23% of the maximum activity measured in the wt strain). Surprisingly, despite the Δ*phaP1.2* strain did not contain PHB granules, as determined by GC and fluorescence microscopy, this strain was able to derepress the transcription of *phaP1*, indicating that not only the granules of PHB can derepress phasin transcription, but also newly synthesized chains of polyhydroxybutyrate could sequester PhbF from DNA. As expected, in the Δ*phaC1* mutant, which is unable to synthesize PHB, the transcription of *phaP1* was totally repressed in all the time points measured. Finally, the mutant with a precise deletion in *phbF*, named Δ*phbF*, presented a constitutive pattern of transcription for *phaP1*. The level of PHB

accumulated in this mutant was measured and, in the same growth conditions used to determine the transcription of *phaP1*, no PHB was detected in this strain (data not shown), indicating that the deletion of *phbF* derepressed *phaP1* transcription.

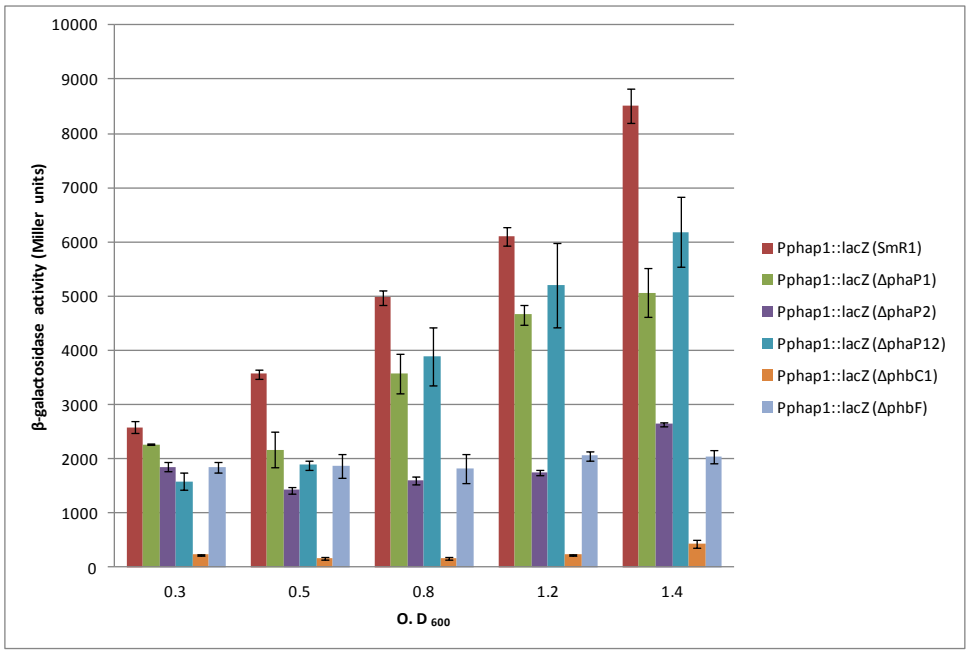
The transcription of *phaP2* was also evaluated in different genetic backgrounds. Interestingly, the transcription of *phaP2* was very low in comparison to the transcriptional level reached for the *phaP1-lacZ* fusion in wt strain (8,500 Miller units, Fig. 5A and B). However, the transcription of *phaP2* was activated in the Δ*phaP1* (Fig. 5B), accordingly with showed previously in the lacking of PhaP1 the homologous PhaP2 was found associated to the granules (34). In the same way, the transcription of *phaP2* was activated in Δ*phaP2* and Δ*phaP1.2* strains (Fig. 5B). As expected, since *phaP2* is also repressed by PhbF (Fig. 5), in the Δ*phaC1* mutant its transcription was totally repressed (Fig. 5B). Notably, the transcription of *phaP2* was decreased in Δ*phbF* mutant when compared to all other strains evaluated (Fig. 5B).

The transcription profile of *phaP1* and *phaP2* were also measured in the minimal medium containing 25 mM of glucose and 20 mM of ammonium chloride. In this condition, we have measured an increasing in the quantity of PHB accumulated. Therefore, it is an appropriate condition to verify if the *phaP* genes transcription level would be also associated to the quantity of PHB produced. The *phaP1* gene was transcribed in all the

strains analysed (wt, Δ*phaP1* and Δ*phaP2*), showing that even in low OD_{600} value as 0.3 the level of beta-galactosidase activity was high (4,000 Miller units) (Fig. 6A). This high value indicates that the quantity of PHB granules into the cells is enough to derepress almost completely the *phaP1* transcription. As observed to the cultures growing in DL-malate, the transcription of *phaP2* was not fully activated in wt, however in Δ*phaP1* the transcription was 5-fold higher than in wt strain, even in low OD_{600} as 0.3 (Fig. 6B). The transcription of *phaP2* was also activated in Δ*phaP2*, exhibiting a similar profile determined to the culture cultivated in DL-malate (Fig. 5B and 6B). In conclusion, *phaP1* transcription was activated when the bacteria started to accumulate PHB. In this condition, PhbF is sequestered to the granules and transcription of *phaP1* is derepressed. However, in the same conditions transcription of *phaP2* is not fully derepressed. In addition, it seems that *phaP2* is only highly transcribed when one or both phasins are lacking.

Figure 5. Transcriptional pattern of *phaP1-lacZ* (A) and *phaP2-lacZ* (B) in *H. seropedicae*, *phaP* mutants, *phaC1* and *phbF* mutant grown in NFb-malate.

A



B

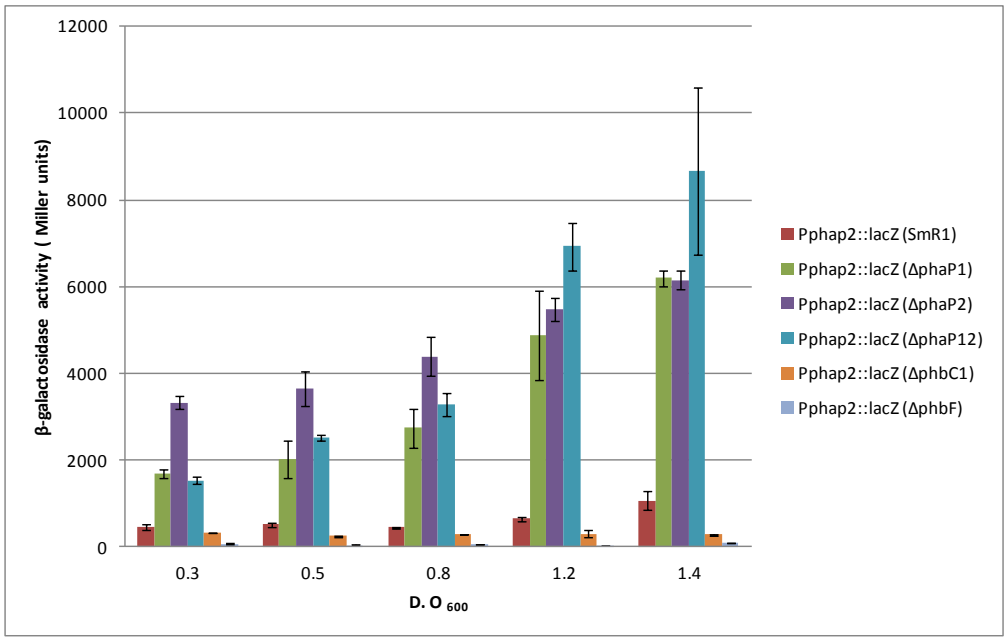
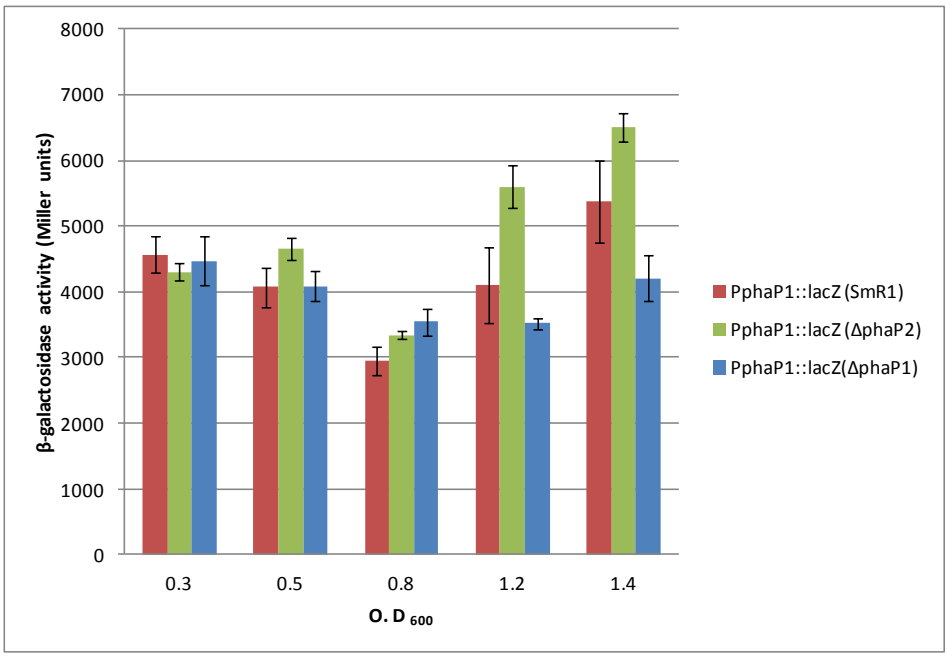
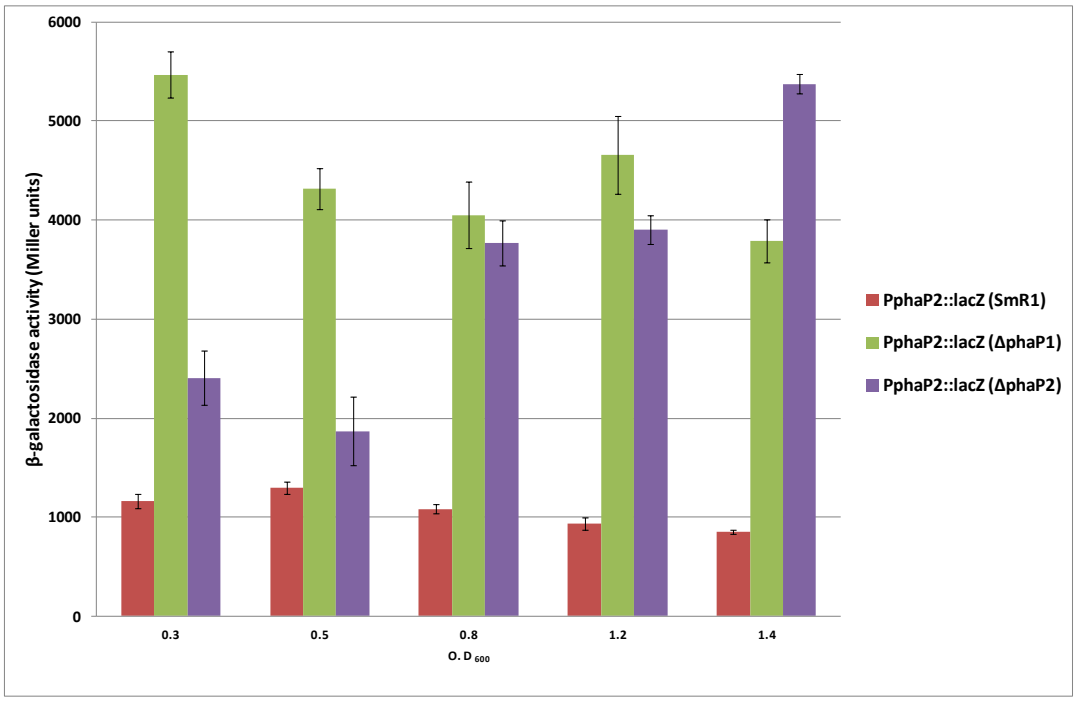


Figure 6. Transcriptional pattern of *phaP1-lacZ* (A)and *phaP2-lacZ* (B) in *H. seropedicae*, Δ*phaP1* and Δ*phaP2* mutants grown in NFb-glucose.

A



B



Discussion

The genome sequencing of *H. seropedicae* SmR1 revealed several genes coding proteins involved in PHB synthesis, degradation and granule biogenesis (4). Two genes coding for putative phasins were located and annotated as *phaP1* (Hsero_1639) and *phaP2* (Hsero_4759). The proteomic analysis of proteins associated to PHB granules in *H. seropedicae* revealed the presence of PhaP1 as the major granule-associated protein (34). Interestingly, when the PhaP1 was lacking, the homologous PhaP2 was mainly found attached to the granules, suggesting that PhaP2 could act as a backup phasin during granule formation.

As an effort to determine the role of these phasins in *H. seropedicae*, in this work we presented a study correlating the influence of phasins in PHB production and in transcription of *phaP1* and *phaP2* genes. We found that the deletion of *phaP1* significantly affected the PHB accumulation, representing a reduction of 50% of PHB/cdw in comparison to the wt (Fig. 2). This result showed clearly that besides the high homology among PhaP1 and PhaP2 (58% of identity, 78% of similarity), PhaP1 seems to be the most important phasin to form and stabilize the PHB granules properly. In order, the deletion of *phaP2* did not affect the quantity of PHB accumulation in the two employed growth media, reinforcing the finding that when PhaP1 is being expressed, the PHB granules are well formed and

stocked. When *phaP1* and *phaP2* genes were deleted in the double mutant, no PHB was detected, indicating that even if *H. seropedicae* has another phasin-like protein, as suggested before (34), PhaP1 and PhaP2 are essential for PHB granules formation in this bacterium. A similar dependence of phasins on accumulation of PHB granules was also related for *Sinorhizobium meliloti* Rm1021 (6). Noticeably, the PhaP1 was the main phasin and, the mutant with *phaP1* deletion presented a reduction of 20% in PHB/cdw and a doubling time of 4 h higher than the wt (6). As observed for *H. seropedicae*, the deletion of *phaP2* has not affected significantly the growth and PHB production (6). Despite the similar behaviour, the phasins from *H. seropedicae* and *S. meliloti* did not present a significant homology among them (data not shown). In comparison to the well studied model *R. eutropha* H16, the behaviour of PHB storage from *H. seropedicae* and *phaP* mutants was quite different. In the case of *R. eutropha*, even the deletion of the four phasin genes has not completely abolished the PHB accumulation (26). Indeed, the *R. eutropha* multiple mutant Δ*phaP1234* reached a maximum of 35% of cdw in comparison to the wt which reached 45% of cdw, at the same growth conditions (26). Although, the authors have reported a fast degradation of PHB in *phaP* mutants of *R. eutropha* H16, particularly those ones defective in *phaP1* and *phaP2* genes, indicating that phasins could also act a relevant role during PHB mobilization (26). In this study, we have not addressed the PHB

mobilization in *phaP* mutants of *H. seropedicae*, however we agree that it will be an important piece of information to further understand the function of phasins in *H. seropedicae* and other bacteria.

The growth profile of the mutants in minimal media containing DL-malate or glucose revealed interesting observations. In general, the deletion of *phaP1* (strains Δ*phaP1* and Δ*phaP1.2*) impaired the growth in both media. However, an intriguing result was the significant difficulty to growth in glucose as the sole carbon source. This phenotype was also observed, and even more drastic, to the Δ*phaC1* mutant of *H. seropedicae* SmR1, which is completely impaired in PHB synthesis (data not shown). These results indicate that in somehow the decreasing or lacking of PHB granules in *H. seropedicae* affects negatively its capacity to metabolize glucose properly. The bases for the emergence of this phenotype are not known until now. As already reported in the literature, the production of PHB in the absence of phasins can potentially generate a stress condition that lead to protein denaturation and aggregation, that ultimately might negatively affect the growth rate as well (49-53). As reported in this work, the production of PHB was higher in glucose as carbon source, then it was expected that when growing in this medium the bacterial strains unable to express the PhaP1 (Δ*phaP1* and Δ*phaP1.2*) have faced a more stressful condition to growth.

A well accepted model for transcriptional regulation of *phaP* genes was established in *R. eutropha* H16 and *Paracoccus denitrificans* (45-48, 54, 55). In this model the transcriptional repressor PhaR (PhbF in *H. seropedicae* SmR1) binds in specific DNA sites into the regulatory region upstream of *phaR* and *phaP* genes. When the bacterium reaches the onset to PHB synthesis, the PhaR repressor is sequestered from the DNA and transcription is released. Thus, PhaR is generally recognized as a device coupling of PHB synthesis to the phasin expression (48). In *H. seropedicae* SmR1, we have previously characterized its activity *in vitro* and showed the transcriptional repressor activity of PhbF (3). In this work, we showed that PhbF acts as a transcriptional repressor of *phaP1* and *phaP2* transcription, expressing PhbF heterologously in *E. coli* (Fig. 4A and B). In *H. seropedicae*, the pattern of transcription of *phaP1* indicates that when the culture is producing and accumulating PHB in form of granules, PhbF is sequestered and *phaP1* transcription is released (Fig. 5A and 6A). Intriguingly, the transcription of *phaP2* is around 8-fold lower than *phaP1* transcription when *H. seropedicae* reaches the maximum quantity of PHB (Fig. 5B and 6B**).** However, when the strain is defective in *phaP1*, the transcription of *phaP2* is active (Fig. 5B and 6B). Assuming that both genes are repressed by PhbF, and that in the initial stage of PHB synthesis PhbF is sequestered from both regulatory sites, it seems that *phaP2* needs an specific activator to be transcribed**.** In fact, in the Δ*phbF* mutant we

have measured a constitutive expression of *phaP1* (around 2,000 Miller units in all measured points) (Fig. 5A), while a very low beta-galactosidase activity was recorded to the *phap2-lacZ* fusion in the same strain in all points measured (Fig. 5B). This result will prompt us to investigate in the future if some kind of stress response, as for instance protein aggregation, is mediating *phaP2* transcription.

Finally, the existence of two highly homologous genes coding for proteins with the predicted same function has raised the question whether the phasins of *H. seropedicae* does not represent a genuine case of genetic redundancy. Genetic redundancy is a term that describes a situation when two or more genes coding for gene products performing the same function in one organism and that inactivation of one of these genes has little or no effect on its phenotype. Redundant genes are frequently eliminated if the selective pressure is removed, however in other cases the gene duplication can evolve phenotypes to be more fit and robust (56). In particular, it is unlikely that PhaP1 and PhaP2 represent a genuine case of genetic redundancy, since the deletion of *phaP1* clearly resulted in a less fit phenotype, in accordance with its PHB accumulation pattern and growth in glucose. The fact that *phaP2* is differentially regulated suggested that during evolution PhaP2 has diverged its function not just to aid PhaP1 during granule biogenesis, but also to perform a role of support when unfavorable conditions for PHB storage are encountered.

Acknowledgments

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CONSIDERAÇÕES FINAIS

Os resultados deste trabalho de Mestrado apontam que na ausência de PhaP1 a produção de grânulos de PHB em *H. seropedicae* SmR1 é prejudicada, embora a fasina PhaP2 possa parcialmente complementar a função de sua homóloga PhaP1. Sendo assim, ainda nos resta investigar possíveis alterações morfológicas que ocorram na formação dos grânulos na ausência de PhaP1 através de microscopia eletrônica de transmissão.

Além disso, os fenótipos apresentados neste trabalho para os mutantes *phaP*, nos levam a acreditar que outros processos fisiológicos importantes em *H. seropedicae* como a fixação de nitrogênio possam ser igualmente afetados, devido a redução de PHB na forma de grânulos. Outro estudo que poderá ser desenvolvido futuramente é endereçar a investigação não somente para a produção de PHB, mas também observar o que ocorre na degradação do polímero em mutantes *phaP*.

CONCLUSÕES

- PhaP1 é a principal fasina em *H. seropedicae* SmR1, e na sua ausência, a PhaP2 consegue substituí-la parcialmente.
- A ausência de PhaP1 e PhaP2 prejudica de forma relevante o crescimento da bactéria.
- Há uma maior produção de grânulos de PHB em meio contendo como única fonte de carbono glucose, do que em malato, para todas as estirpes.

- O regulador PhbF reprime a transcrição dos genes *phaP1* e *phaP2*.
- O perfil transcricional de *phaP1* e *phaP2* revelou que na ausência de PhaP1 a transcrição de *phaP2* é ativada.

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ANEXO I

METODOLOGIA

BACTÉRIAS E CONDIÇÕES DE CULTIVOS

Durante todo o trabalho foi utilizada a bactéria *Herbaspirillum seropedicae* SmR1 e os mutantes *ΔphaP1*, *ΔphaP2*, *ΔphaP12*, *ΔphbC1* e *ΔphbF*. As estirpes foram normalmente cultivadas em meio NFbHPN com 37 mM de malato, 25 mM de glucose e 20mM de NH4Cl a 30° C. (PEDROSA, *et al*., 1984). A bactéria *Escherichia coli* estirpe ET8000 e S17. 1 foram crescidas a 37° em meio Luria – Bertani (LB) (SAMBROOK *et al*., 1989).

Para os experimentos de curva de crescimento, as culturas foram medidas por densidade optica a 600nm a cada hora, e então foram obtidas as curvas de crescimento e posteriormente através de regressão linear da fase exponencial de crescimento do microrganismo foram obtidas as taxas de crescimento.

QUANTIFICAÇÃO DE PHB

Para a quantificação de polihidroxibutirato as amostras foram determinadas por cromatografia gasosa através de detector por ionização de chama (FID) segundo o método de Braunegg *et al.* (1978).

Durante os cultivos, a quantidade de PHA nas amostras foram determinadas por cromatografia gasosa com detector por ionização de chama (FID) baseado no método de Braunegg *et al.* (1978). O precipitado

de células foi liofilizado e pesado em um tubo Pyrex® com tampa para ser metanolizado com 2 ml de clorofórmio e 2 ml de solução de metanol contendo 15% de ácido sulfúrico. As reações foram incubadas a 100°C durante 3,5 horas para assegurar que todo o material seria esterificado. Após esse tempo os tubos foram resfriados em gelo por 30 a 60 minutos, e então foram misturados por 30 segundos com água MiliQ em vortex. A fase aquosa e a fase orgânica se separaram por 30 minutos, e a fase aquosa foi retirada totalmente com uma pipeta Pasteur. Após separação das fases, a fase inferior que contém o éster metílico de ácido 3-hidróxibutírico foi seca com Na_2SO_4. Os ésteres foram quantificados em um cromatógrafo a gás. Foi utilizada uma coluna capilar CP-Sil-5CB (Varian Inc.) de 30 m x 0.25 mm. Como gás de arraste foi utilizado N_2 em um fluxo de 0,7 ml/min. A temperatura do injetor foi mantida em 250ºC, com rampa de temperatura na coluna iniciando em 30ºC, subindo até 200ºC a 40ºC/min. A quantificação foi realizada utilizando uma curva padrão feita com padrão autêntico de 3-hidroxibutirato (Sigma Aldrich-Co).

DETERMINAÇÃO DA PRESENÇA DOS GRÂNULOS POR MICROSCOPIA DE FLUORESCÊNCIA

Para investigar em maiores detalhes a morfologia dos grânulos de PHB em *H. seropedicae* SmR1 e mutantes *phaP*, análise de microscopia de fluorescência foi realizada após marcação das bactérias com a sonda fluorescente vermelho Nilo, específica para lipídeos neutros.

As culturas (1ml) de todas as estirpes foram centrifugadas por 60 segundos a 14, 5 rpm em eppendorfs de 1,5 ml. Após isso o sobrenadante foi descartado, ressuspenso em etanol 30% em salina, então adicionado 3µL do corante vermelho Nilo e foram incubados por 5 minutos no escuro.

Por fim, as amostras foram centrifugadas novamente por 60 segundos a 14,5 rpm, ressuspensas em salina e enviadas para a analise de microscopia. As amostras foram colocadas em lâminas próprias para tal experimento.

ATIVIDADE β-GALACTOSIDASE

Todas as análises transcricionais foram realizadas segundo o Método de Miller (1972). Seguindo esse modelo o sistema de reação continha 950 µL de tampão Z completo, 100 µL de clorofórmio e 50 µL de cultura, e então foram misturados em vortex e incubados por 5 minutos em banho a 30°C.

A reação teve início com a adição de 200 µL do substrato ONPG e interrompida quando foi adicionado 500 µL de Na2CO3. Por fim as amostras foram submetidas a leitura de absorbância do o-nitrofenol (420nm), turbidez da mistura (550nm) , além disto foi realizada a leitura de 600 nm da densidade celular.

Seguindo a fórmula, a atividade foi expressa em Unidades Miller:

Unidades Miller = 1000 x (DO 420nm – 1,75 x DO 550nm) / (D.O. 600nm x t x v)

t = tempo de reação (minutos) e v = volume de cultura (mL).

ANÁLISE TRANSCRICIONAL DOS GENES *phaP1* E *phaP2* EM *E. coli* ET 8000

Atividade β-galactosidase foi determinada em *Escherichia coli* ET8000 com a fusão dos plasmídeos (pdK6) na presença ou ausência do plasmídeo

pMMS31, o qual expressa PhbF, e foram crescidas em meio Luria – Bertani.

ANÁLISE TRANSCRICIONAL DOS GENES *phaP1* E *phaP2* EM *Herbaspirillum seropedicae* SMR1

As fusões transcricionais das regiões regulatórias dos genes *phaP1* e *phaP2* com o gene repórter lacZ que expressa a enzima β-galactosidase foram obtidas clonando a região a montante do gene no vetor pMP220 (SPAINK et al., 1987). Os plasmídeos obtidos foram transformados em *E. coli* S17-1 e conjugados para *H. seropedicae* SmR1 e mutantes *ΔphaP1, ΔphaP2*, *ΔphaP1/phaP2, ΔphbC1 e ΔphbF* . O nível de transcrição das fusões foi avaliado pela atividade β-galactosidase determinada pelo método de Miller (1972) utilizando como substrato o-nitrofenil-β-galactosídeo (ONPG).