UNIVERSIDADE FEDERAL DO PARANÁ

LUIS PAULO SILVEIRA ALVES

INVESTIGAÇÃO DO PAPEL DO METABOLISMO DE POLIHIDROXIBUTIRATO NA RESPOSTA ANTI-ESTRESSE E NA COLONIZAÇÃO DE PLANTAS EM *Herbaspirillum seropedicae* SmR1.

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

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RESUMO

Herbaspirillum seropedicae é uma bactéria fixadora de nitrogênio, endofítica, promotora de crescimento vegetal e produtora de um polímero estocado internamente, denominado polihidroxibutirato (PHB). O PHB é produzido a partir da condensação de monômeros de ácido 3-hidroxibutírico e estocado na forma de grânulos insolúveis. Bactérias acumulam maior quantidade de PHB, geralmente, em situações de excesso de carbono ou falta de algum outro nutriente essencial como fosfato e nitrogênio. O PHB estocado pode ser utilizado como uma reserva energética, sendo mobilizado quando a bactéria se encontra em situações de estresse. A superfície dos grânulos de PHB é revestida por várias proteínas, principalmente aquelas envolvidas na síntese e na degradação do polímero. As proteínas de superfície mais abundantes são as fasinas, que têm um papel fundamental na manutenção do número e do tamanho dos grânulos no interior da célula. Além das fasinas, PHA sintases, PHA depolimerases e o regulador transcricional PhaR também são encontrados associados à superfície. Recentemente, o papel do PHB na interação planta-bactéria passou a ser novamente discutido, devido a alguns microrganismos mobilizarem os grânulos de PHB como forma de defesa antioxidante durante a colonização de raízes de plantas. Além disso, dados de análise transcriptômica apontam que a síntese e a degradação de PHB podem ocorrer durante a colonização de plantas por *H. seropedicae* e *Azospirillum brasilense*.

Sendo assim, nesse trabalho de tese objetivou-se avançar no entendimento do papel do PHB para a proteção de *H. seropedicae* contra o estresse celular e para a colonização de plantas. Nessa tese serão apresentados resultados relacionados a (i) uma metodologia criada para uma rápida quantificação de PHB através da técnica de citometria de fluxo, (ii) a influência das fasinas na produção de PHB em *H. seropedicae*, (iii) o papel do PHB frente a diferentes tipos de estresse *in vitro* e (iv) a relevância do PHB durante o processo de colonização de plantas.

Os resultados do Capítulo 1 mostram que é possível substituir a técnica laboriosa de quantificação de PHB baseada em metanólise e cromatografia a gás pela citometria de fluxo de células coradas com Vermelho Nilo. Desta forma o processo de quantificação de PHB pode ser realizado em intervalos curtos de 5 min, além de permitir a quantificação de concentrações diminutas do polímero, requerendo poucas células. Tal metodologia é de extrema importância para se determinar a quantidade de PHB em populações de bactérias colonizando a superfície de raízes, o que seria inviável

utilizando a metodologia por cromatografia a gás devido ao grande número de plantas necessárias para se obter a quantidade de biomassa bacteriana necessária.

No Capítulo 2, demonstrou-se o papel das fasinas para a síntese de PHB e a importância da redundância genética entre os genes *phaP1* e *phaP2*, assegurando a síntese de PHB e o *fitness* de *H. seropedicae*.

No Capítulo 3, os resultados mostram que durante o choque térmico, tanto a síntese quanto a degradação do polímero são importantes para a sobrevivência da bactéria. Sendo que, as bactérias que não produzem o grânulo possuem uma taxa de sobrevivência muito menor comparado à estirpe selvagem e o mesmo ocorre com as estirpes que não conseguem mobilizar os grânulos durante o estresse.

No Capítulo 4, apresentam-se resultados que demonstram a importância do metabolismo de PHB para o processo de colonização de plantas pela bactéria *H. seropedicae* SmR1. Realizou-se ensaios de interação planta-bactéria utilizando as estirpes mutantes defectivas na produção e degradação do polímero, e novamente foram observados resultados significativos quanto ao desenvolvimento das raízes de plantas inoculadas, assim como uma significativa redução da população endofítica das bactérias incapazes de produzir ou degradar PHB adequadamente.

Palavras-chave: *Herbaspirillum seropedicae*, polihidroxibutirato, fasinas, interação planta-bactéria, citometria de fluxo.

ABSTRACT

Herbaspirillum seropedicae is a nitrogen-fixing, endophytic, plant growth promoter and producer of an internally stored polymer, called polyhydroxybutyrate (PHB). This polymer is produced by the condensation of monomers of 3-hydroxybutyric acid and stored as insoluble granules. Bacteria accumulate more PHB in situations of excess carbon or lack of some other essential nutrients like phosphate and nitrogen. In this way, PHB can be used as an energy reserve and mobilized when the bacteria are in a stressful situation. On the surface of the PHB granules, there are many proteins involved in the synthesis and degradation of the polymer. The most abundant are the phasins, which play a key role in maintaining the number and size of the granules inside the cell. In addition to the phasins, PHA synthases, PHA depolymerases and the transcriptional regulator PhaR are also found associated with the surface. Recently, the role of PHB in plant-bacteria interaction has been actively discussed, because some bacteria mobilize PHB granules as a form of antioxidant defense during the colonization of plant roots. Also, data from transcriptomic analysis indicate that the synthesis and degradation of the PHB occur during the colonization of plants by *H. seropedicae* and *Azospirillum brasilense*.

Thus, the objective of this thesis was to advance the understanding of the role of the PHB protecting *H. seropedicae* against stressful conditions and for the colonization of plants. This thesis will present results related to (i) the methodology established for a rapid quantification of PHB through the flow cytometry technique, (ii) the influence of the phasins on PHB production in *H. seropedicae*, (iii) the protecting role of PHB against different types of stress in *H. seropedicae* and (iv) the relevance of the polymer during the plant colonization process.

The results of Chapter 1 showed that it is possible to replace the labour-intensive technique of quantification of PHB based on methanolysis and gas chromatography by flow cytometry of cells stained with Nile Red. In this way, the PHB quantification process can be performed at 5-min intervals, whilst allowing the quantification of PHB at low concentrations and applying a few number of cells. Such methodology is critical to determine the amount of PHB in bacterial populations colonising the root surface, which would be impracticable using the methodology by gas chromatography due to the high biomass content required (~ 5 mg of liophylized bacteria).

In Chapter 2, the role of phasins for PHB synthesis and the importance of genetic redundancy between *phaP1* and *phaP2* genes were demonstrated, ensuring PHB synthesis and the fitness of *H. seropedicae* SmR1.

In Chapter 3, the results show that during thermal shock, both the synthesis and the degradation of the polymer are essential for the survival of the bacterium. The bacteria that do not produce the granule have a much lower survival rate than the wild-type strain, and so do the strains that cannot mobilise the granules during stress.

In Chapter 4, we present results that demonstrate the importance of PHB metabolism for the process of colonization of plants by *H. seropedicae* SmR1. Plant-bacteria colonization assays were performed using mutants defective in the production and degradation of the polymer, and again significative results were determined for PHB-producing strains on the development of inoculated plant roots, while a significative reduction in the endophytic population of bacteria unable to produce or degrade PHB properly.

Keywords: *Herbaspirillum seropedicae,* polyhydroxybutyrate, phasin, plant-bacteria interaction, flow cytometry.

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1. INTRODUÇÃO

1.1 *Herbaspirillum seropedicae* como modelo endofítico para o estudo do metabolismo de PHB

Herbaspirillum seropedicae é uma bactéria da classe Betaproteobacteria aeróbica, prototrófica, endofítica, fixadora de nitrogênio e promotora de crescimento vegetal (BALDANI et al., 1986). A ocorrência de *H. seropedicae* no interior de plantas de interesse comercial, tais como milho, cana-de-açúcar, arroz e sorgo, sem desenvolvimento de doença e promovendo o crescimento vegetal, já foi relatada previamente por diversos estudos (MONTEIRO et al., 2012). Portanto, há um grande interesse no estudo de aspectos fisiológicos desta bactéria e da sua associação com plantas, visando sua potencial aplicação na agricultura como um biofertilizante.

Diversas bactérias associadas a plantas, incluso *H. seropedicae*, apresentam como característica fenotípica a produção de polihidroxialcanoatos (PHA), em crescimento planctônico e/ou durante a colonização de tecidos vegetais. PHA são poliésteres formados pela condensação enzimática de 3-hidroxiacil-CoA, sendo 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). O PHB é formado pela condensação enzimática de 3-hidroxibutiril-CoA, produzido a partir de acetil-CoA (MADISON & HUISMAN, 1999). A síntese de PHB a partir de acetil-CoA ocorre através de uma via de três passos: 1) condensação de duas moléculas de acetil-CoA formando acetoacetil-CoA através da ação da beta-cetotiolase (PhaA); 2) redução de acetoacetil-CoA a R-3-hidróxibutiril-CoA pela enzima acetoacetil-CoA redutase NADPH dependente (PhaB) e 3) polimerização de R-3-hidróxibutiril-CoA pela enzima PHA sintase (PhaC) (STEINBUCHEL & HEIN, 2001). Além das proteínas envolvidas na síntese, também tem papel ativo no metabolismo de PHB o regulador negativo PhaR (anteriormente denominado PhbF em *H. seropedicae*) e a proteína estrutural fasina (PhaP) que reveste o grânulo recém-formado (POTTER et al., 2004; KADOWAKI et al., 2011).

O sequenciamento do genoma de *H. seropedicae* SmR1, revelou a presença de 13 genes potencialmente envolvidos na síntese e formação de grânulos de PHB, assim

como na sua degradação, através das PHA depolimerases (PhaZ) (PEDROSA et al., 2011).

1.2 Avanços na caracterização do metabolismo de PHB em *H. seropedicae* SmR1

Alguns mutantes de *H. seropedicae* SmR1 para genes potencialmente envolvidos no metabolismo de PHB foram construídos no Núcleo de Fixação Biológica de Nitrogênio (NFIX) da UFPR, entre eles: mutantes defectivos na expressão das fasinas PhaP1 e PhaP2 (Δ*phaP1*, Δ*phaP2* e Δ*phaP1.2*), defectivo na expressão da PHA sintase PhaC1 (Δ*phaC1*), defectivos na expressão das PHA depolimerases PhaZ1 e PhaZ2 (Δ*phaZ1*, Δ*phaZ2* e Δ*phaZ1.2*), defectivo na expressão do principal regulador transcricional PhaR (Δ*phaR*) e defectivos na expressão de genes que podem estar indiretamente envolvidos no metabolismo de PHB tal como na expressão do fator sigma RpoS ou σ^S (Δ*rpoS*).

A deleção do gene *phaC1* aboliu por completo o acúmulo de grânulos de PHB em *H. seropedicae* (TIRAPELLE et al., 2013; ALVES et al., 2016). Nesse mutante, há alta propagação de espécies reativas de oxigênio (ROS), afetando a expressão de genes controlados pelo regulador transcricional Fnr e diminuindo significativamente a fixação de nitrogênio (TEIXEIRA, 2015). Acredita-se que o ambiente mais oxidativo seja decorrência da ausência do PHB e que afeta de forma generalizada metaloproteínas sensíveis a oxigênio e ROS. Além disso, dados de colonização de plântulas de milho mostraram que o mutante Δ*phaC1* teve uma redução de 8 vezes na colonização epifítica no estágio inicial de colonização (1 dia após inoculação) em comparação a estirpe selvagem (BALSANELLI et al., 2015). Esses dados mostram claramente que a produção de PHB é um fator importante para o *fitness* e possivelmente para a competitividade da bactéria durante a colonização de plantas.

A deleção do gene *phaP1* reduziu em cerca de 50% o PHB acumulado, como no caso do mutante Δ*phaP1*, ou aboliu totalmente o acúmulo de PHB no duplo mutante Δ*phaP1.2* (ALVES et al., 2016). Já o mutante Δ*phaP2* apresentou um fenótipo similar a estirpe parental SmR1, quanto a produção de PHB e outras características. Para os mutantes Δ*phaP1* e Δ*phaP1.2*, também se observou uma drástica redução no crescimento em D-glucose, o que indica que a falta de produção e acúmulo de PHB pode afetar outras vias metabolicas e potencialmente o balanço redox da célula. O fato

de esses mutantes apresentarem diferentes níveis de acúmulo de PHB os tornam uma ferramenta interessante para se estudar a correlação entre a produção de PHB e a capacidade de colonização de plantas.

A deleção dos genes *phaZ1* e *phaZ2* não afeta a síntese de PHB, entretanto o duplo mutante *phaZ1.2* é incapaz de mobilizar o PHB estocado quando submetido a condições de limitação nutricional ou a estresse como choque térmico a 45ºC (Capítulo 3 dessa Tese). Análise transcriptômica de raízes de trigo (*Triticum aestivum*) colonizadas com *H. seropedicae* SmR1 mostrou um aumento de 2,7 a 1,5 vezes na transcrição dos genes de biossíntese de PHB (*phaABC1*) e de 2 vezes para o gene *phaZ1*, que expressa uma das PHA depolimerases envolvidas na mobilização de PHB (PANKIEVICZ et al., 2016). Além disso, *H. seropedicae* SmR1 colonizando raízes de milho (*Zea mays*) teve um aumento de 12 e 16 vezes na transcrição do gene *phaZ2* após 1 dia de inoculação, tanto para bactérias planctônicas quanto para epifíticas (BALSANELLI et al., 2015). Esses resultados mostram que provavelmente o metabolismo de PHB ativo seja vantajoso para que *H. seropedicae* se estabeleça e colonize plantas.

A deleção do gene *phaR* que codifica o repressor transcricional PhaR em *H. seropedicae* SmR1 (KADOWAKI et al., 2011) reduziu em cerca de 90% a quantidade de PHB acumulado (GAVIDIA, 2017)

Alguns autores mencionam que o fator sigma S (RpoS) pode ser responsável por ativar genes durante algum tipo de estresse que a bactéria possa sofrer, como choque térmico ou oxidativo (HENGGE-ARONIS, 2002; RAMOS-GONZÁLEZ et al., 1998). A partir disto, foi realizada a construção do mutante Δ*rpoS* com o intuito de verificar se o fator σ^S pode ser ativado apartir do choque térmico e como se comporta a produção do PHB neste mutante no decorrer do estresse. Esse mutante será explorado no capítulo 3 dessa Tese. Os resultados apontam o envolvimento de RpoS na mobilização de PHB em *Herbaspirillum seropedicae* SmR1.

1.3 O papel do PHB na interação planta-bactéria

Apesar de várias bactérias associativas de plantas terem sido descritas como produtoras de PHB, até o momento poucos trabalhos avaliaram o papel desse polímero no mecanismo de interação planta-bactéria. Essa relação foi mais bem estudada em modelos de interação entre leguminosas e *Rhizobium* sp. ou *Sinorhizobium* sp.

(CEVALLOS et al., 1996; MANDON et al., 1998; LODWIG et al., 2005). Portanto, para a interação de outras bactérias com gramíneas ainda há um grande campo de estudo a ser explorado. Recentemente, Koskimäki et al. demonstraram que a degradação de PHB em *Methylobacterium extorquens* gera oligômeros de ácido 3-hidroxibutírico que atuam como defesa antioxidante durante a infecção de raízes de *Pinus sylvestris* (pinheiro-silvestre) (KOSKIMAKI et al., 2016). Esses resultados abrem um precedente importante para investigar se o mecanismo proposto por Koskimäki et al. (2016) aplica-se também a bactérias associadas a outros tipos de plantas. Análises transcriptômicas de raízes de milho e trigo colonizadas por *Herbaspirillum seropedicae* SmR1 apontam que tanto a síntese quanto a mobilização de PHB devem estar ocorrendo durante a colonização (BALSANELLI et al., 2015; PANKIEVICZ et al., 2016). Isso estaria de acordo com o mecanismo de defesa antioxidante proposto por Koskimäki e colaboradores no caso de *Methylobacterium extorquens*. Além disso, o ciclo de síntese e degradação de PHB tem sido sugerido como um fator importante na interação planta-bactéria também no caso de rizóbios-leguminosas (TRAINER & CHARLES, 2006).

Na próxima seção será apresentada a revisão bibliográfica sobre os principais tópicos apresentados na introdução desse trabalho.

2. REVISÃO BIBLIOGRÁFICA

2.1 Gênero *Herbaspirillum*

Herbaspirillum é o gênero referente a bactérias que podem se associar a plantas que não possuem a propriedade de produzir tecido lenhoso. Além disto, Baldani et al., (1986) descrevem esta bactéria como pertencente a um gênero que se caracteriza por serem bactérias gram-negativas, na maioria dos casos vibróide e algumas vezes helicoidais. Como característica, também, apresentam 1 a 3 flagelos em um único polo da célula ou em ambos os polos **(Figura 1)**, configurando certa motilidade a este gênero. Seu tamanho tem aproximadamente 0,6 a 0,7 μm de diâmetro e o comprimento varia de 0,5 a 5 μm. Bactérias pertencentes a este gênero fixam nitrogênio atmosférico sob condições microaeróbicas e podem crescer utilizando N_2 como única fonte de nitrogênio. *Herbaspirillum* tem como habitat natural o solo e as raízes de plantas pertencentes à classe das Gramíneas (BALDANI et al., 1996).

2.2 Herbaspirillum seropedicae

O nome da espécie *seropedicae* remete a uma homenagem à cidade de Seropédica no estado do Rio de Janeiro, onde foi isolada pela primeira vez (BALDANI et al., 1986). Assim como as bactérias do seu gênero, esta espécie também é vibróide, helicoidal e móvel quando há concentração ideal de O_2. Baldani et al. (1982) descrevem esta espécie como uma bactéria que possui um crescimento mais lento na presença de N_2 se comparada ao crescimento de *Azospirillum* spp, no entanto na presença de 2% de cloreto de sódio *H. seropedicae* não consegue crescer. Além de fixar nitrogênio sob condições restritas de amônio e oxigênio, esta bactéria é prototrófica, endofítica, consegue promover o crescimento vegetal, pertence a classe das β-proteobacteria e pode ser encontrada dentro de tecidos vegetais de arroz, sorgo, cana-de-açúcar e milho sem causar danos a planta (PIMENTEL et al., 1991; BALDANI et al., 1992, 1996; GYANESHWAR et al., 2002; OLIVARES et al., 1996, 1997; JAMES et al., 1997 e 2002; RONCATO-MACCARI et aal., 2003; MONTEIRO et al., 2012).

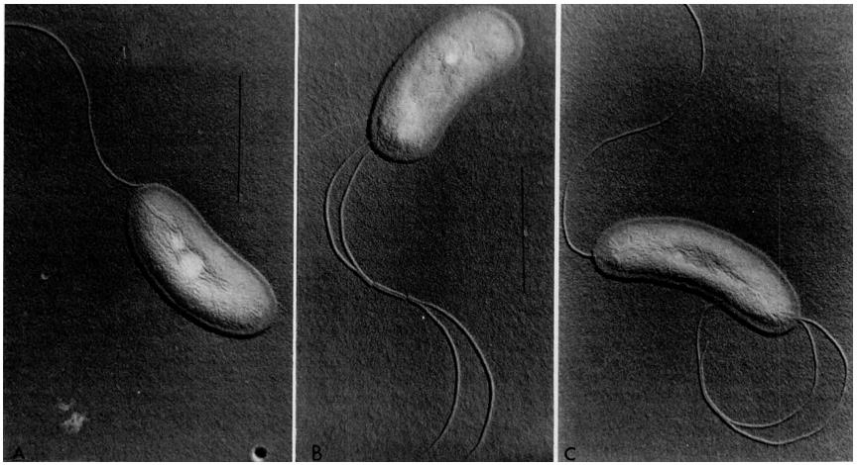


Figura 1. Eletromicrografia de transmissão de *H. seropedicae* crescendo em meio com ágar (Fonte: BALDANI et al., 1986).

Por isso, a utilização destas bactérias fixadoras de nitrogênio e promotoras de crescimento vegetal como *H. seropedicae*, pode ser uma alternativa para substituir os fertilizantes químicos que prejudicam não somente a natureza, mas também a saúde humana (LADHA et al., 1997).

2.3 POLIHIDROXIALCANOATOS

Os PHA, os quais são estudados desde 1920, são macromoléculas sintetizadas por bactérias e são compostos de monômeros unidos por ligações ésteres **(Figura 2)**. Estes polímeros possuem uma alta massa molecular, entre 50.000 e 100.000 Da. (CATALAN et al., 2006). Algumas bactérias acumulam grânulos de PHA no seu interior como um armazenamento de carbono quando algum outro nutriente, tal como fósforo, enxofre, nitrogênio ou oxigênio é diminuído ou restrito devido a alguma situação ambiental (STEINBÜCHEL, 1991; ANDERSON & DAWES, 1990). Em *Ralstonia eutropha* H16, um organismo modelo para o estudo do metabolismo de PHA, encontrou-se a presença de PHB ocupando 90% do peso seco da célula (SCHLEGEL et al., 1961; WILDE et al., 1962). Dentre toda a família de PHA já caracterizada, o PHB é o PHA mais abundante em bactérias, incluindo *H. seropedicae* SmR1.

Os PHA podem ser utilizados como reserva de carbono e energia, uma vez que durante a síntese há o consumo de equivalentes reduzidos na forma de NADPH (MADISON et al*.,* 1999). No entanto, quando a bactéria se encontra em condições inóspitas ao seu desenvolvimento, como falta de carbono, a tendência é que o microrganismo utilize o polímero para posterior produção de acetil-CoA e assim haja a regeneração de NADPH (REN et al., 2009).

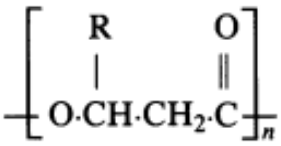


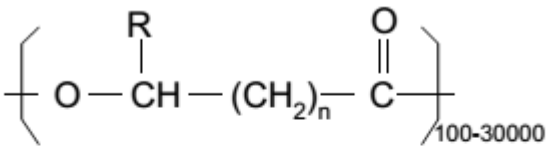
Figura 2. Estrutura química de um polihidroxialcanoato genérico (Anderson & Dawes, 1990). R representa um radical que normalmente varia de 1 a 12 átomos de carbono. PHB é o polímero que possui 1 carbono como cadeia lateral R e é o mais produzido por bactérias.

Além de atrair o interesse do meio acadêmico, polihidroxialcanoatos têm chamado à atenção da área industrial e da sociedade, pois são nítidos os problemas com descarte de materiais plásticos que provem da indústria petroquímica. Assim, desenvolver materiais plásticos com propriedades biodegradáveis é cada vez mais

necessário. Por isso o estudo do metabolismo de PHA em bactérias é relevante tanto do ponto de vista acadêmico quanto da biotecnologia. Esses polímeros, após passarem por processos industriais, possuem características semelhantes aos plásticos de origem petroquímica como o polietileno e polipropileno, podendo assim ser um substituinte adequado para o uso e também para a produção industrial (HOLMES 1988; LEE 1996a; STEINBÜCHEL 1991a; 1992). Algumas empresas já fabricam materiais utilizando PHA, como garrafas, canetas e embalagens para cosméticos (WEBB, 1990). Entretanto, ainda há um alto custo para a produção destes polímeros, uma vez que há gastos com as fontes de carbono, manipulação genética da estirpe capaz de sintetizar o PHA, métodos eficazes para purificação do grânulo, entre outros custos (REDDY et al., 2003). Contudo, para tentar reduzir os custos da produção do PHA e assim aumentar a produção e o rendimento, alguns estudos mencionam que são importantes novas estratégias de cultivos com substratos de carbono renovável e de baixo custo, além do modo de como ocorre o processo de recuperação do PHA, uma vez que isto pode elevar todo o processo econômico (LEE, 1996; BYROM, 1992; KIM et al., 1994, PARK & DAMODARAU, 1994; ISHIZAKI & TANAKA, 1991; POIRIER et al., 1995; DOI et al., 1988). Para isto ocorrer é necessária a presença de um solvente aquoso que quebra a parede celular e assim ocorre a extração do PHA e após isso a purificação do polímero. No entanto, este processo diminui o peso molecular final do grânulo (LUZIER et al., 1992). Entre os solventes que podem ser utilizados, estão: clorofórmio, cloreto de metileno, carbonato de propileno e dicloroetano (BAPTIST, 1962; LAFFERTY et al., 1988; RAMSAY et al., 1994). Faz-se importante ressaltar que a necessidade de se utilizar um solvente é devido a viscosidade que o polihidroxialcanoato apresenta, o que de certa maneira pode elevar o custo do processo. Contudo, após a otimização de extração do polímero, isto pode diminuir o custo total do processo de síntese de produtos que provem do PHA (LEE et al., 1996).

Alguns estudos mencionam que os substituintes da cadeia lateral podem ser modificados de forma química e que esta variação no comprimento da cadeia e a mudança na composição das cadeias laterais pode causar uma mudança no tipo de polihidroxialcanoato que uma bactéria pode produzir. PHAs de cadeias curtas geralmente possuem de 2 a 5 átomos de carbono, cadeias médias têm 6 a 12 átomos de carbono, cadeias longas podem possuir mais de 12 átomos de carbonos, podem ainda serem hetero ou homopolímeros **(Figura 3)** (BYROM 1987; STEINBUCHEL, 1995;

WILLIAMS & PEOPLES, 1996; MADISON & HUISMAN, 1999). Além disso, como as bactérias podem utilizar diversas fontes de carbono para sintetizar PHA, isto pode fazer com que alterem o tipo de PHA a ser produzido dependendo das rotas metabólicas que utilizam para produzir os 3-hidroxialcanoil-CoA indispensáveis para a síntese do polímero. Contudo, o PHA mais comumente produzido por bactérias gram-negativas e que tem despertado interesse comercial devido as suas inúmeras propriedades químicas e físicas, benéficas ao meio ambiente e é produzido em grande quantidade é o polihidroxibutirato (CHOI & LEE, 1999; REDDY, GHAI & KHALIA, 2003).



n =1	R =	hydrogen	poly (-3-hydroxypropionate)
		methyl	poly (-3-hydroxybutyrate)
		ethyl	poly(3-horoxyvalerate)
		propyl	poly (-3-hydroxyhexanoate)
		pentyl	poly (-3-hydroxyoctanoate)
		nonyl	poly (-3-hydroxydodecanoate)
n = 2	R =	hydrogen	poly (-4-hydroxybutyrate)
n = 3	R =	hydrogen	poly (-5-hydroxyvalerate)

Figura 3 – Estrutura geral do Polihidroxialcanoatos e suas variações conforme o tamanho da cadeia lateral (MARVI et al., 2013).

2.4 POLIHIDROXIBUTIRATO

PHB é sintetizado quando as condições para o crescimento bacteriano são desfavoráveis, como limitação de nitrogênio, fósforo, oxigênio, magnésio e excesso de fonte de carbono (DU et al., 2001a, DU & YU, 2002a; LEE, 1996). Além disso, o PHB é um polímero de armazenamento de carbono e energia para muitas bactérias quando se encontram em condições de estresse (KADOURI et al., 2005). PHB é um poliéster alifático e estudos relatam que é possível relacionar a produção do polímero com a sobrevivência bacteriana em ambientes competitivos (KADOURI et al., 2003; RATCLIFF et al., 2008). O grânulo de PHB é armazenado na forma insolúvel dentro da célula, é revestido por inúmeras proteínas e isto pode elevar o peso do PHB em torno de 0,5 a 2%. Estudos ainda indicam que lipídios podem se ligar ao grânulo, revestindo toda a sua estrutura (BEEBY et al., 2012).

O início da síntese do polihidroxibutirato ocorre pela condensação de duas moléculas de acetil-CoA levando a formação de um intermediário acetoacetil-CoA através da ação da enzima beta-cetotiolase (PhaA). O intermediário acetoacetil-CoA é reduzido pela enzima acetoacetil-CoA redutase (PhaB), formando então 3-hidroxi-butiril-CoA. Nesta reação há o consumo de um equivalente redutor na forma de NADPH. Por fim, o intermediário 3-hidroxi-butiril-CoA serve de substrato para a enzima PHA sintase (PhaC), a qual polimeriza 3-hidroxi-butiril-CoA formando o polímero polihidroxibutirato **(Figura 4)**. Ao redor do grânulo há algumas proteínas estruturais responsáveis por diversas funções, as quais são denominadas do inglês de PGAPs (*PHB granule-associated proteins*) (GRIEBEL et al., 1968; PFEIFFER et al., 2011).

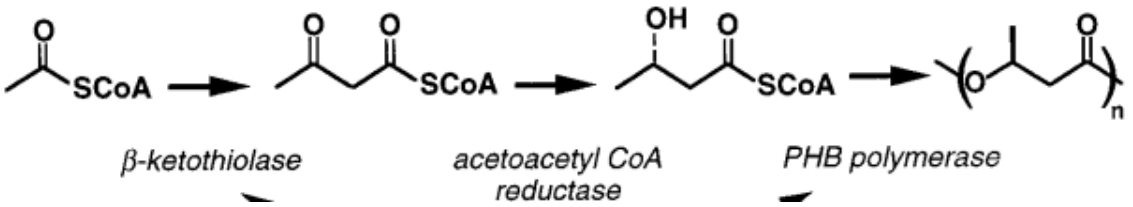


Figura 4– Via Biosintética do PHB

Síntese do polímero segue três passos sob ação de três enzimas a β-cetotiolase (PhaA), acetoacetil-CoA redutase (PhaB) e por fim PHB polimerase ou sintase (PhaC) **(**MADISON & HUISMAN, 1999).

Jendrossek e colaboradores (2009) mostraram que existem várias proteínas ao redor dos grânulos de PHB em *Raltoni eutropha* H16. O mesmo foi verificado por Tirapelle et al. (2013) utilizando grânulos de PHB produzidos por *H. seropedicae* SmR1 como bactéria de estudo. Segundo Jendrossek, tais proteínas são essenciais, pois evitam a ligação inespecífica de outras proteínas que podem prejudicar a síntese do polímero, como foi verificado por Liebergesell e colaboradores (1992) que encontraram proteínas incomuns ligadas ao PHB, por exemplo, a lisozima. Além disso, Jendrossek e colaboradores (2009) mencionam que quando as proteínas que fazem parte do metabolismo do PHB estão ligadas ao grânulo, isto faz com que o polímero permaneça como uma estrutura estável e bem organizada, funcionando como uma "pseudo-organela" **(Figura 5)**.

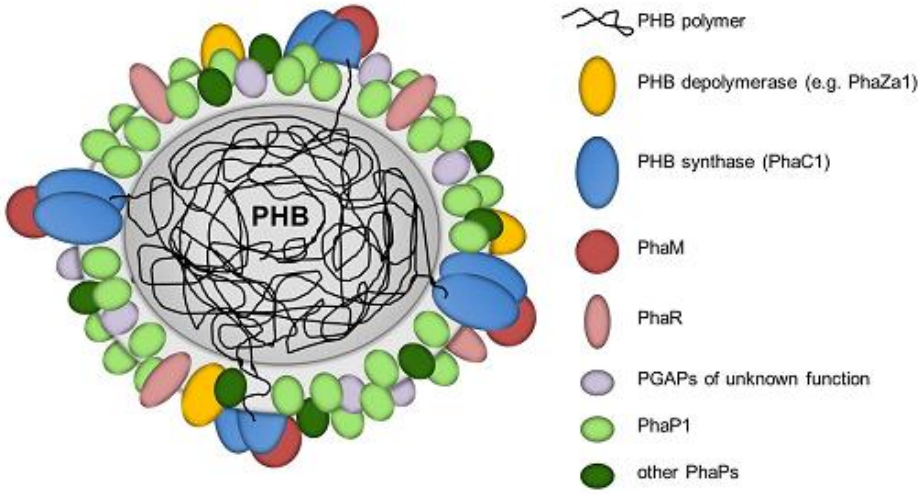


Figura 5 - Modelo da estrutura do grânulo de PHB de *Ralstonia eutropha* H16(Jendrossek et al., 2016).

Contudo, este autor descreve que entre todas as proteínas as mais abundantes são as fasinas (PhaP) e que quando as fasinas não estão presentes há uma diminuição na produção de grânulos. Além das proteínas envolvidas na síntese, há também proteínas que atuam na mobilização (catabolismo) dos grânulos de PHB, que pode gerar acetil-CoA e NADPH (JENDROSSEK & HANDRICK, 2002). Segundo Hayward et al., (1959) a produção de PHB em bactérias do gênero *Rhizobium, Spirillum* e *Pseudomonas* conseguem produzir uma quantidade de bactérias em quantidades máximas, mas durante a fase estacionária a tendência é que haja uma diminuição na produção. Para que isto ocorra é necessário a presença da enzima PHB depolimerase (PhaZ), a qual participa do importante ciclo de produção e degradação do grânulo, hidrolisando o polímero e formando o ácido 3-hidroxibutírico (3HB). Este 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 (PhaA) **(Figura 6)**.

Para a bactéria produtora de PHB sintetizar o polímero é necessária uma alta metabolização da fonte de carbono, para gerar acetil-CoA suficiente que não seja requerido pelo ciclo do ácido cítrico, e também disponibilidade de NADPH. Mas para a mobilização do grânulo a bactéria precisa passar por um estado no qual tenha baixa

concentração da fonte de carbono disponível, além de concentrações baixas de nitrogênio. Assim ela começa a degradar o grânulo para suprir suas necessidades e poder sobreviver por um tempo mais longo (JENDROSSEK & HANDRICK, 2002).

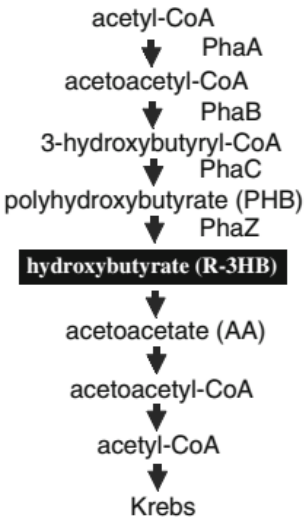


Figura 6-Biossíntese e degradação do Polihidroxibutirato (Soto et al., 2012).

Alves et al. (2016) observaram que *Herbaspirillum seropedicae* SmR1, é capaz de produzir PHB **(Figura 7)**, em torno de 14% do peso seco da célula, utilizando DL-malato como fonte de carbono. Sacomboio et al. (2017) mostraram que esta mesma bactéria consegue acumular grânulos de PHB utilizando outras fontes de carbono, como: glucose, xilose e frutose, demonstrando desta forma que esta bactéria pode ser um organismo modelo para a produção e estudo do polímero.

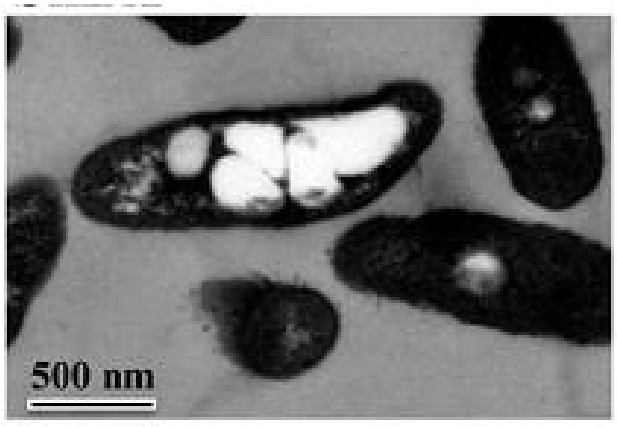


Figura 7 - Microscopia eletrônica de transmissão de H. seropedicae SmR1 (ALVES et al., 2016).

2.5 BIOSSÍNTESE DE PHB

Estudos relatam que para bactérias como *Alcaligenes eutrophus* (POIRIER et al., 1995; LEE et al., 1996), a biossíntese do grânulo de PHB é uma via de 3 etapas. Esta mesma via de síntese foi relatada para outros microrganismos que são organismos modelos para o estudo da biossíntese do PHB. Alguns autores mencionam que para *Ralstonia eutropha*, os genes envolvidos na síntese deste polímero encontram-se organizados em um operon (SCHUBERT et al., 1988; PEOPLES et al., 1989; SLATER et al., 1988; LIEBERGESELL et al., 1992), **(Figura 8).**

Pesquisas utilizando microscopia de fluorescência e que envolvem o início da síntese de polihidroxibutirato revelam que nos estágios iniciais da formação do polímero os grânulos se encontram na periferia ou nos polos da célula (JENDROSSEK et al., 2005). Esses dados foram confirmados por microscopia confocal utilizando bactérias com tamanhos maiores como *Caryophanon latum* e *Azotobacter vinelandii* (HERMAWAN et al., 2007; JENDROSSEK et al., 2007). Sabe-se também que diferentes polihidroxialcanoatos podem ser obtidos através de diferentes processos biológicos, como foi relatado por Steinbüchel & Füchtenbusch (1998), os quais mencionam que algumas bactérias podem formar o polímero através do processo de fermentação. Estes mesmos autores, descrevem que os PHAs podem ser obtidos através de produção química, produção biotecnológica e isolamento a partir de fontes naturais.

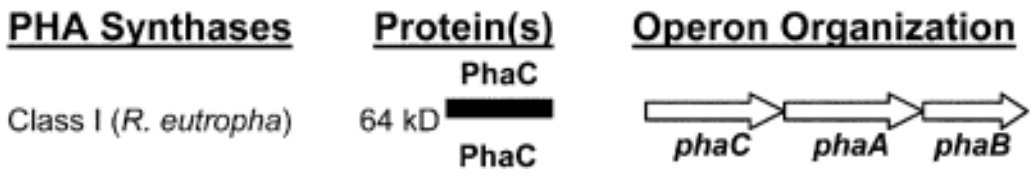


Figura 8.Operon que codifica os genes envolvidos na síntese de PHB em *Ralstonia eutropha* (STUBBE & TIAN, 2003).

Contudo, além dos genes envolvidos na síntese do PHA, existem outros genes que são importantes para o metabolismo e na homeostase do polímero de uma forma geral. Existem proteínas envolvidas no controle do tamanho dos grânulos (PhaP), na mobilização do polímero quando há necessidade do consumo (PhaZ), proteínas

regulatórias da expressão das fasinas (PhaR), (SAEGUSA et al., 2001; WIECZOREK et al., 1995; MAEHARA et al., 1999; MAEHARA et al., 2001; PÖTTER et al., 2002; STUBBE & TIAN, 2003)

2.6 FASINAS

Na primeira metade dos anos 90, alguns pesquisadores identificaram proteínas que estavam associadas aos grânulos de PHB e que, pela primeira vez, não eram PHA sintases e PHA depolimerases (PIEPER-FÜRST et al., 1994; STEINBÜCHEL et al., 1995; WIECZOREK et al., 1995). Essas proteínas foram nomeadas de Fasinas (PhaP), em analogia as oleosinas presentes em inclusões lipídicas em plantas. Alguns autores resumiram as propriedades destas proteínas como sendo: baixo peso molecular (11 a 25 kDa), alta afinidade por inclusões de PHA, que contribuem com grande parte das proteínas totais do polímero, localizadas na superfície do grânulo e ocorrem em qualquer bactéria que consegue produzir PHB de forma natural (STEINBÜCHEL et al., 1995). Para *Ralstonia eutropha*, a quantidade de fasinas encontrada ao redor do polímero foi muito alta, isto pode sugerir que o PHB, embora tenha outras proteínas presentes ao seu redor, o qual é coberto em sua maior parte por fasinas. Além disso, estudos realizados com mutantes defectivos na expressão destes genes mostraram que a quantidade de PHB produzido por algumas bactérias diminuiu drasticamente (WIECZOREK et al., 1995; ALVES et al., 2016). Portanto, além de cobrir o grânulo e influenciar na quantidade do polímero produzido, estas proteínas controlam a relação superfície-volume do PHB, uma vez que na ausência delas pode ocorrer a coalescência dos grânulos (WIECZOREK et al., 1995).

Desta forma, depois da síntese da cadeia do polímero através das PHA sintases, os grânulos estão envoltos por proteínas e fosfolipídios. A única esturutra tridimensional de fasina relatada até o momento (ZHAO et al., 2016) e experimentos adicionais mostram que estas proteínas são anfipáticas (interagem com um solvente aquoso e com uma estrutura hidrofóbica) (NEUMANN et al., 2008). Contudo, quando as regiões hidrofóbicas das fasinas foram removidas, essas proteínas mutantes foram incapazes de se ligar ao polímero. Portanto, sabendo da importância da estrutura completa das fasinas, quando o grânulo é produzido a parte hidrofóbica das fasinas interage com o polímero e a parte hidrofílica fica exposta se associando com o

citoplasma da bactéria, estabilizando o grânulo de PHB (PIEPER & FÜRST, 1995; STEINBÜCHEL et al., 1995). Sabe-se também que na presença das fasinas (superexpressão ou em mutantes complementados com genes *phaP*) há uma influência positiva na síntese do polímero, no número e no tamanho dos grânulos (PÖTTER *et al*., 2004; STEINBÜCHEL *et al*., 1995; WIECZOREK *et al*.,1995; ALVES et al., 2016).

Experimentos *in vitro* mostram que podem ser gerados grânulos de PHA incubando-se o substrato 3-hidroxi-acil-CoA com PHA sintase. A adição de fasinas acelera a síntese do polímero e produz grânulos de menor tamanho (JOSSEK & STEINBÜCHEL, 1998). Em um outro estudo, na presença de uma fasina de *Ralstonia eutropha* aumentou a atividade da PHA sintase de classe II de *Pseudomonas aeruginosa* em quase 50% (QI et al., 2000).

Sabendo que uma das funções das fasinas é formar uma camada de isolamento entre o citoplasma hidrofílico e o núcleo hidrofóbico dos grânulos de PHA (STEINBÜCHEL et al., 1995), alguns estudos relatam que desta forma, as fasinas podem impedir a ligação inespecífica de outras proteínas que causam estresse ou impedem a formação adequada do polímero. Isto foi provado através de experimentos utilizando *Escherichia coli* como organismo heterólogo que não produz PHB naturalmente, mas que através da inserção do operon *phaABC* de *R. eutropha* permitiu a produção mesmo sem a presença das fasinas. No entanto constatou-se um aumento na expressão de genes relacionados ao estresse como *dnaK*, *groEL* e *groES* (HAN et al., 2001). A resposta de estresse pode se justificar uma vez que o PHB não é um produto metabólico natural nesta bactéria e, além disso, sem as fasinas os grânulos ficam com suas partes hidrofóbicas expostas ao citosol da célula, causando ligação inespecífica de proteínas citoplasmáticas e problemas ao metabolismo da bactéria. Em 2005, Han e colaboradores observaram através de análises de proteoma, varias proteínas associadas aos grânulos de PHB produzido em *E. coli* (sem a expressão de fasinas). Entre as proteínas mais destacadas encontraram IbpA/B, a qual previne a agregação de proteínas durante o choque térmico ou algum outro estresse, e observaram que esta proteína pode se ligar ao PHB e atuar como uma fasina, impedindo a ligação de outras proteínas citosólicas. Em 2007, Tessmer e colaboradores observaram o mesmo para a proteína HspA que agia de forma a substituir as fasinas na síntese de PHB ou politioésteres. Relatos indicam que após a iniciação da formação das cadeias do polímero e sem a presença das fasinas, há uma grande interação inespecífica com outras proteínas que

podem prejudicar a formação do grânulo e levar a desnaturação de proteínas relevantes para o todo o processo de polimerização, desencadeando uma resposta de estresse e fazendo com que outras proteínas tentem substituir as fasinas (STEINBÜCHEL et al., 1995; LIEBERGESELL et al., 1992; HOROWITZ & SANDERS, 1995; TESSMER et al, 2007).

Fasinas já foram relatadas ou caracterizadas em vários microrganismos produtores de polihidroxialcanoatos, 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), *Pseudomonas oleovorans* (PRIETO et al., 1999) e *Pseudomonas* sp. 61-3 (MATSUMOTO et al., 2002) e *Herbaspirillum seropedicae* (TIRAPELLE et al., 2013).

Mutantes que não expressam fasinas em *Ralstonia eutropha* tiveram 50% menos PHB quando comparados a estirpe selvagem (WIECZOREK et al., 1995; PIEPER-FURST et al., 1994; FULLER et al., 1992; PIEPER-FURST et al., 1995; KUCHTA et al., 2007). O mesmo ocorreu quando Alves e colaboradores (2016) caracterizaram a produção de PHB no simples mutante (Δ*phaP1*) e no duplo mutante (Δ*phaP1.2*) de *Herbaspirillum seropedicae.* O mutante Δ*phaP1* apresentou uma redução de 50% de PHB em relação a estirpe selvagem, já o duplo mutante não acumulou o polímero.

Ralstonia eutropha, assim como *Herbaspirillum seropedicae*, possui mais de uma fasina, porém a principal é a PhaP1 (PÖTTER et al., 2004; PÖTTER et al., 2005; KUCHTA et al., 2007, ALVES et al., 2016). Além disso, os resultados apresentados por Wieczorek et al. (1995), revelam que na ausência de PhaP1, *R. eutropha* possuía um único e grande grânulo dentro da célula ao contrário do que se vê na estirpe selvagem **(Figura 9)**. De fato, isto nos mostra a importância das fasinas para a produção do grânulo em bactérias gram-negativas.

Wang e colaboradores (2007) identificaram dois genes que codificam fasinas no genoma de *Sinorhizobium meliloti* 1021. A estirpe duplo mutante Δ*phaP1phaP2* não produziu grânulos de PHB, mas produziu o dobro de grânulos de glicogênio que a estirpe selvagem. Também, o duplo mutante apresentou 4 horas de atraso no seu

crescimento comparado a estirpe selvagem. Quanto a fixação de nitrogênio, a estirpe Δ*phaP1phaP2* teve uma redução significativa na atividade nitrogenase em torno de 20% em relação a estirpe selvagem. Portanto, a partir destes resultados é possível sugerir que as fasinas têm importantes funções controlando o metabolismo de PHB, e que esse por sua vez pode afetar diversos aspectos da fisiologia bacteriana.

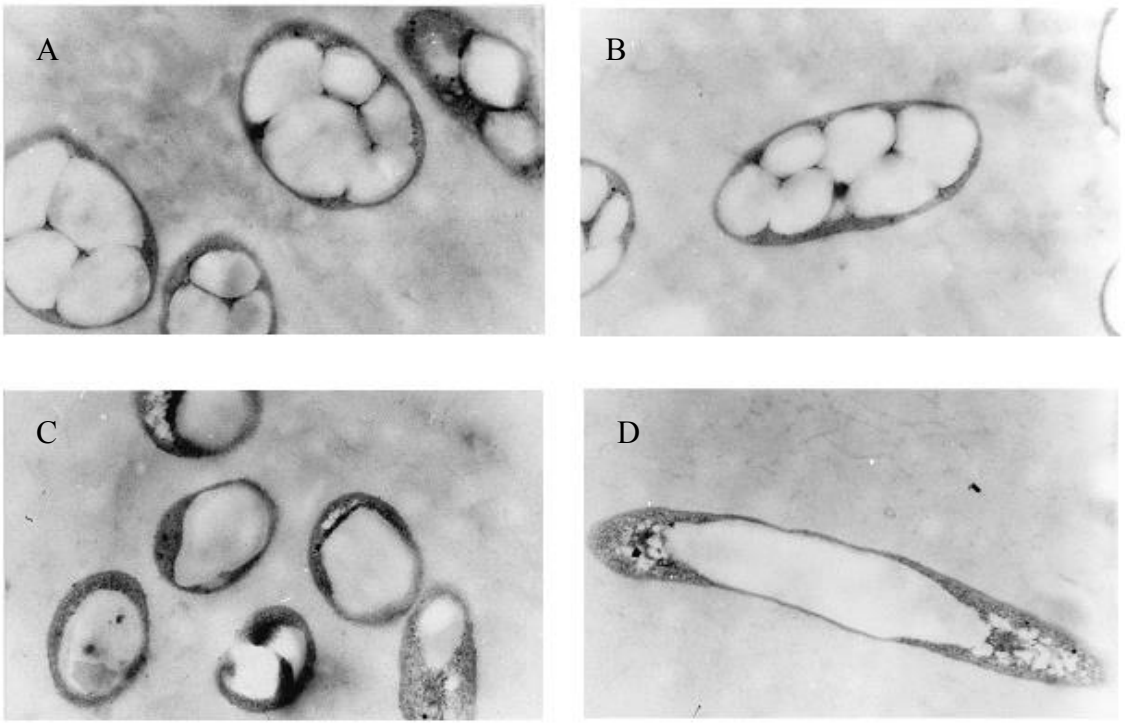


Figura 9.Morfologia das células de *Ralstonia eutropha* com grânulos de PHB acumulados no citoplasma. Estirpe selvagem contendo grânulos de tamanhos normais (A e B), e estirpe mutante defectiva na expressão de *phaP1* apresentando somente um grânulo ocupando toda a célula (C e D) (Wieczorek et al., 1995).

2.7 PROTEÍNA REGULATÓRIA DA EXPRESSÃO DAS FASINAS (PhaR)

Muitas bactérias produtoras de PHA, como: *R. eutropha, H. seropedicae, S. meliloti, P. denitrificans, A. vinosum,* entre outras, possuem um sistema de regulação da expressão das fasinas, o qual é controlado pela proteína PhaR. Para *Ralstonia eutropha* foi identificado que PhaR é um repressor transcricional que regula a expressão de *phaP1* (PÖTTER et al., 2002; YORK et al., 2002). Assim PhaR consegue se ligar na região a jusante do promotor do gene *phaP1*, bloqueando o início da transcrição.

Pötter e Steinbüchel (2005) descreveram um modelo de funcionamento do sistema de repressão por PhaR em *Ralstonia eutropha*. Segundo os autores, quando não há produção de grânulos de PHB, PhaR não consegue se ligar ao polímero, a concentração citoplasmática de PhaR é alta, sendo capaz de reprimir a expressão de *phaP1* e sua própria expressão. No entanto, quando há o início da síntese das cadeias de PHB pela PHA sintase (PhaC), proteínas PhaR ligam-se ao polímero, fazendo com que a concentração de PhaR no citoplasma diminua consideravelmente, desta forma há a desrepressão do gene *phaP1*. Após o grânulo de PHB atingir seu tamanho máximo, e possuir uma grande quantidade de fasinas ao redor do polímero, PhaR é deslocada do grânulo para o citoplasma, liga-se ao DNA e reprime a expressão de *phaP1*. Além disso, PhaR liga-se a sua própria região promotora, funcionando como um auto-regulador.

Na Figura 10 é apresentado o modelo da atuação do regulador PhaR em *R. eutropha*. Kadowaki e colaboradores (2011) reportaram o mesmo sistema regulatório para a atuação de PhaR (anteriormente PhbF) em *H. seropedicae* SmR1.

Segundo York et al. (2002), a proteína PhaR pode promover a síntese de PHB através da regulação da expressão das fasinas, além de regular a expressão de outras proteínas. Em um experimento realizado por Maehara e colaboradores em 2013, eles transferiram os genes *phaR* e *phaP* de *P. denitrificans* para *E. coli* e verificaram que quando PhaR está presente há uma grande diminuição da expressão de *phaP*.

Para *H. seropedicae*, a expressão da principal fasina (PhaP1) é regulada por PhaR e pela produção de PHB, pois quando há o início da produção do polímero há um aumento da expressão da fusão transcricional *phaP1-lacZ* na estirpe selvagem, mas quando expressão desta mesma fusão foi avaliada no mutante Δ*phaC1* (sem produção de PHB), não houve expressão do promotor (Alves et al., 2016).

Portanto, como descrito primeiramente por York e colaboradores (2002), PhaR atua como um sensor de cadeias de PHB e que permite a síntese de plena de PHB somente após a síntese de algumas cadeias de PHB na bactéria. Isso indica que a expressão não regulada dos genes *phaP*, e outros possíveis genes do regulon PhaR, possa levar a redução na síntese de PHB.

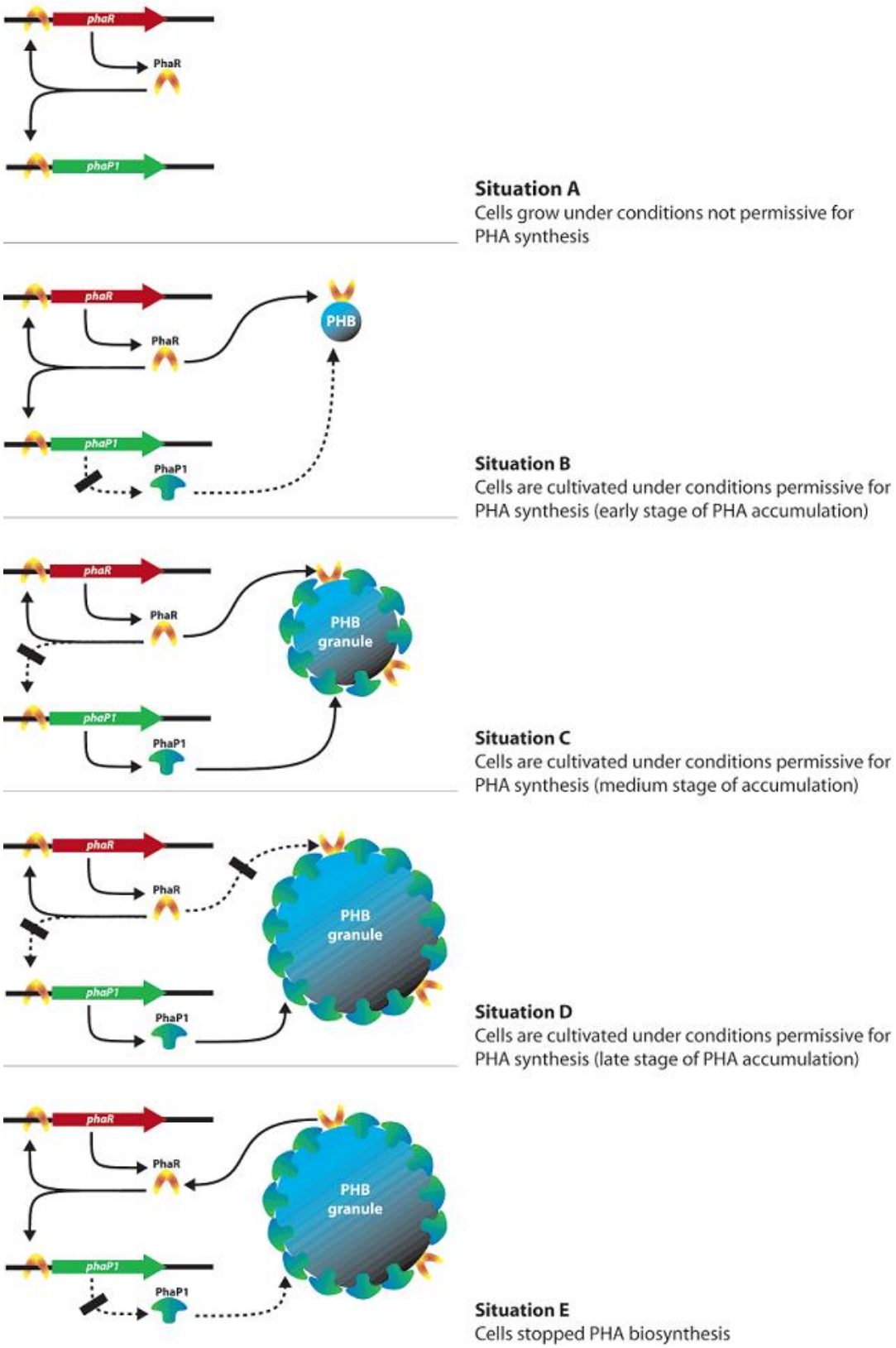


Figura 10.Sistema de regulação das fasinas por PhaR em *Ralstonia eutropha* (Pötter & Steinbuchel, 2005).

2.8 PHA SINTASES DA CLASSE I (PhaC1)

PHA sintase é a terceira enzima da via de biossíntese do PHB. O primeiro gene *phaC* estudado foi sequenciado a partir de *Ralstonia eutropha.* O gene *phaC* foi isolado através de estudos de complementação de mutantes de *R. eutropha* defectivos na produção de PHB (PEOPLES et al., 1989; SCHUBERT et al., 1988). Com o decorrer dos anos e do avanço no sequenciamento genômico, várias PHA sintases foram identificadas, clonadas e sequenciadas a partir de diferentes microrganismos.

Atualmente as PHA sintases são divididas em quatro classes. A classificação depende da composição em subunidades e especificidade ao substrato (**Figura 11**). Em *R. eutropha*, assim como na maioria das bactérias, incluindo *H. seropedicae* SmR1, as PHA sintases são da classe I. Em espécies dos gêneros *Pseudomonas*, *Aeromonas* e *Burkholderia* encontram-se as PHA sintases da classe II. Apesar das classes I e II apresentarem subunidades com peso molecular próximo, PHA sintases da classe I utilizam preferencialmente como substratos tioésteres de ácidos (R)-3-hidroxialcanóicos com 3 a 5 átomos de carbonos, enquanto PHA sintases de classe II preferem tio ésteres de ácidos (R)-3-hidroxialcanóicos de 6-14 carbonos (REHM, 2003; REN et al., 2009).

As PHA sintases de classe III e IV ocorrem nas bactérias *Allochromatium vinosum* e *Bacillus megaterium*, respectivamente. PHA sintases de classe III possuem a subunidade catalítica PhaC e a subunidade regulatória PhaE. Assim como na classe I, as PHA sintases da classe III preferem tio ésteres de ácidos (R)-3-hidroxialcanóicos com 3 a 5 átomos de carbono (LIEBERGESELL & STEINBÜCHEL, 1992). Para PHA sintases de classe IV, as quais se assemelham as PHA da classe III, a diferença está na subunidade regulatória denominada PhaR que substitui a subunidade PhaE da classe III e tem apenas 22 kDa de peso molecular (McCOOL et al., 1999; McCOOL & CANNON et al., 2001) **(Figura 11).**

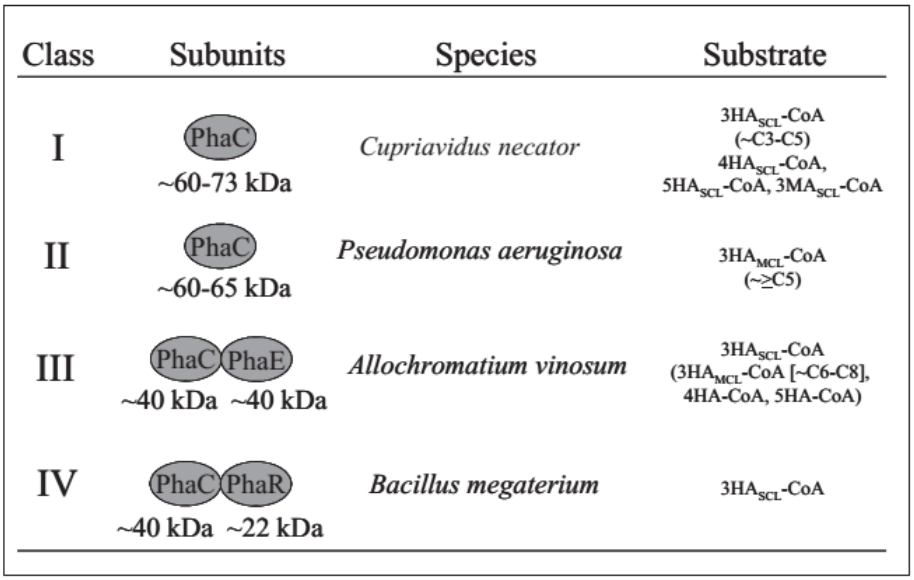


Figura 11.PHA sintases divididas em quatro classes (REHM et al., 2003)

A importância da PHA sintase foi demonstrada através de experimentos que relatam que esta proteína pode se ligar diretamente a superfície do grânulo de PHA (HAYWOOD et al., 1989; GERNGROSS et al., 1993; LIEBERGESELL et al., 1992). Entretanto, em estirpes mutantes de *R. eutropha* que não produzem PHB, a PHA sintase encontra-se solúvel no citoplasma da célula (HAYWOOD et al., 1989). Além disso, Pötter & Steinbüchel (2005) relataram que a PHA sintase é a enzima chave e mais importante na síntese do PHA, pois esta enzima não pode ser substituída, diferentemente das outras 2 enzimas PhaA e PhaB que podem ser substituídas por outras isoenzimas.

Alguns fatores podem influenciar o peso molecular do PHA sintetizado pelas PHA sintases, sendo esses: a concentração intracelular destas enzimas, a relação enzima-substrato e principalmente o nível de expressão da PhaC, já que altas concentrações de PHA sintases podem resultar em moléculas de PHA com baixa massa molecular (SIM et al., 1997; KRAAK et al., 1997). Além disso, enzimas capazes de hidrolisar PHA ou a ausência delas, como: esterases, lipases e principalmente PHA depolimerases, também podem influenciar o peso molecular do PHA acumulado (MUKAI et al., 1993; JAEGER et al., 1995).

2.9 PHA DEPOLIMERASES

Existem pelo menos 500 PHA depolimerases identificadas de vários microrganismos, as quais possuem oito diferentes superfamílias e 38 famílias homólogas baseadas na similaridade das suas sequências (KNOLL et al., 2009). As bactérias que são mais utilizadas como modelo de estudo para a enzima depolimerase pertencem ao gênero *Ralstonia, Alcaligenes, Comamonas* e *Pseudomonas* (JENDROSSEK, 2002; SAITO et al., 1989; BACHMANN et al., 1999; KASUYA et al., 1994; MUKAI et al., 1994, SCHÖBER et al., 2000, CALABIA et al., 2004).

Algumas PHA depolimerases são específicas para PHA de cadeia curta como 3-hidrobutirato, 3-hidroxivalerato ou 4-hidroxibutirato (JENDROSSEK et al., 1993; MÜLLER et al., 1998; SAITO et al., 1991). Bactérias mobilizam PHA durante um determinado estresse, em condições desbalanceadas para sua sobrevivência ou ainda quando necessitam de uma fonte de carbono e quando outros nutrientes possuem concentrações diminuídas. Sendo assim, o polímero é clivado em monômeros e a energia liberada pode ser utilizada pela bactéria para poder sobreviver (STEINBÜCHEL & HEIN, 2001; VIGNESWARI et al., 2015).

Mukai e colaboradores (1993) identificaram alguns fatores que contribuem para a taxa de biodegradação do polímero, as quais são: condições ambientais como temperatura e pH, propriedades físicas do polímero e a população microbiana. Para *Acidovorax* sp. DP5 uma bactéria isolada na Malásia, foi observado à completa hidrólise de PHB. A enzima PHA depolimerase desta estirpe mostrou alta porcentagem de degradação do polímero em ambiente com pH alcalino (~9) e em temperatura de 40°C (VIGNESWARI et al., 2015). Em outras bactérias tais como *B. megaterium, Z. ramigera, S. meliloti* e *R. eutropha* determinou-se que a atividade PHA depolimerase teve um papel importante na degradação e no metabolismo do PHA (SAITO et al., 1995; CHARLES et al., 1997; SAEGUSA et al., 2001). Alguns autores relatam que tanto o acúmulo como a degradação do polímero são importantes para aumentar a habilidade competitiva, tolerância ao estresse e aumento do *fitness* durante mudanças ambientais (LOPEZ et al. 1995; RUIZ et al. 2001; KADOURI et al. 2003; PHAM et al. 2004; KADOURI et al. 2005).

Para *R. eutropha*, foram identificadas ao menos sete PHA depolimerases (PhaZ1 - 7) e duas 3HB oligômero hidrolases (PhaY1 e PhaY2), as quais são expressas na falta

de nitrogênio e com excesso de fonte de carbono (SAEGUSA et al., 2001; YORK et al., 2003; SCHWARTZ et al., 2003; PÖTTER et al., 2004). Porém somente as PhaZ1 e PhaZ6 foram encontradas ligadas aos grânulos. Estirpes simples mutantes foram construídas, gerando deleções em cada uma das PHA depolimerases de *R. eutropha*, para verificar a importância destas enzimas para a degradação do polímero. Os resultados destes experimentos mostram que todas as estirpes mutantes tinham problema na degradação do PHB, embora as PHA depolimerase 1 e 2 (PhaZ1 e PhaZ2) tiveram um maior impacto na degradação do polímero (YORK et al., 2003; BRIGHAM et al., 2012).

Tirapelle et al., (2013) constatou que nos grânulos de PHB de *H. seropedicae* SmR1 estava ligada a PHA depolimerase (PhaZ1), além de outras proteínas como PHA sintase, fasinas e PhaR. Desta forma, estudos com mutantes que não expressem as PHA depolimerases são relevantes para verificar a influência da degradação do polímero para a resistência ao estresse e em condições de colonização de plantas.

2.10 RpoS – Fator sigma S

Em grande parte das bactérias gram-negativas, o gene *rpoS* (σ^S), o qual codifica uma subunidade sigma alternativa da RNA polimerase, pode ativar a transcrição de alguns genes que estão relacionados a proteção celular contra estresses tais como calor, alta osmolaridade, variações de pH e estresse oxidativo (HENGGE-ARONIS, 2002; RAMOS-GONZÁLEZ et al., 1998). Em *Escherichia coli* o fator sigma RpoS foi identificado como um regulador central durante a fase estacionária (LANGE et al., 1991). Durante o crescimento normal em meio rico não foi possível detectar a expressão de *rpoS*, contudo foi possível observar sua expressão em meio mínimo e em condições estressantes **(Figura 12)**. Sabe-se que a expressão do gene *rpoS* pode ser controlada a nível transcricional, traducional e através da estabilidade da proteína (HENGGE-ARONIS, 2002). Alguns trabalhos relatam que o nucleotídeo guanosina tetrafosfato (ppGpp), o qual está envolvido no aumento da sobrevivência e resistência ao estresse, pode estar envolvido no controle de expressão de *rpoS.* Além disso, sabe-se que a degradação de PHA faz aumentar a concentração intracelular de ppGpp, sugerindo que o polímero pode possuir um papel essencial na sobrevivência de bactérias (BROWN et al., 2002; DiRUSSO et al., 1998; GENTRY et al., 1993).

Ruiz e colaboradores (2003) observaram que houve um aumento da tolerância ao estresse durante a mobilização de PHA e isto pode ter ocorrido devido ao aumento da concentração intracelular de RpoS. Segundos estes autores, isto confirma a existência da presença de um regulador global que afeta o ciclo do PHA.

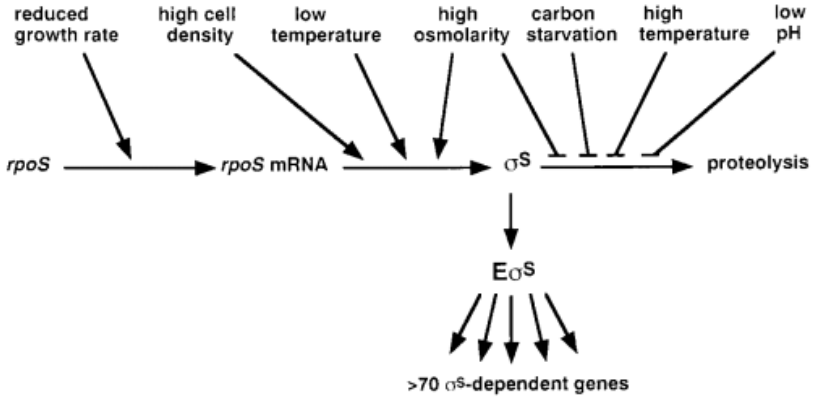


Figura 12. Condições de estresse que podem afetar a expressão do gene *rpoS* (HENGGE-ARONIS, 2002).

2.11 Influência do PHB na interação planta-bactéria

PHA são importantes polímeros para reserva de energia e carbono para bactérias em situações adversas a sua sobrevivência. Estudos com mutantes de *Azospirillum brasilense* Sp7 nos genes *phaC* e *phaZ* mostram que a tolerância e a sobrevivência destas bactérias ao choque térmico, radiação UV, estresse oxidativo ou choque osmótico, diminuiu drasticamente (KADOURI et al., 2003, 2005). Além disso, foi verificado que quando bactérias que produziam PHA foram inoculadas em plantas, ocorreu um aumento na colonização da raíz e na promoção do crescimento vegetal, como no milho, utilizando-se *A. brasilense.* Especificamente, foi observado um aumento no crescimento do comprimento da panícula e também no peso seco da planta(FALLIK & OKON, 1996).

Algumas bactérias que fixam nitrogênio acumulam PHB e glicogênio como reserva de carbono (LODWIG & POOLE, 2003; MANDONET al., 1998; MARROQUI et al., 2001; TRAINER & CHARLES, 2006). Para a interação entre *S. meliloti* e *Medicago sativa* o mutante *phaC* foi capaz de fixar nitrogênio no mesmo nível que a estirpe selvagem, porém este mutante tornou-se menos competitivo do que a estirpe selvagem na colonização da planta (ANEJA et al., 2005; WILLIS &WALKER, 1998). Lodwig e

colaboradores (2005) reportaram que o acúmulo de PHB pela bactéria pode prepará-la para a infecção da planta e aumentar a diferenciação bacteriana durante a colonização. Segundo Wang et al. (2007), plantas inoculadas com o mutante *phaC* de *S. meliloti* mostraram propriedades simbióticas diminuídas, tais como: formação cinética dos nódulos, peso seco nodular e da parte aérea das plantas. Desta forma a produção de PHB pode resultar, além do aumento da infecção, em uma melhora na fixação de nitrogênio. Além disto, no mutante *phaC* a redução na relação simbiótica pode ser explicada pela diminuição de exopolissacarideos neste mutante, pois estes compostos são essenciais para o início do processo infeccioso (FRAYSSE et al., 2003).

Em *H. seropedicae* SmR1, Pankievicz et al. (2016) observaram um aumento na expressão de genes relacionados a síntese de PHB durante a colonização de raízes de trigo. O mesmo foi observado por Balsanelli et al. (2015) em *H. seropedicae* SmR1 colonizando raízes de milho. Segundo Pankievicz et al. (2016), isto ocorreu devido à alta disponibilidade de fonte de carbono para as células que estão realizando o processo de infecção. Portanto o metabolismo de PHB pode ser requerido pela bactéria para que ocorra a colonização de forma eficiente.

Koskimäki et al. (2016) demonstraram que *Methylobacterium extorquens* produziu oligômeros de 3-hidroxibutirato durante a colonização de *Pinus sylvestris* e que os oligômeros apresentavam forte atividade antioxidante contra radicais hidroxila. A análise transcricional *in planta* dos genes *phaC1* e *phaZ1* mostrou que ambos os genes estavam sendo transcritos durante os estágios iniciais de infecção. Nos sítios de infecção foi determinada uma alta atividade oxidativa gerada por radicais hidroxila e provavelmente a degradação do PHB em oligômeros de 3-hidroxibutirato protegeu as bactérias do estresse oxidativo. Os autores ainda mencionam que como o PHB representa grande parte do peso seco da célula, ele pode servir como uma reserva antioxidante para a bactéria ao longo da colonização. Portanto, estudos com bactérias endofíticas e produtoras de PHB são relevantes para a área de interação planta-bactéria, pois este polímero pode auxiliar tanto a colonização quanto a promoção do crescimento vegetal.

3. Objetivos

3.1 Objetivo Geral

Investigar os fatores envolvidos na síntese e na degradação de PHB em *H. seropedicae* SmR1 e o impacto do metabolismo de PHB para a resistência ao estresse abiótico e colonização de plantas.

3.2 Objetivos específicos

1. Investigar os mecanismos da regulação transcricional dos genes *phaP1* e *phaP2* de *H. seropedicae* SmR1;

2. Investigar o papel das fasinas PhaP1 e PhaP2 na promoção da síntese de PHB em *H. seropedicae* SmR1;

3. Investigar se mutantes defectivos na síntese ou na degradação de PHB em *H. seropedicae* SmR1 resistem menos aos estresses térmico, oxidativo e osmótico;

4. Investigar o efeito da síntese e degradação de PHB na interação planta-*H. seropedicae*;

CAPÍTULO I

Manuscrito publicado

A simple and efficient method for poly-3-hydroxybutyrate quantification in diazotrophic bacteria within 5 minutes using flow cytometry

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A simple and efficient method for poly-3-hydroxybutyrate quantification in diazotrophic bacteria within 5 minutes using flow cytometry

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Abstract

The conventional method for quantification of polyhydroxyalkanoates based on whole-cell methanolysis and gas chromatography (GC) is laborious and time-consuming. In this work, a method based on flow cytometry of Nile red stained bacterial cells was established to quantify poly-3-hydroxybutyrate (PHB) production by the diazotrophic and plant-associated bacteria, *Herbaspirillum seropedicae* and *Azospirillum brasilense*. The method consists of three steps: i) cell permeabilization, ii) Nile red staining, and iii) analysis by flow cytometry. The method was optimized step-by-step and can be carried out in less than 5 min. The final results indicated a high correlation coefficient (R^2=0.99) compared to a standard method based on methanolysis and GC. This method was successfully applied to the quantification of PHB in epiphytic bacteria isolated from rice roots.

Keywords: Flow cytometry; Nile red; Poly-3-hydroxybutyrate; *Herbaspirillum seropedicae; Azospirillum brasilense*

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Running title: PHB quantification by flow cytometry

1. Introduction

Polyhydroxyalkanoates (PHAs) are biodegradable polymers that can provide an environmentally friendly alternative to replace petroleum-based plastics (1). Poly-3-hydroxybutyrate (PHB) is the most abundant naturally-occurring polyester produced by bacteria in response to carbon oversupply and other nutrient limitations, such as low nitrogen levels (2). PHB is stored as cytoplasmic granules with a diameter ranging from 0.2 to 0.5 μm, and can provide carbon and energy for the bacteria under certain conditions (3).

The initial studies concerning PHAs detection were based on gravimetric and infrared spectroscopy methods, and the earliest research in PHB quantification was performed using gas chromatography (GC) (3). Although several methods have been described for this purpose, such as HPLC (4), fluorescent dyes (5), ionic chromatography, and enzymatic methods (6), GC is still the preferred standard method for PHB quantification, showing high sensitivity, accuracy, and reproducibility. However, there are three major drawbacks associated with GC-based methods: i) the use of hazardous solvents at high temperature, ii) a long time requirement for sample processing, and iii) the need for large amounts of bacterial cells. Thus the need for alternatives to GC for PHB quantification.

In the early 1980's, Nile red [9-diethylamino-5H-benzo(α)phenoxazine-5-one] was described as a promising fluorescent dye for detection of intracellular lipid droplets by flow cytometry in aortic smooth muscle cells and on cultured peritoneal macrophages (7). In the 1990's, a method based on Nile red (NR) staining was applied for the detection of PHB production in *Alcaligenes eutrophus* (8) and *Ralstonia eutropha* H16 (5). To date, several protocols established for NR staining and PHB quantification by flow cytometry in *Saccharomyces cerevisae*, *Cupriavidus necator* (9), *Synechocystis* sp. strain PCC6803, *Escherichia coli* (10), and three *Pseudomonas sp.* (11) have been reported. The optimization of a flow cytometry protocol for the quantification of PHB requires the determination of conditions for efficient cell permeabilization and optimal NR concentration, parameters that are dependent on cell type, membrane properties and bacterium size.

Herbaspirillum seropedicae and *Azospirillum brasilense* are plant growth-promoting diazotrophic bacteria (12,13). While there are several indications that PHB plays important roles in nitrogen fixation and plant-bacteria interactions (14–17), the real significance of PHB for bacteria during plant-colonization remains unknown. The development of techniques allowing PHB quantification in small volumes and low cell numbers will allow data collection in restrictive conditions – such as in bacteria colonizing roots and other plant tissues – and will contribute to the determination of the true role of PHB in plant-bacteria association. In this study, we describe the optimization and validation of a simple, fast and accurate method for the quantification of PHB production in *H. seropedicae* and *A. brasilense*, based on NR staining and flow cytometry.

2. Material and Methods

2.2 Reagents and buffers

Phosphate-buffered saline (PBS) contained 137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4 and 1.4 mM KH_2PO_4 at pH 7.2 NR (Sigma Aldrich, USA) was dissolved in DMSO to a final concentration of 3.14 mM (1 mg/mL) and kept in the dark. NR was further diluted in different buffers as indicated in the legend of Figures. TBAC buffer [PBS containing 1 mM EDTA and 0.01% (v/v) Tween 20] was used to avoid the formation of bacterial aggregates that could potentially perturb light-scattering and fluorescence signals in flow cytometric analysis. TSE buffer [10 mM Tris-HCl pH 7.5, 20% (wt/vol) sucrose, 2.5 mM EDTA]. All other reagents were commercial products of the highest purity grade available.

2.3 Bacterial strains and growth conditions

H. seropedicae strain SmR1 (wild type) (18) and strain Δ*phaC1*, an SmR1 mutant deficient in PHB synthesis, previously described as Δ*phbC1* (19), were cultivated in NFbHP-malate medium containing 0.5% of DL-malic acid and 20 or 5 mM of NH_4Cl. *A. brasilense* strain FP2 (20) and *A. brasilense* Sp7 mutant strain *phbC* (21) were cultivated in NFbHP-lactate medium containing 0.5% of DL-lactic acid and 20 or 5 mM of NH_4Cl. *A. brasilense* FP2 is a spontaneous mutant strain from *A. brasilense* Sp7

resistant to nalidixic acid and streptomycin (20). Antibiotics were added to the growth media in the following concentrations: streptomycin (80 µg/mL) for *H. seropedicae*, streptomycin (80 µg/mL) and nalidixic acid (10 µg/mL) for *A. brasilense* strain FP2, and kanamycin (100 µg/mL) for *A. brasilense* Sp7 mutant strain *phbC*.

2.4 PHB quantification by gas chromatography

The bacterial PHB amount was determined by acid methanolysis followed by GC coupled to a flame-ionization detector as previously described (22). Methanolysis was performed 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. For each reaction, 0.5 mg of benzoic acid was added as internal standard. Reaction mixtures 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 vortexed 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 equipped with a CP-Sil-5 CB column (10 m x 0.53 mm ID). Argon was used as carrier gas at 0.9 mL/min. The injector was set at 250°C and the detector at 275°C. The oven temperature program was: initial temperature 50°C for 2 min, then from 50°C up to 110ºC at a rate of 20°C/min and finally up to 250°C at a rate of 20°C/min. The PHB amount in each sample was normalized by the weight of the lyophilized bacteria and expressed as a percentage of PHB/cell dry weight.

2.5 PHB quantification by flow cytometry

Flow cytometry experiments were performed in a BD Accuri C5® Flow Cytometer equipped with a 488-nm laser for fluorescence excitation. For each sample, 100,000 events were acquired, and the median fluorescence intensities were obtained from histograms of FL2-H 585/40 nm channel. Flow cytometry calibration was performed using spherothech 8-peak beads (BD Accuri™) according to the manufacturer's recommendations and instructions.

2.6 Optimization of the flow cytometric protocol for PHB quantification

For all steps of the optimization process, an aliquot of 100 µL (~10^6–10^7 cells/mL) of the cell culture was centrifuged for 1 min at 13,400 *g*, the supernatant solution was discarded and the cell pellet was treated according to each specific condition. For all conditions, after staining with NR, the cells were collected by centrifugation for 1 min at 13,400 *g* and resuspended in TBAC buffer for analysis by flow cytometry. During the optimization process the following steps were carried out in order of description:

1) Cell permeabilization conditions: the cell pellet was resuspended in 1 mL of each of the evaluated membrane permeabilization solutions (TBAC containing 30% of ethanol, TBAC containing 0.1% of Triton X-100 and TSE buffer). The bacterial suspensions were stained with NR (9.42 µM) for 5 min and analyzed by flow cytometry.

2) Optimization of the ethanol concentration for cell permeabilization: the cell pellet was resuspended in 1 mL of TBAC buffer containing increasing concentrations of ethanol (up to 70%). After 5 min of ethanol exposure, cells were stained with NR (9.42 µM) for 5 min.

3) Bacterial cell permeabilization time: the cell pellet was resuspended in 1 mL of TBAC buffer containing 50% of ethanol and incubated up to 30 min. After ethanol exposure, cells were stained with NR (9.42 µM) for 5 min.

4) Optimization of NR staining: the cell pellet was resuspended in 1 mL of TBAC buffer containing 50% of ethanol for 1 min. After ethanol exposure, cells were stained with NR (9.42 µM) for up to 30 min.

5) Determination of the optimal NR concentration: the cell pellet was resuspended in 1 mL of TBAC buffer containing 50% of ethanol for 1 min. After ethanol exposure, cells were stained with NR (0 to 500 µM) for 1 min.

6) Fluorescence stability: the cell pellet was resuspended in 1 mL of TBAC buffer containing 50% of ethanol for 1 min. Cells were subsequently stained with NR (31.25 µM) for 1 min, centrifuged (1 min at 13,400 *g*), and resuspended in TBAC buffer for analysis by flow cytometry. The NR fluorescence was monitored during 90 min by flow cytometry. In addition, samples were stored at 4°C in permeabilization solution (TBAC buffer containing 50% of EtOH), until analysis.

2.7 PHB quantification by flow cytometry using NR fluorescence

The optimized protocol for the quantification of PHB by flow cytometry is described as follows: an aliquot of 100 µL (~10^6–10^7 cells/mL) of a bacterial culture is centrifuged for 1 min at 13,400 x *g*. The supernatant solution is discarded and the cell pellet is resuspended in 1 mL of TBAC containing 50% of ethanol. After 1 min of incubation, samples are stained with 31.25 µM of NR for 1 min in the dark, centrifuged 1 min at 13,400 *g*, and the supernatant solution discarded. The pellet is then resuspended in 1 mL of TBAC solution and immediately analyzed by flow cytometry.

2.8 PHB staining for fluorescence microscopy

The same optimized protocol to prepare bacterial cells stained with NR for flow cytometry was applied to prepare cells for fluorescence microscopy. The non-optimized protocol (9) differed from the optimized one mainly in the ethanol (30%) and NR concentration (9.42 µM). The fluorescent images were obtained using the Axio Imager Z2 microscope (Carl Zeiss, USA), equipped with the scanning platform Metafer 4 and CoolCube 1 camera (Metasystems, USA) magnifying 100 times.

2.9 PHB production by epiphytic rice bacteria

Rice experiments were performed according to Valdameri et al. (23). PHB measurements in the epiphytic bacterial populations were performed in bacteria detached from plants 7 days after inoculation. Bacteria was removed from roots by vortexing for 1 min in 1 mL of TBAC containing 50% of ethanol. The suspension was stained with NR following the optimized protocol.

3 Results

3.1 Screening for permeabilization solutions

To determine the best cell permeabilization solution to stain *H. seropedicae* strain SmR1 and *A. brasilense* strain FP2 with NR, we initially compared three different conditions: i) TBAC containing 30% of ethanol, ii) TBAC containing 0.1% of Triton X-100, and iii) TSE buffer. TBAC buffer was previously applied to determine bacterial cell concentration by flow cytometry (23).

As shown in Figure 1A and B, intracellular fluorescence of samples stained with NR in TBAC (TBAC+) or TBAC containing detergent (0.1% triton X-100), did not differ

from non-stained (TBAC–) samples. In addition, a sucrose-buffer (TSE) produced only a partial permeabilization effect. The representative histograms of *A. brasilense* strain FP2 permeabilized with TSE buffer clearly showed a heterogeneous cell population (Figure 1A). The same heterogeneous distribution was observed with the *H. seropedicae* strain SmR1 treated with TSE buffer (data not shown). TBAC buffer containing 30% of ethanol (EtOH) permeabilized both *H. seropedicae* strain SmR1 and *A. brasilense* strain FP2, producing a single peak of higher fluorescence, denoting a homogeneous and full membrane permeabilization.

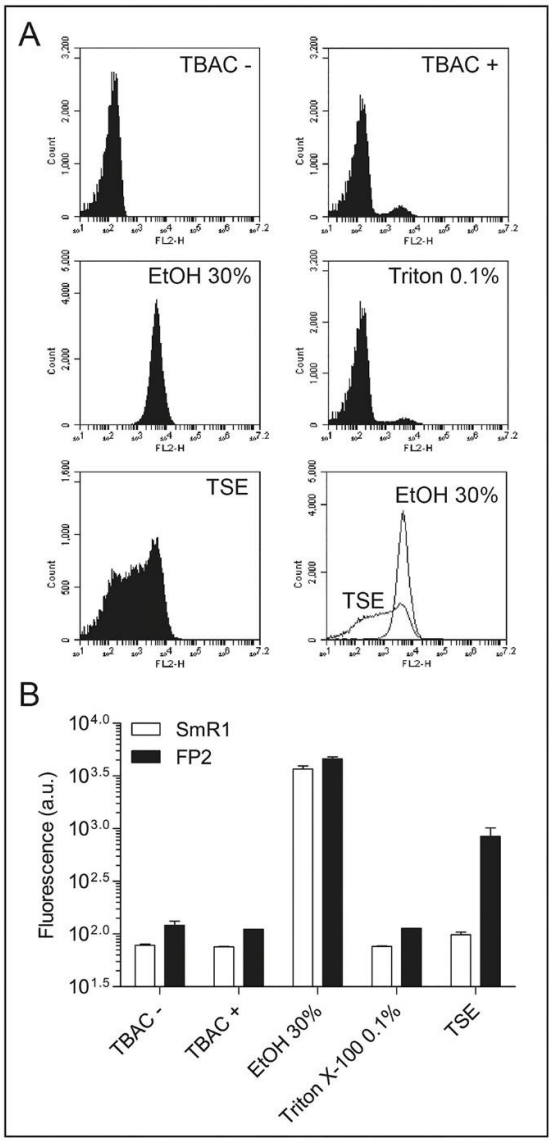

Figure 1. Screening for cell permeabilization solutions

Bacteria were grown at an OD600 of 1.2, 0.1 mL was centrifuged (1 min at 13,400 g), and resuspended in 1 mL of each solution evaluated. The bacterial suspensions were stained with NR (9.42 μM) during 5 min, and analyzed by flow cytometry. A,

Histograms are representative of *A. brasilense* strain FP2. Conditions: TBAC – corresponds to (Nile Red) NR non-stained samples, used as a blank. All other samples were stained with NR. TBAC + corresponds to non-permeabilized samples in TBAC. EtOH 30% corresponds to samples in TBAC containing 30% of ethanol. Triton 0.1% corresponds to samples in TBAC containing 0.1% of Triton X-100. TSE corresponds to samples in TSE buffer. TSE/EtOH 30% corresponds to overlay of two histograms, TSE and EtOH 30%, respectively. B, Fluorescence (arbitrary units, a.u.) data are reported as means±SD of 3 independent experiments with *H. seropedicae* strain SmR1 and A. brasilense strain FP2, by using the median fluorescence intensity values in the FL2-H channel.

3.2 Optimization of ethanol percentage for cell permeabilization

Since TBAC buffer containing 30% of ethanol permeabilized both bacteria, the effect of ethanol concentration on NR staining was evaluated. Fluorescence histograms of *A. brasilense* strain FP2 reveals that an increase in ethanol concentration increased the amount of permeabilized cells. Indeed, for both bacteria, TBAC buffer containing 50% of ethanol was the best condition for permeabilization considering the increase in NR fluorescence, and a single distinct peak (Figure 2A and B).

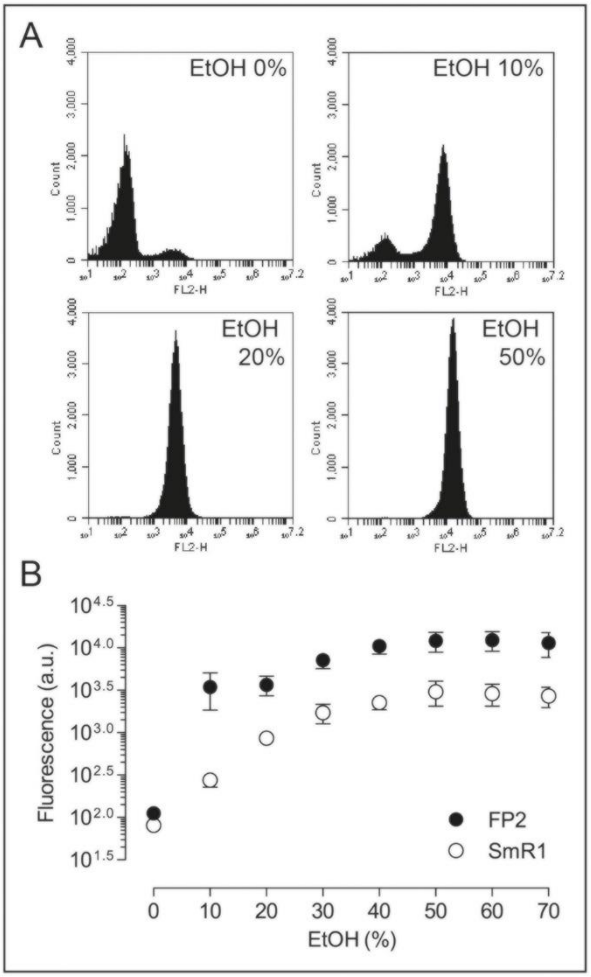

Figure 2. Optimization of ethanol (EtOH) percentage in TBAC buffer for cell permeabilization

Bacteria were grown at an OD_{600} of 1.2, 0.1 mL was centrifuged (1 min at 13,400 g), and resuspended in 1 mL of TBAC buffer containing different percentages of EtOH (0–70%). After 5 min of EtOH exposure, cells were stained with Nile red (9.42 µM) during 5 min, centrifuged (1 min at 13,400 g), and resuspended in TBAC buffer for analysis by flow cytometry. A, Histograms are representative of *A. brasilense* strain FP2 using different percentages of EtOH in TBAC buffer, as indicated. B, Fluorescence (arbitrary units, a.u.) data are reported as means±SD of 3 independent experiments with *H. seropedicae* strain SmR1 and *A. brasilense* strain FP2, by using the median fluorescence intensity values in the FL2-H channel.

3.3 Permeabilization time, Nile red exposure and optimal concentration

Cells were incubated in TBAC buffer containing 50% of ethanol up to 30 min before NR staining. As shown in Figure 3A, NR fluorescence levels were similar regardless the

incubation time. Therefore, 1 min of permeabilization was used in further experiments, allowing the manipulation of five samples simultaneously. A range of NR incubation periods from 0 to 30 min was also evaluated, and the results showed a similar pattern observed previously for the permeabilization time experiments, with no significant variation in fluorescence values for all sampling times (Figure 3B). Based on these results, the NR time exposure was established as 1 min. It is noteworthy that both *H. seropedicae* strain SmR1 and *A. brasilense* strain FP2 showed the same behavior, allowing the use of the same protocol for both species.

To determine the optimal NR concentration to stain cells with higher fluorescence values, a range from 0 to 500 μM was assayed. Since NR is not a PHB-specific dye, it was necessary to measure the NR fluorescence background in non-PHB producing cells. The *H. seropedicae* SmR1 derived mutant Δ*phaC1* defective in PHB production was tested against the wild type SmR1, both at OD_{600} 1.2. For *A. brasilense* strain FP2, the wild type at low (0.3) and high (1.2) OD_{600} were compared. These conditions were selected based on our GC data (not shown) that have shown a high production of PHB at OD_{600} 1.2 for both wild type strains, whereas both *H. seropedicae* Δ*phaC1* mutant strain and *A. brasilense* strain FP2 at OD_{600} 0.3 did not produce PHB, as detected by the GC method. The optimal NR concentration was 31.25 μM for both bacteria (Figure 3C and D).

3.4 Fluorescence emission stability

The last parameter to be optimized was the NR fluorescence stability in TBAC buffer and in non-stained and stored permeabilized samples. The fluorescence emission stability of NR stained samples of *A. brasilense* strain FP2 was monitored for a duration of 90 min (Figure 4A). The NR fluorescence started to decrease 5 min after staining. After 30 min, the fluorescence levels were similar to those of the background level. However, it is worth noting that unstained permeabilized cells can be stored under refrigeration (2 to 8°C) without significant loss of NR staining capacity for at least 5 days (Figure 4B). Therefore, one can store samples prior to NR staining for at least 5 days and then stain cells and capture the emitted fluorescence.

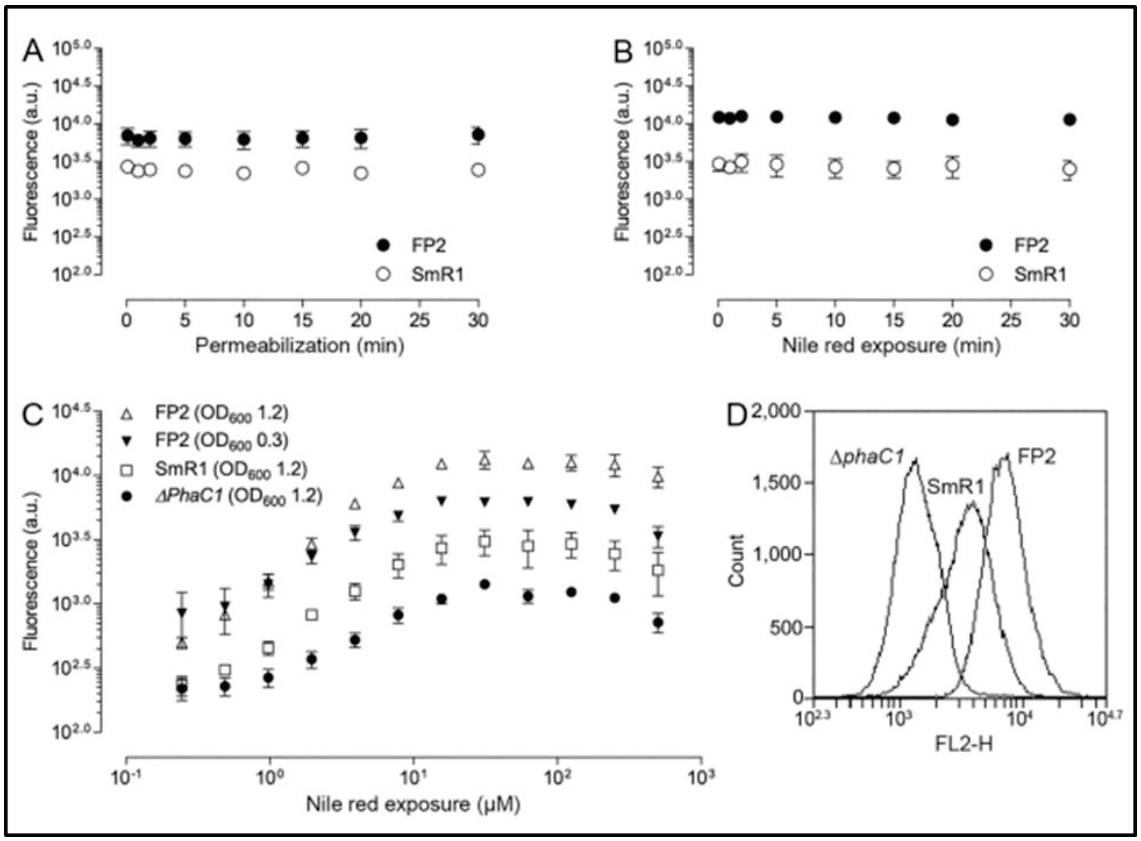

Figure 3. Incubation time required for cell permeabilization and Nile red (NR) staining

Bacteria were grown at an OD_{600} of 1.2, 0.1 mL was centrifuged (1 min at 13,400 g), and resuspended in 1 mL of TBAC buffer containing 50% of EtOH. A, exposure with EtOH during 0 to 30 min and cells stained with NR (9.42 μM). B, after 1 min of exposure in TBAC buffer containing 50% of EtOH, the bacterial suspensions were incubated with NR (9.42 μM) during 0 to 30 min. C, after 1 min of exposure of cells in TBAC buffer containing 50% of EtOH, the bacterial suspensions were stained with NR (0 to 500 μM) during 1 min, centrifuged (1 min at 13,400 g), and resuspended in TBAC buffer for analysis by flow cytometry. Fluorescence (arbitrary units, a.u.) data are reported as means±SD of 3 independent experiments with *H. seropedicae* strain SmR1 and *A. brasilense* strain FP2, by using the median fluorescence intensity values in the FL2-H channel. D, histogram overlay of three samples, a mutant strain of H. seropedicae, ΔphaC1, *H. seropedicae* strain SmR1, and *A. brasilense* strain FP2, using the optimized concentration of NR (31.25 μM).

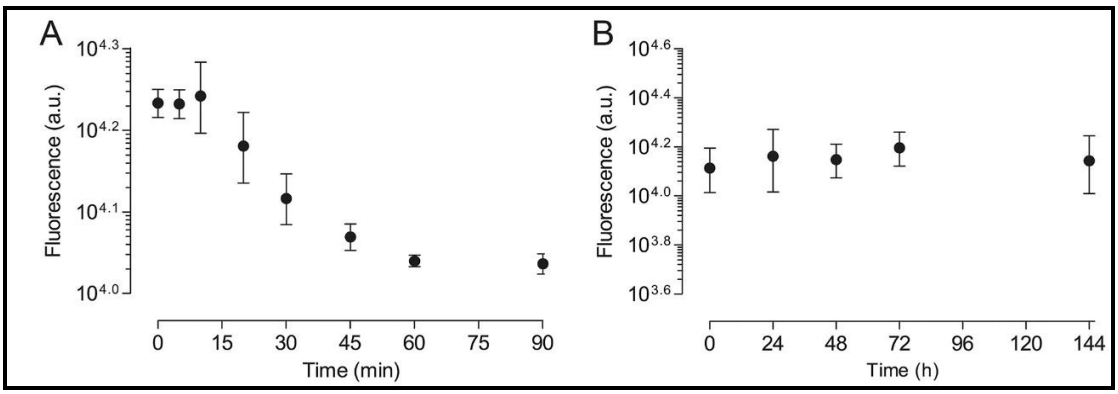

Figure 4. Nile red (NR) fluorescence stability.

A. brasilense strain FP2 was grown until reaching OD600 of 1.4. PHB measurements were performed using the optimized procedure, as described in the Materials and Methods. A, After resuspension of cells in TBAC buffer, NR fluorescence was monitored during 90 min by flow cytometry. B, Samples were stored at 4°C in permeabilization solution (TBAC buffer containing 50% of EtOH), until analysis, as indicated. For analysis, samples were stained with NR (31.25 µM) during 1 min, centrifuged (1 min at 13,400 g), resuspended in TBAC buffer and analyzed immediately by flow cytometry. Fluorescence (arbitrary units, a.u.) data are reported as means±SD of 3 independent experiments with *H. seropedicae* strain SmR1 and *A. brasilense* strain FP2, by using the median fluorescence intensity values in the FL2-H channel.

3.5 Flow cytometry *versus* gas chromatography

Several reports have demonstrated a linear correlation between the amount of PHB and the fluorescence emission intensity of NR stained cells. In order to validate the optimized method for PHB quantification by flow cytometry, the standard gas chromatography method was applied to the same bacterial cultures. *H. seropedicae* SmR1 and Δ*phaC1* strains, *A. brasilense* strain FP2, and mutant of *A. brasilense* strain Sp7, identified as *phbC* Sp7, impaired in the production of PHB, were grown in a medium containing 5 and 20 mM NH_4Cl, since PHB production has been correlated with carbon availability and nitrogen limitation.

The results revealed that the kinetic curves of NR fluorescence as a function of OD_{600} varied among bacteria. As shown in Figure 5A, *H. seropedicae* strain SmR1 grown in 5 mM NH_4Cl produced more PHB as compared to the growth in 20 mM NH_4Cl, results

also confirmed by GC. For *A. brasilense* strain FP2, low NH_4Cl levels also triggered PHB production; however, *A. brasilense* strain FP2 grown in 20 mM NH_4Cl did not produce any detectable PHB (results also confirmed by GC; Figure 5B). These data highlighted important differences between PHB accumulation in *H. seropedicae* strain SmR1 and *A. brasilense* strain FP2, since *H. seropedicae* strain SmR1 seems to always produce PHB, even at low OD_{600}, whereas *A. brasilense* strain FP2 produces PHB only in OD_{600} 1.0 or higher, with limiting nitrogen concentration in the growth medium (25). As shown in Figure 5C, flow cytometry and GC present a very high correlation coefficient (R^2) of 0.99 for both bacteria.

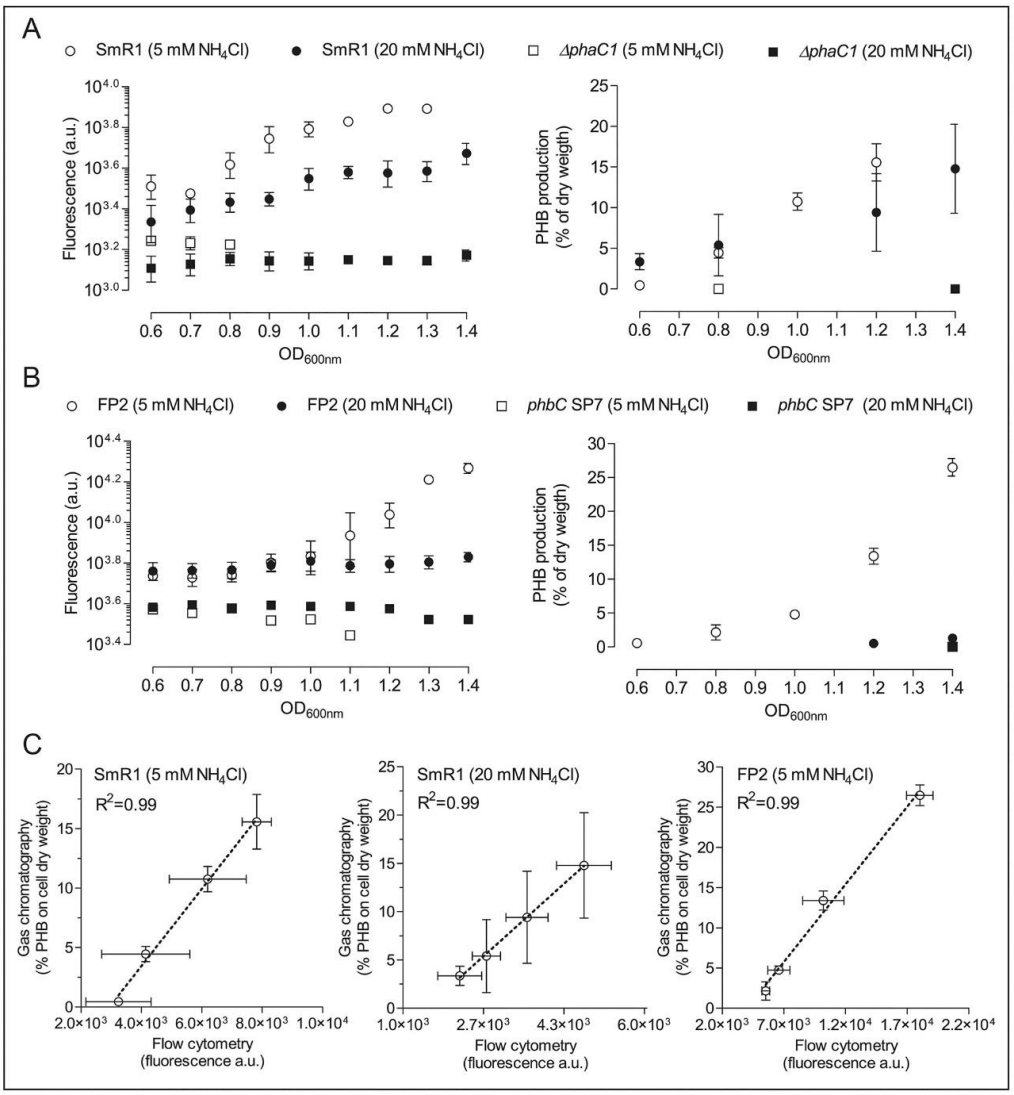

Figure 5. Correlation between flow cytometry and gas chromatography (GC) analysis for PHB quantification.

PHB measurements by flow cytometry using the optimized procedure and the standard method based on GC were applied on *H. seropedicae* strain SmR1, a mutant strain of *H.*

seropedicae, Δ*phaC1*, *A. brasilense* strain FP2, and a mutant strain of *A. brasilense*, phbC SP7, OD_{600} ranging from 0.6 to 1.4 using two NH4Cl concentrations in growth medium, as indicated. A, *H. seropedicae*. B, *A. brasilense*. C, correlation between flow cytometry and GC. Fluorescence (arbitrary units, a.u.) data are reported as means±SD of 3 independent experiments with *H. seropedicae* strain SmR1 and *A. brasilense* strain FP2, by using the median fluorescence intensity values in the FL2-H channel.

3.6 Fluorescence microscopy analysis

Although this method has been optimized with every precaution possible to ensure high accuracy in flow cytometry determination of PHB, the same protocol can be successfully applied to stain cells for fluorescence microscopy analysis. To confirm this assumption, *H. seropedicae* and *A. brasilense* samples stained with NR using the optimized protocol were analyzed by fluorescence microscopy. In addition, the non-optimized *versus* optimized procedures were compared. Fluorescent micrographs of *H. seropedicae* and *A. brasilense* revealed an increase fluorescence emission intensity in samples stained using the optimized protocol (Figure 6).

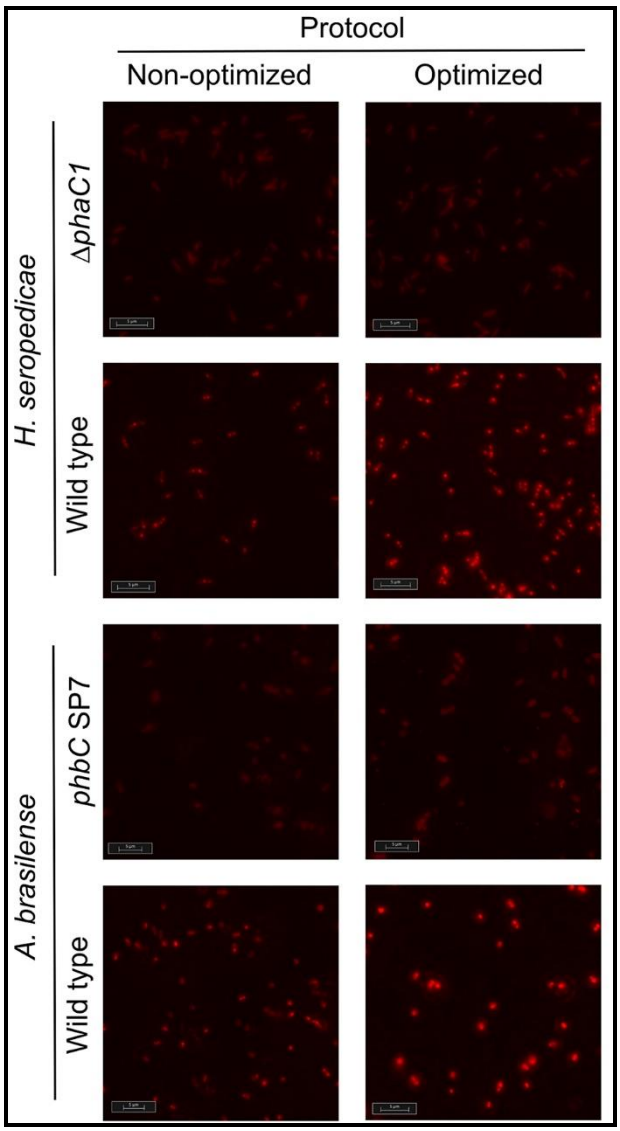

Figure 6. Intracellular PHB detection by fluorescence microscopy using the non-optimized and optimized protocols.

H. seropedicae strain SmR1, a mutant strain of *H. seropedicae*, Δ*phaC1*, *A. brasilense* strain FP2, and a mutant strain of *A. brasilense*, phbC SP7 were grown to OD600 of 1.4 using 5 mM NH4Cl in growth medium. Fluorescence microscopy analysis was performed using the procedure described in the Material and Methods.

3.7 PHB production in epiphytic bacteria analyzed by flow cytometry

To determine whether this optimized method could be applied to quantify PHB in small-cell-number samples, *H. seropedicae* strain SmR1 and *A. brasilense* strain FP2 cells epiphytically growing on rice roots were detached, stained and PHB was quantified by flow cytometry. After 7 days of rice inoculation, the results showed that both *H. seropedicae* SmR1 and *A. brasilense* FP2 grown epiphytically on rice produced

PHB during colonization (Figure 7). This is a first-time demonstration of what can be considered an easy and reliable approach to follow the kinetics of PHB production by epiphytic bacteria. Since the amount of epiphytic cells is usually insufficient to be determined by GC-based methods, the optimized protocol developed in the present work constitutes an important tool to monitor the production of PHB during plant-bacteria interaction, to screen for potential PHB producers among plant-associated bacteria, and in biotechnological studies to evaluate and improve PHB production by bacteria.

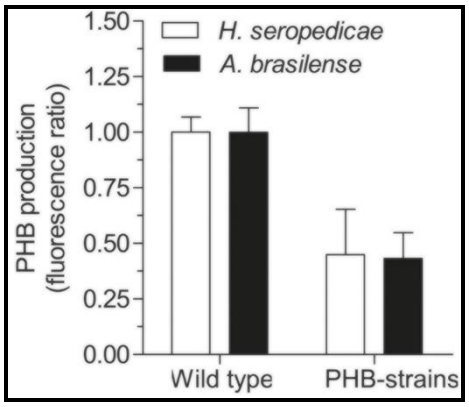

Figure 7. PHB production of epiphytic bacteria analyzed by flow cytometry.
PHB measurements were performed using the optimized procedure on rice epiphytic *H. seropedicae* strain SmR1, a mutant strain of *H. seropedicae*, Δ*phaC1*, *A. brasilense* strain FP2, and a mutant strain of *A. brasilense*, phbC SP7, seven days after inoculation, as described in the Materials and Methods. Fluorescence ratio data are reported as means±SD of 2 independent experiments performed in triplicate, using the median fluorescence intensity values in the FL2-H channel.

4 Discussion

For the feasibility of a NR-based method, the dye must cross the bacterial membranes in order to stain the intracellular PHB. Full uptake of fluorescent dyes appears to be critical for a complete intracellular target staining. Besides the hydrophobicity of NR, the bacterial uptake varies widely among different species, essentially due to differences in membrane permeability. Several strategies can be applied to improve the entry of NR, such as those using buffers containing ethanol (9), or sucrose-based buffers, such as the TSE buffer (10).

The sucrose-buffer TSE was successfully applied to permeabilize cells of *Synechocystis* sp. PCC6803 and *Escherichia coli* to NR staining in PHB-producing conditions (10). In

the present work the TSE buffer failed to efficiently permeabilize *H. seropedicae* strain SmR1, and produced only a partial effect on *A. brasilense* strain FP2 (Figure 1A and B). The TBAC buffer, on the other hand, containing 50% ethanol was found to be the best permeabilization solution for *H. seropedicae* and *A. brasilense*.

Apparently, the optimal NR concentration can vary among species, and therefore the adjustment of the concentration according to species is a critical step for optimization. For both *H. seropedicae* and *A. brasilense* strains, fluorescence emission increased up to the NR concentration of 31.25 μM, decreasing at higher concentrations of NR (Figure 4A). Despite the very low concentration of NR (0.032 μM) described for both *Saccharomyces cerevisae* and *Cupriavidus necator* (9), most bacteria require higher NR concentrations, as demonstrated for *Synechocystis* sp. strain PCC6803 (3.3 μg/mL – 9.42 μM), *Escherichia coli* (33 μg/mL – 94.2 μM) (10) and *Ralstonia pickettii* AR1 (20 μg/mL –62.8 μM) (24). Such variations clearly show that protocols must be optimized for each microbe under study before the introduction of NR-fluorescence flow cytometry as a technique to quantify PHB or other kinds of neutral lipids.

Compared to *H. seropedicae* strain SmR1, in all conditions assayed here, *A. brasilense* strain FP2 always produced higher basal fluorescence values (Figures 2, 3, and 5). Two major reasons could explain this observation: i) NR binding to different intracellular lipids droplets, and ii) the difference in size between the *H. seropedicae* strain SmR1 and *A. brasilense* strain FP2, which was almost twice the size. In view of this, we hypothesize that the higher fluorescence emission values observed for *A. brasilense* strain FP2 are probably due to the bacteria size.

Despite the fact that PHA quantification by GC is largely used in microbial PHA research, this methodology is quite laborious and requires hazardous solvent manipulations. The standard method for PHB quantification involving methanolysis followed by GC analysis is a well-established and reproducible technique, however with a main drawback: the long time needed to analyze sample by sample. A typical procedure of methanolysis followed by GC analysis requires 16 h of lyophilization, 5 h of methanolysis and 20 min for GC data acquisition for each sample – at least 250 times longer than our NR optimized protocol using flow cytometry, which was less than 5 min. In addition, our flow cytometry protocol is even faster than other flow cytometry methodologies that require 25 to 50 min to be completed (9,10,24). Another advantage of the NR flow cytometry protocol reported here is the low amount of cells required for

analysis, which unlike other methodologies allows one to perform a larger number of experiments in different conditions. While the quantification of other PHAs, such as polyhydroxyhexanoate and polyhydroxyoctanoate, was not tested in this study, we believe our protocol can be successfully adapted to quantify other PHAs.

The methodologies used here, flow cytometry and GC, applied to PHB quantification presented a very high correlation coefficient (R^2) of 0.99 for both bacteria. This level of correlation is in agreement with methods optimized for other microorganisms, such as *E. coli* (R^2=0.96) (10), *S. cerevisiae* (R^2=0.99), and *C. necator* (R^2=0.99) (9).

In summary, a reliable and relatively fast flow cytometric procedure was developed for PHB quantification in *H. seropedicae* SmR1 and *A. brasilense* FP2 grown in cultures or in cells isolated from grass root surfaces. PHB production can be quantified with accuracy and precision using NR staining, following the six optimized steps detailed in this paper. This protocol has potential to be used in other studies involving PHB metabolism in these and other bacterial species, as well as in quality control of inoculant, since PHB production has been reported as an important feature to maintain the fitness of plant-associated bacteria.

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CAPÍTULO II

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Backup expression of the PhaP2 phasin compensates for *phaP1* deletion in *Herbaspirillum seropedicae*, maintaining fitness and PHB accumulation

Backup expression of the PhaP2 phasin compensates for phaP1 deletion in *Herbaspirillum seropedicae*, maintaining fitness and PHB accumulation

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Abstract

Phasins are important proteins controlling PHB granules formation, their number into the cell and stability. The genome sequencing of the endophytic and diazotrophic bacterium *Herbaspirillum seropedicae* SmR1 revealed two homologous phasin genes. To verify the role of the phasins on PHB accumulation in the parental strain *H. seropedicae* SmR1, isogenic strains defective in the expression of *phaP1*, *phaP2* or both genes were obtained by gene deletion and characterized in this work. Despite of the high sequence similarity between PhaP1 and PhaP2, PhaP1 is the major phasin in *H. seropedicae*, since its deletion reduced PHB accumulation by ≈ 50 % in comparison to the parental and Δ*phaP2*. Upon deletion of *phaP1*, the expression of *phaP2* was 6-fold enhanced in the Δ*phaP1* strain. The responsive backup expression of *phaP2* partially rescued the Δ*phaP1* mutant, maintaining about 50% of the parental PHB level. The

double mutant Δ*phaP1.2* did not accumulate PHB in any growth stage and showed a severe reduction of growth when glucose was the carbon source, a clear demonstration of negative impact in the fitness. The co-occurrence of *phaP1* and *phaP2* homologous in bacteria relatives of *H. seropedicae*, including other endophytes, indicates that the mechanism of phasin compensation by *phaP2* expression may be operating in other organisms, showing that PHB metabolism is a key factor to adaptation and efficiency of endophytic bacteria.

1. Introduction

Herbaspirillum seropedicae SmR1 is a diazotrophic β-Proteobacterium that associates beneficially with economically relevant species of Gramineae (Baldani et al., 1986) and produces poly-3-hydroxybutyrate (PHB) granules as means of carbon and energy storage (Catalan et al., 2007; Kadowaki et al., 2011). Therefore, *H. seropedicae* SmR1 is an important model to study the impact of PHB metabolism on endophytic growth and adaptation. Thirteen genes probably involved in PHB metabolism were identified in the strain SmR1 (Kadowaki et al., 2011; Pedrosa et al., 2011), including four *phaC*, two *phaZ* and two *phaP* genes encoding PHA synthases, PHA depolymerases and phasins, respectively. Although *H. seropedicae* SmR1 possesses three genes encoding proteins homologous to PHA synthases (Pedrosa et al., 2011), PHB synthesis is supported by the PHA synthase expressed by *phaC1*, since its deletion abolishes PHB accumulation (Tirapelle et al., 2013). Furthermore, *phbF* (hereafter *phaR*) which encodes a transcriptional repressor protein was identified and characterized (Kadowaki et al., 2011). Probably, PHB plays important roles in nitrogen fixation and plant-bacteria interactions (Mandon et al., 1998; Kadouri et al., 2003; Wang et al., 2007; Quelas et al., 2013), but its action in *H. seropedicae* was not been fully characterized.

PHB is an aliphatic polyester member of the polyhydroxyalkanoates (PHA) family that some bacteria synthesize to store carbon and reducing equivalents (Anderson and Dawes, 1990; Madison and Huisman, 1999). In addition, the production of PHB is a hot topic in biotechnology due to its physicochemical properties very close to oil-based plastics, while PHB is readily degradable in the environment (Chen, 2009; Urtuvia et al., 2014). Therefore, PHB is a bio-sustainable alternative for synthetic plastic materials. PHB is usually produced under conditions of carbon excess and low levels of essential

nutrients including nitrogen, phosphate and oxygen (Hervas et al., 2008). At least three enzymes: 3-ketothiolase, acetoacetyl-CoA reductase and PHA synthase encoded by *phaA*, *phaB* and *phaC* respectively are involved in its synthesis (Babel et al., 2001), which occurs by condensation of acetyl-CoA forming acetoacetyl-CoA, then reduction of acetoacetyl-CoA to 3-hydroxybutyryl-CoA (3HB-CoA) and finally polymerization of 3HB-CoA to yield PHB (Steinbuchel and Hein, 2001). When carbon/energy is required, the polymer is mobilized by PHA depolymerazes encoded by *phaZ* genes (Babel et al., 2001). Polymeric PHB is stored as insoluble, intracellular granules that are coated with proteins (totaling 0.5 to 2 % of the granule weight) (Grage et al., 2009; Jendrossek, 2009). Phasins are small amphiphilic proteins attached on the surface of polyhydroxyalkanoate inclusions in Bacteria and Archaea (Neumann et al., 2008; Jendrossek, 2009; Cai et al., 2012). These proteins control the size and number of PHB granules (Wieczorek et al., 1995; Jurasek and Marchessault, 2002; 2004; Potter et al., 2004; Cho et al., 2012) and are present in all PHA producing bacteria. Although not highly conserved in terms of amino acid sequence, phasins perform similar functions in promoting granule formation and stabilization of PHA in different microbes (York et al., 2001a; York et al., 2001b; Jurasek and Marchessault, 2002). *Ralstonia eutropha* H16, a well-studied model of PHB metabolism, contains seven phasin genes (Potter et al., 2005; Kuchta et al., 2007; Pfeiffer and Jendrossek, 2011; 2012) but it seems that PhaP1 is the major phasin affecting PHB accumulation (Potter et al., 2005). In plant-associated bacterium *Sinorhizobium meliloti* Rm1021, the genes SMc00777 and SMc02111 encode the phasins PhaP1 and PhaP2, respectively (Wang et al., 2007). The deletion of both genes resulted in a mutant defective in PHB production and plants of *Medicago truncatula* inoculated with this mutant exhibited reduced shoot dry weight. The occurrence of phasin-expressing genes in the genome of other plant-associated bacteria as *Azospirillum brasilense* Sp245, *Azospirillum lipoferum* 4B, *Azoarcus* sp. BH72 and *Pseudomonas stutzeri* A1501 (Krause et al., 2006; Yan et al., 2008; Wisniewski-Dye et al., 2011) indicates that PHB metabolism is important to bacteria during plant colonization, as previously suggested (Trainer and Charles, 2006). The genome sequencing of *H. seropedicae* SmR1 revealed two paralogous phasin genes (75% similarity, 59% identity), the products of which had been shown by proteomic analyses to be the main phasins associated to PHB granules (Pedrosa et al., 2011; Tirapelle et al., 2013). Nevertheless, in the Δ*phaP1* mutant, PhaP2 replaced PhaP1 on

the surface of the granules (Tirapelle et al., 2013). To see if this functional complementarity was also reflected in conservation of function, we deleted *phaP1* or *phaP2* or both genes to analyze the effect of phasin absence on PHB accumulation in the mutants. At the same time, we determined the expression patterns of the genes in transcriptional fusions to a *lacZ* reporter-gene housed in different genetic backgrounds, to address if *phaP2* expression enhances upon deletion of *phaP1*, as related previously for other examples of redundant genes (Bratlie et al., 2010; Kondrashov, 2012).

2. Materials and Methods

2.1 Bacterial strains, plasmids and growth conditions

Strains and plasmids used in this work are listed in Table 1. *Escherichia coli* strain Top10 (Thermo Fisher Scientific Inc., Waltham, MA, USA) and S17.1 (Simon et al., 1983) were used for cloning and conjugation procedures, respectively, while strain ET8000 (MacNeil et al., 1982) was used in expression assays with *lacZ* fusions. *E. coli* strains were grown at 37°C in LB medium and shaken at 160 rpm. *Herbaspirillum seropedicae* parental strain SmR1 (Souza et al., 2000) and mutant strains were grown in NFbHP media with 37 mM DL-malate or 25 mM glucose and 20 mM NH_4Cl at 30°C and shaken at 120 rpm (Pedrosa and Yates, 1984).

Tabela 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	(Simon et al., 1983)
ET8000	Wild-type strain	(MacNeil et al., 1982)
H. seropedicae strains		
SmR1	Parental strain, Nif^+, Sm^R	(Souza et al., 2000)
Δ*phaP1*	Chromosomal deletion of *phaP1*	(Tirapelle et al.,

		2013)
Δ*phaP2*	Chromosomal deletion of *phaP2*	This work
Δ*phaP1.2*	Chromosomal deletion of *phaP1* and *phaP2*	(Tirapelle et al., 2013)
Δ*phaC*1	Chromosomal deletion of *phaC1*	(Tirapelle et al., 2013)
Plasmids		
pTZ18R	Cloning plasmid	(Mead et al., 1986)
pTZ57R/T	T/A cloning plasmid	Fermentas
pDK6	Expression vector *tac* promoter *lacI*^{q}, Km^{R}	(Kleiner et al., 1988)
pMMS31	Derivative of pDK6 encoding PhbF from *H. seropedicae* SmR1	(Kadowaki et al., 2011)
pMP220	RK2 derivative, low-copy number, promoterless *lacZ* containing vector used to construct transcriptional fusions; Tc^{R}	(Spaink et al., 1987)
pEFT11	pMP220 harbouring the 5'-flanking region of *phaP*1 cloned upstream of *lacZ*	This work
pEFT12	pMP220 harbouring the 5'-flanking region of *phaP*2 cloned upstream of *lacZ*	This work
pK18*mobsacB*	Suicide vector; Km^{R}, *sacB*, mobilizable plasmid	(Schafer et al., 1994)
pEFT13	Deletion product Δ*phaP*2 cloned into the pK18*mobsacB*	This work
pBBR1MCS3	Broad-host-range vector	(Kovach et al., 1995)
pLPA01	pBBR1MCS3 harbouring *phaP*1 of *H. seropedicae*. Over-expression of PhaP1	This work
pLPA02	pBBR1MCS3 harbouring the *phaP*2 of *H. seropedicae*. Over-	This work

	expression of PhaP2	

2.2 Quantification of PHB

PHB was quantified by methanolysis and GC-FID (gas chromatography coupled to flame-ionization detector) analyses as described previously (Braunegg et al., 1978) on 5 to 10 mg of lyophilized bacteria. Amounts of PHB in each sample were normalized to the cell dry weight (cdw; weight of the lyophilized bacterial pellet) and expressed as % of PHB.cell dry weight^{-1}.

2.3 Construction of mutants of *H. seropedicae* SmR1

The ΔphaP1 and ΔphaC1 mutants were constructed by in-frame deletion of the phaP1 (Hsero_1639, GenBank: 9402240) and phaC1 (Hsero_2999, GenBank: 9403600) in H. seropedicae SmR1, as previously described (Tirapelle et al., 2013). The in-frame marker-less deletion of phaP2 (Hsero_4759, GenBank: 9405360) was obtained by cloning upstream and downstream fragments of the gene into the non-replicating plasmid pK18mobsacB, which carries a kanamycin resistance cassette along with sacB that confers sucrose sensitivity (Schafer et al., 1994). Briefly, 500 bp fragments to either flank of phaP2 were amplified by PCR with primers Fw_phaP2_UP and Rev_phaP2_UP (sequences showed in Supplementary table 2) for the upstream region whereas Fw_phaP2_DOWN and Rev_phaP2_DOWN were used for the downstream region. The PCR products were cloned into pTZ57R/T and sequenced using universal and reverse M13 primers. The downstream fragment was ligated to the upstream fragment using the KpnI site, resulting in the deletion product ΔphaP2. The entire construction was digested with BamHI and SalI, in order to ligate the ΔphaP2 fragment into pK18mobsacB digested with the same enzymes, yielding pEFT13. E. coli S17-1 was transformed with pEFT13 and the plasmid conjugated to H. seropedicae SmR1 using bi-parental mating. Single-recombinants were selected on NFb-malate agar containing streptomycin 80 µg.mL-1, nalidixic acid 5 µg.mL-1 and kanamycin 500 µg.mL-1. A single-recombinant colony was collected in 3 mL NFb-malate and cultivated overnight without antibiotics. The culture was serially diluted and plated on NFb-malate agar containing 10 % (w/v) sucrose. Colonies that grew on sucrose were

screened for deletion by PCR using the primers Fw_phaP2_UP and Rev_phaP2_DOWN. To obtain the double mutant ΔphaP1.2, the plasmid pEFT13 was conjugated in the ΔphaP1 mutant of *H. seropedicae* and double-recombinants were selected as described above.

2.4 Construction of transcriptional fusions

The intergenic regions of *phaP1* (333 bp including 28 bp of the *phaP1* coding sequence) and *phaP2* (224 bp including 54 bp of the *phaP*2 coding sequence) were amplified from *H. seropedicae* SmR1 genomic DNA by PCR (primer sequences showed in Supplementary table 1) and cloned into pMP220 (Spaink et al., 1987) upstream of a promoterless, rbs-containing *lacZ* to yield plasmids pEFT11 and pEFT12, respectively.

2.5 RNA extraction and RT-PCR

Strains were grown on NFb-malate medium with 20 mM of ammonium chloride at 30°C and shaken at 120 rpm. Cells from 1.5 mL of culture at OD_{600} of 1.0 were collected by centrifugation (10,000 x *g*, 4°C, 5 minutes) and re-suspended in 1 mL of TRIzol® Reagent (Life Technologies, USA). The homogenized sample was incubated for 5 minutes at room temperature and then, extracted with 0.25 mL of chloroform. After centrifugation (10,000 x *g*, 4°C, 5 min), the aqueous phase was precipitated with 0.5 mL of isopropanol. The pellet was collected by centrifugation (10,000 x *g*, 4°C, 5 minutes) and washed with 1 mL of ethanol 80%. The air dried RNA pellet was resuspended in 30 µL of RNAse-free water and the quality of RNA preparation was determined by A_{260}/A_{280} ratio and checked by electrophoresis in 1.0% agarose gel. For cDNA preparation, 100 ng of total RNA was used in 20 µL reactions applying the High Capacity RNA-to-cDNA kit (Applied Biosystems, USA) according to the manufacturer's instructions. From the cDNA preparations, 1 µL was used as template in a 20 µL PCR reaction with PfuX7 as described previously (Norholm, 2010), applying the specific primers described in Supplementary table 1, 60°C as annealing temperature and 30 cycles of reaction. From each reaction, 2 µL were applied on a 1.5% agarose gel to visualize the intensity of the bands after ethidium bromide staining. The *rrsA* gene encoding the 16S rRNA was used as endogenous control. Negative controls were performed using as template samples of RNA untreated with reverse transcriptase.

2.6 Complementation of *phaP* mutants

*phaP*1 (Hsero_1639) plus 333 bp of the intergenic region upstream of its start codon was amplified by PCR with Fw_Pro*_phaP1* and Rev_Gen*_phaP1* primers (Supplementary table 1) from *H. seropedicae* SmR1 genomic DNA and cloned into the XhoI and XbaI sites of pBBR1MCS-3 (Kovach et al., 1995), generating pLPA01. Similarly, *phaP*2 (Hsero_4759) plus 224 bp of the intergenic region upstream of its start codon was amplified with Fw_Pro*_phaP2* and Rev_Gen*_phaP2* primers (Supplementary table 1) by PCR and cloned into the XhoI and XbaI sites of the pBBR1MCS-3, generating pLPA02. Conjugation was performed by bi-parental mating between *H. seropedicae* SmR1 and *E. coli* S17-1.

2.7 β-galactosidase activity assay

β-galactosidase activity was determined in *E. coli* ET8000 (grown in LB media) carrying the transcriptional fusion plasmids pEFT11 or pEFT12 in the presence of pMMS31 (that expresses the PhaR from *H. seropedicae*) or pDK6 (negative control). To perform similar analyses in *H. seropedicae* parental strain SmR1 and mutant strains, pEFT11 or pEFT12 was introduced by bi-parental mating with *E. coli* S17-1. Transconjugants were selected on NFb-malate agar containing 20 mM of ammonium chloride and tetracycline (10 $\mu g.mL^{-1}$). β-galactosidase activity was assayed following previous protocol (Miller, 1972).

2.8 Fluorescence and transmission electron microscopy

To visualize PHB granules in SmR1 and its *phaP* mutants, fluorescence microscopy was performed after staining with the fluorescent probe Nile Red, which stains neutral lipids (Spiekermann et al., 1999). Bacterial cultures (1 mL) were harvested by centrifugation for 60 seconds at 10,000 x *g*. The pellets were resuspended in 30 % (v/v) ethanol in PBS (Na_2HPO_4 10 mM, KH_2PO_4 1.8 mM, NaCl 130 mM) and 3 µL of 1.6 mM Nile Red (λ_{ex} 586 – 579 nm; λ_{em} 637 – 597 nm) dissolved in DMSO was added and incubated in the dark for 5 min. Then, the samples were centrifuged again for 60 s at 10,000 x *g*, resuspended in PBS and viewed under a fluorescent microscope. The images were obtained using an Axio Imager Z2 microscope (Carl Zeiss Microscopy GmbH, Jena, Germany), equipped with 4 Metafer automated capture software (Metasystems GmbH, Altlussheim, Germany) and a CoolCube 1 camera (with 100 x

magnification). To TEM analyses, cell pellets from *H. seropedicae* cultures were fixed with Karnovsky's fixative (Karnovsky, 1965), post-fixed with 2% OsO_4 in 0.1 M cacodylic acid buffer (pH 7.2) for 1 h and embedded in Epon 812 (Luft, 1961). After contrasting with 2% uranyl acetate (Watson, 1958) and lead citrate (Reynolds, 1963), samples were examined with a JEOL-JEM 1200 EX II transmission electron microscope.

2.9 Phylogenetic analysis of phasin sequences

The amino acid sequences used in the analysis are listed in the Supplementary figure 1. The alignment was performed by Muscle (Edgar, 2004) into the MEGA software package (Tamura et al., 2013), using default parameters. The resulting alignment was cured by Gblocks to remove poorly aligned positions and highly divergent regions (Castresana, 2000). The phylogeny reconstruction of the sequences in the cured alignment was obtained by the Neighbor-joining method and tested by bootstrap (10,000 replicates).

2.10 Statistical analysis

Where appropriate, statistical analysis was carried out using independent two-sample t-test with the R package (R Development Core Team, 2015).

3 Results

3.1 *H. seropedicae* SmR1 contains two paralogous phasin and a third less conserved putative phasin

Three genes coding putative phasins (*phaP1*, locus-tag Hsero_1639; *phaP2*, Hsero_4759 and *phaP3*, Hsero_2402) (Kadowaki et al., 2011; Pedrosa et al., 2011) are present in *H. seropedicae* SmR1. The alignments and pair-wise distances between PhaP1 and PhaP2 showed a short evolutionary distance (0.412 using the p-distance method), but PhaP3 was further removed, indicating significant sequence divergence and possibly also function (Figure 1A-D). Indeed, PhaP3 was only detected on PHB granules when *phaP1* was deleted and, it was clearly less abundant than PhaP2, the main phasin in the absence of PhaP1. The *phaP1* and *phaP2* genes could have been

generated by gene duplication based on 59 % identity between their encoded amino acid sequences, as well as a region of more than 150 amino acids that can be aligned (Figure 1A), according to the classification of duplicated genes proposed by Gevers *et al.* (Gevers et al., 2004). Nevertheless, the horizontal gene transfer (HGT) of an additional phasin gene cannot be totally ruled out, as intra-genome homologs are also acquired via HGT (Maerk et al., 2014). Accordingly, the co-occurrence of *phaP1* and *phaP2* homologs was found in other bacteria phylogenetically close to *H. seropedicae* SmR1 (Figure 1E). The phylogenetic analysis of PhaP1 and PhaP2 homolog sequences showed that the appearance of a second phasin occurred early in evolution of the *Herbaspirillum* genus and was conserved in subsequent speciation events (Figure 1E), indicating that the presence of both phasins should be important for PHB accumulation.

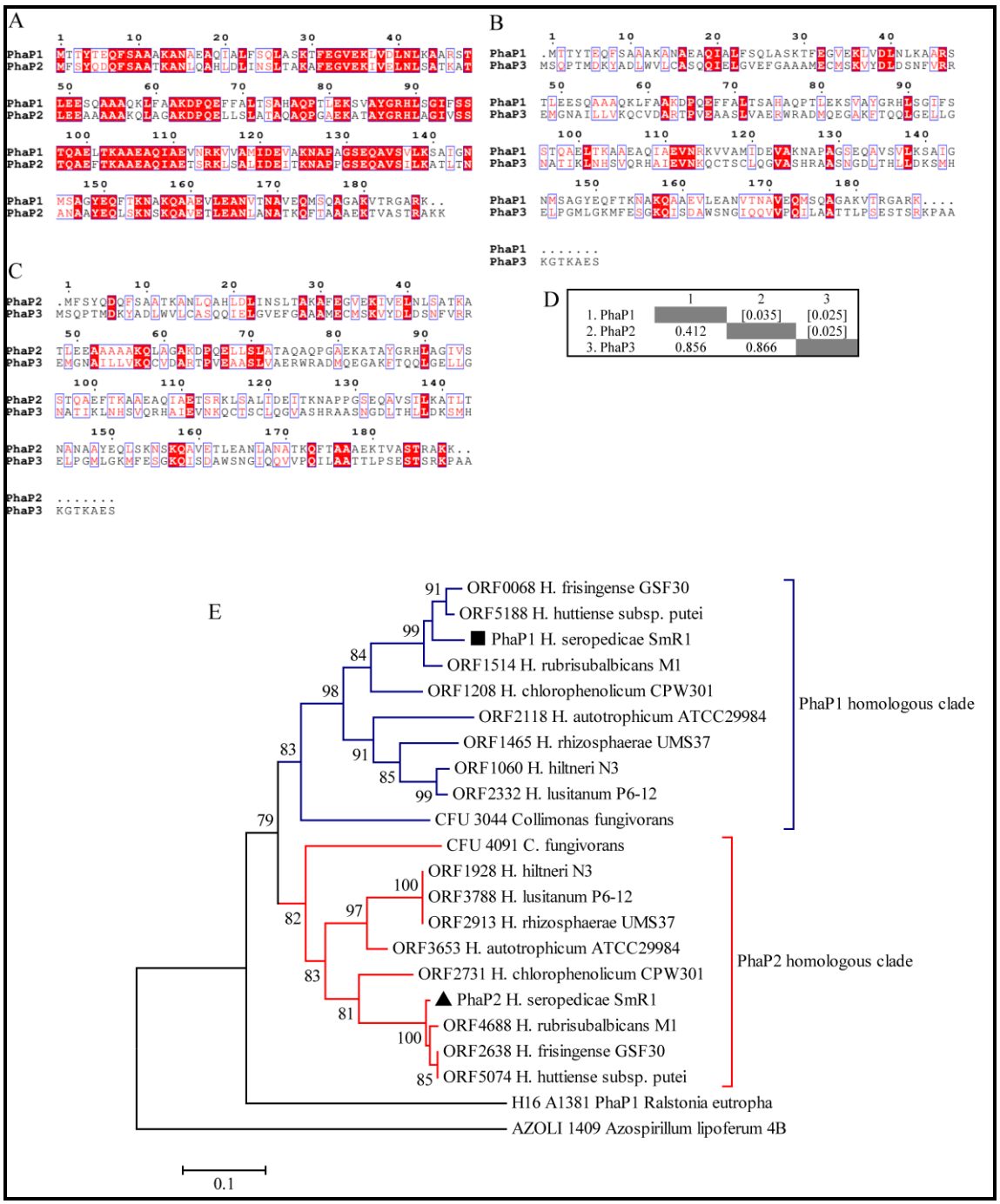

Figure 1. Protein sequence alignments of phasins from *H. seropedicae* SmR1 and phylogenetic analysis of homologous sequences. Multiple alignments of protein sequences of (A) PhaP1 (Hsero_1639) and PhaP2 (Hsero_4759), (B) PhaP1 and PhaP3 (Hsero_2402) and, (C) PhaP2 and PhaP3 were carried out using Espript 3.0 and the default parameters (Robert and Gouet, 2014). The red shaded letters indicate identical residues, while red letters represent conserved residues. (D) The number of amino acid differences per site from between PhaP1, PhaP2 and PhaP3 sequences are shown. Standard error estimate(s) are shown above the diagonal (enclosed within brackets) and were obtained by a bootstrap procedure (10000 replicates). All ambiguous positions were removed for each sequence pair. There were a total of 199 positions in the final dataset. Evolutionary analyses

were conducted in MEGA6 (Tamura et al., 2013). (E) The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The putative phasin of *Azospirillum lipoferum* encoded by the AZOLI 1409 (Wisniewski-Dye et al., 2011) was applied as an outgroup. Only nodes with bootstrap test (10000 replicates) bigger than 70% are shown next to the branches (Efron et al., 1996). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the p-distance method and are in the units of the number of amino acid differences per site. Evolutionary analyses were conducted in MEGA6 (Tamura et al., 2013). The PhaP sequences used to construct the tree are shown in Supplementary figure 1.

3.2 Deletion of *phaP1* reduces PHB accumulation

To verify the roles of PhaP1 and PhaP2 in PHB accumulation, three isogenic deletion mutants - Δ*phaP1*, Δ*phaP2* and Δ*phaP1.2* - were constructed. The mutant strains grew equally well as the parental strain on NFb-malate medium (Figure 2A and Table 2). Since cells accumulating PHB are less translucent than those without PHB, which can affect optical density measurements, the growth of parental and mutant strains was also determined by counting the number of viable cells in culture (Supplementary figure 2). Intracellular accumulation of PHB in the strains grown on malate was determined: (i) in early exponential growth (OD_{600} = 0.6); in mid-exponential growth (OD_{600} = 0.8); (iii) early stationary phase (OD_{600} = 1.2) and, (iv) in late stationary growth (OD_{600} = 1.4). The parental strain accumulated PHB up to 13 % (w/w) of cell dry weight (cdw) after 12 h of growth (early stationary phase) (Figure 2B), but decreased to 11 % of PHB at late stationary phase. Similar behavior was observed with Δ*phaP2* which reached 15 % of cdw at early stationary phase. On the other hand, Δ*phaP1* only accumulated approximately 50 % of parental levels of PHB at all tested growth phases (Figure 2B). Deletion of both phasins (Δ*phaP1.2*) drastically reduced PHB accumulation to < 1 % of cdw (Figure 2B).

To exclude the possibility that it was growth on malate rather than the mutations *per se* that affected PHB accumulation, similar experiments were performed using glucose as the carbon source. Under these conditions, SmR1 also accumulated PHB as previously reported for the strains Z67, Z69 and Z78 of *H. seropedicae* (Catalan et al., 2007) and the parental strain reached a maximum of 40 % of cdw in late-stationary phase. The production of PHB by Δ*phaP2* was similar to the parental strain at the early stat-phase and reached 56 % of cdw at the late stat-phase. On the other hand, the

Δ*phaP1* accumulated only 18 % of PHB as its maximum production, corresponding to 50 % of the PHB content of the parental strain (Fig. 2C). In Δ*phaP1.2*, PHB accumulation was not detected when cultivated on glucose. Noteworthy, the Δ*phaP1.2* strain presented a growth penalty when cultivated in glucose (Table 2), suggesting that the absence of PHB affects the glucose metabolism in *H. seropedicae*. Despite the growth rate in glucose of the Δ*phaP1* mutant did not present a statistical difference (Table 2), its growth pattern was atypical, since it stopped to grow early than parental strain (Figure 2A). This finding is also in agreement that reduction in PHB affects growth in glucose. In conclusion, regardless of the carbon source employed and the growth phase, the absence of PhaP1 or both phasins negatively affected PHB accumulation.

Tabela 2. Growth rates of *phaP* mutants in NFb media containing malate or glucose as carbon source.

Strain	**Growth rate (ΔOD_{595}/h) [a]**	
	Malate[b]	Glucose
SmR1 (wt)	0.237 ± 0.007	0.184 ± 0.026[c]
Δ*phaP1*	0.182 ± 0.029	0.123 ± 0.022[d]
Δ*phaP2*	0.200 ± 0.018	0.151 ± 0.017[c]
Δ*phaP1.2*	0.164 ± 0.019	0.055 ± 0.009[e*]

a - The values are averages ± standard errors for two independent experiments. b – growth rate was calculated with the data of OD_{600} between 8 – 13 h of growth for all strains. c – growth rate calculated between 10 – 16 h of growth. d – growth rate calculated between 12 – 19 h of growth. e - growth rate calculated between 7 – 16 h of growth. Where appropriate, statistical significance is shown ([*] p-value ≤ 0.05). The data obtained to the SmR1 strain was used for normalization and determination of statistical significance.

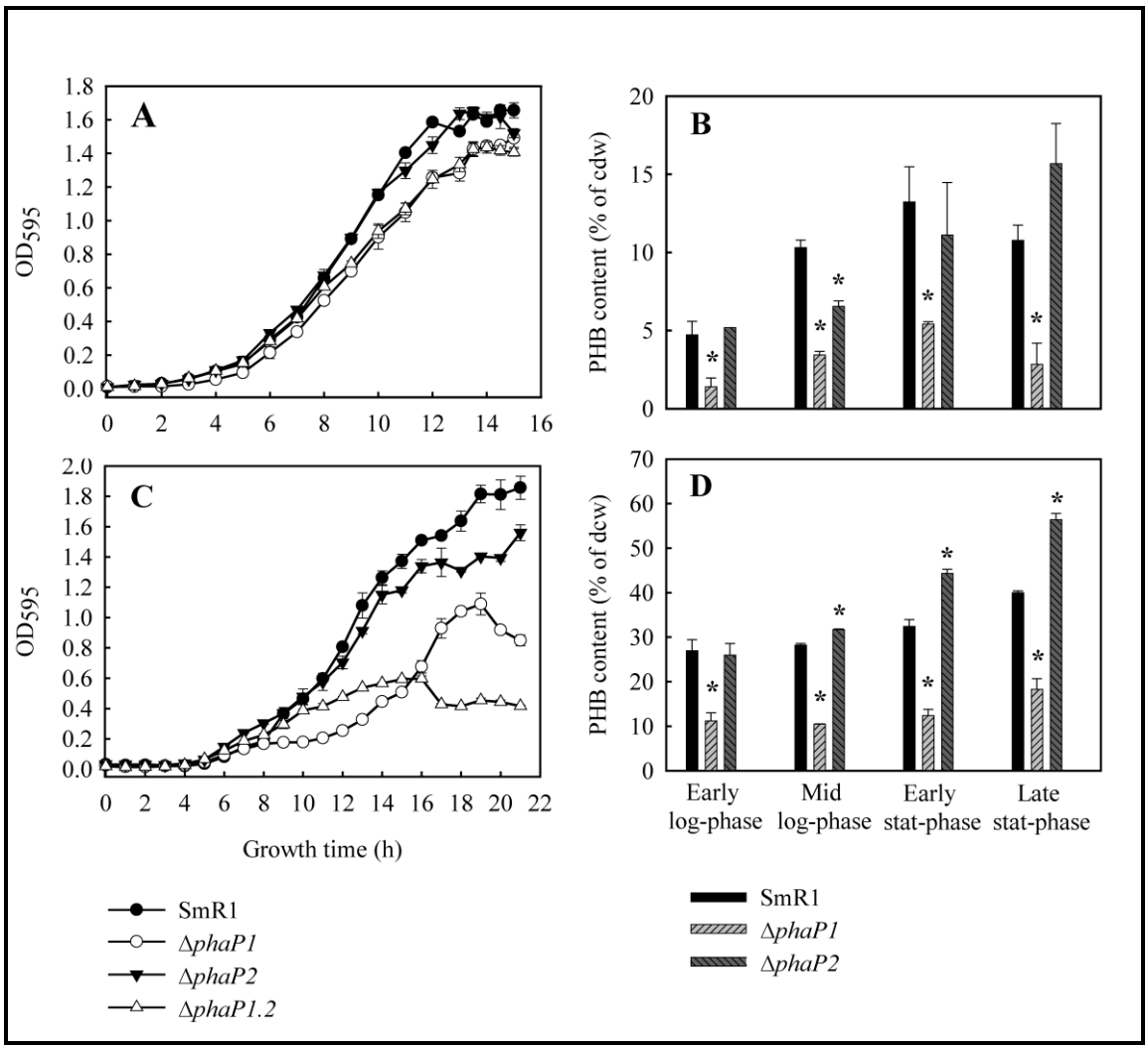

Figure 2.Growth and PHB accumulation profiles of *H. seropedicae* SmR1 (parental strain) and the mutants *ΔphaP1, ΔphaP2 and ΔphaP1.2*. Strains were grown in NFb medium with 20 mM of ammonium chloride and 37 mM DL-malate (A and B) or 25 mM (w/v) D-glucose (C and D) at 30°C (orbital agitation at 120 rpm). OD_{595} growth data were obtained from three independent cultures, while PHB contents were determined on four independent samples. PHB levels in Δ*phaP1.2* were less than 1 % at all growth phases. Where appropriate, statistical significance is shown (* - p-value ≤ 0.05, independent two-sample t-test). The data obtained to SmR1 was used for normalization and determination of statistical significance.

3.3 Microscopic alterations in the number of granules per cell

After staining with Nile Red, 92 % of native cells contained at least two PHB granules per cell (Fig 3) the rest only one (n = 100 counted cells), but in Δ*phaP1* the situation was practically reversed - only 18 % of cells contained two granules and 82 % only one (n = 100 cells). Δ*phaP2* had a similar granular distribution as the parental strain. No cells containing two PHB granules in Δ*phaP1.2* were observed, but 9 % of the counted

cells (n = 100) contained one PHB granule. In the strict sense, it was not clear whether these PHB granules were coated by other proteins or a transient agglomeration of polymer within the cytoplasm, since at all times examined, the level of PHB accumulated in the double mutant was below 1 % of cdw. The absence of granules in Δ*phaP1.2* was also confirmed by TEM analysis (Figure 3B).

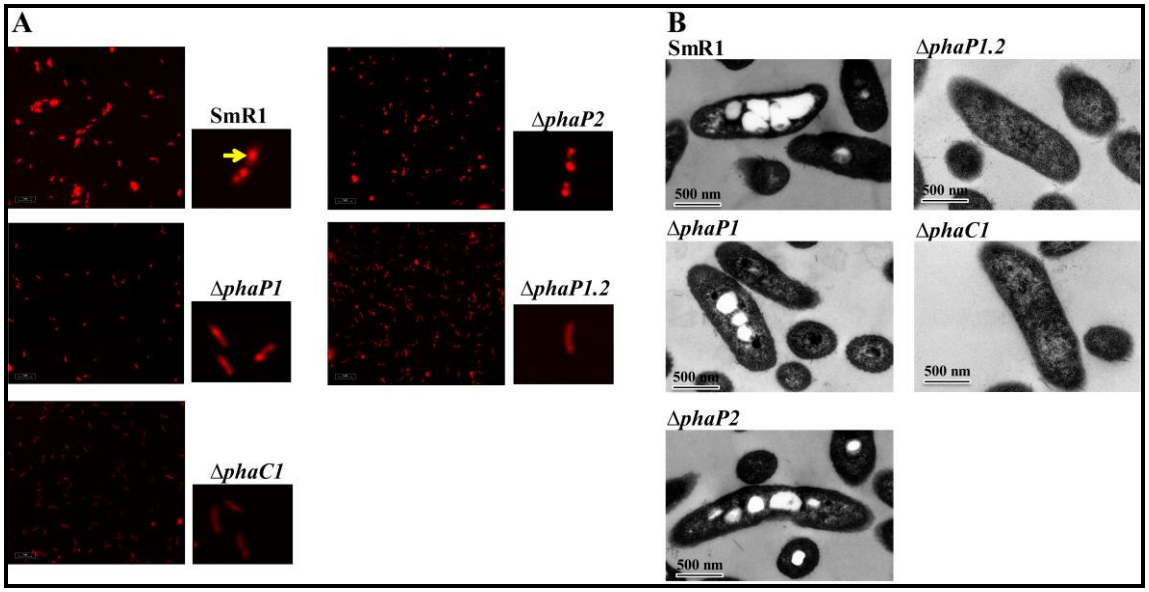

Figure 3. Fluorescence microscopy and transmission electron microscopy of *H. seropedicae* SmR1, phaP and phaC1 mutants. (A) Strains were grown in NFb medium with 37 mM DL-malate and 20 mM NH_4Cl to an OD_{600} of 1.0. Cells were stained with Nile Red and visualized by excitation with 543 nm light. The yellow arrow in the SmR1 panel indicates a typical PHB granule stained with Nile Red. (B) The strains cultivated in NFb medium with 37 mM DL-malate and 20 mM NH_4Cl to an OD_{600} of 1.0 were processed and visualized by TEM. The black bar represents 500 nm in scale.

3.4 Expression of phaP1 and phaP2 is repressed by PhaR

Previously, we showed that the negative regulator PhaR of *H. seropedicae* (a homologue of PhaR in *R. eutropha* H16) binds to the regulatory region of *phaP1* thus repressing its expression (Maehara et al., 2001; Maehara et al., 2002; Pötter et al., 2002; York et al., 2002; Kadowaki et al., 2011). To further examine the role of PhaR on expression of *phaP1* and *phaP2*, we fused a promoterless rbs-containing *lacZ* downstream of the regulatory regions of both genes (hereafter denoted P*phaP1-lacZ* and P*phaP2-lacZ*). We decide to measure the activity of the fusions in *E. coli* as a heterologous non-PHB-producing model, to avoid any perturbation on PhaR activity

caused by PHB production (Maehara et al., 2002). The fusions were transformed into *E. coli* ET8000 expressing or not *H. seropedicae* PhaR. Cells carrying P*phaP1-lacZ* and P*phaP2-lacZ* fusions showed similar, high β-galactosidase activities when PhaR was not expressed. However, cells expressing PhaR had a remarkable repression on expression levels of both P*phaP1-* and P*phaP2-lacZ* (Table 3).

Table 3 .Transcriptional analysis of PphaP1-lacZ and PphaP2-lacZ fusions in E. coli ET8000 expressing PhaR from H. seropedicae SmR1.

Strain	β-galactosidase activity before induction (Miller units)	β-galactosidase activity 2h after induction (Miller units)
ET8000/pDK6/P*phaP1-lacZ*	8,516.5 ± 693.3	11,801.3 ± 642.1
ET8000/pMMS31/P*phaP1-lacZ**	168.0 ± 6.4**	89.7 ± 2.5**
ET8000/pDK6/P*phaP2-lacZ*	11,180.8 ± 142.8	12,061.1 ± 55.5
ET8000/pMMS31/P*phaP2-lacZ**	149.8 ± 3.2**	85.5 ± 2.8**

The cultures were grown in LB medium until OD600 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. * - strains expressing PhaR from *H. seropedicae* under control of the P*tac* promoter. Where appropriate, statistical significance is shown (** p-value ≤ 0.01, independent two-sample t-test). The data obtained to the strains harboring P*phaP1-* or P*phaP2-lacZ* not expressing PhaR were used for normalization and determination of statistical significance.

3.5 The phaP1 and phaP2 genes are dissimilarly expressed

To determine expression levels of *phaP1* and *phaP2*, the P*phaP1-* and P*phaP2-lacZ* fusions were conjugated into the parental and mutant strains. Expression profiles were evaluated during growth in media containing DL-malate as the carbon source. Expression of P*phaP1-lacZ* in the parental strain was dependent on the growth stage, since expression increased after an OD_{600} of 0.5 was achieved and PHB began to accumulate (Figure 4A). Interestingly, expression of P*phaP2-lacZ* was 8-fold lower than that achieved by the P*phaP1-lacZ* fusion in the same strain (Figure 4A). However, expression of P*phaP2-lacZ* increased 6-fold in Δ*phaP1*, showing that upon deletion of the main phasin, PhaP2 can act as a backup phasin (Figure 4B). Since the Δ*phaP1.2* did not accumulate PHB granules, one should anticipate that both fusions would be repressed by PhaR. Nevertheless, the P*phaP1-* and P*phaP2-lacZ* fusions were actives in Δ*phaP1.2*, indicating that not only PHB granules de-repress expression of *phaP*, but

that newly synthesized chains of PHB have the same effect (Figure 4C). As expected, in Δ*phaC1*, which is unable to synthesize PHB, expression of both fusions was repressed (Figure 4D). Expression of *phaP1* and *phaP2* was also assayed in NFb media containing 25 mM glucose and 20 mM ammonium chloride, conditions which stimulated PHB accumulation. High β-galactosidase activities (4,000 Miller units) for P*phaP1-lacZ* were obtained in all strains analyzed (parental and the mutants), showing that even at low cell densities ($OD_{600} \geq 0.3$, Figure 4E) enough PHB granules were present to almost completely de-repress *phaP1* expression. Expression of *phaP2* was not fully activated in the parental strain (Figure 4E), but in Δ*phaP1* it was 5-fold higher even at low cell densities (Figure 4F). The results of expression applying *lacZ* fusions were validated by RT-PCR. The same profile of transcription was observed when the intensities of the amplified bands from *phaP1* and *phaP2* cDNAs were compared in different genetic backgrounds (Figure 4G).

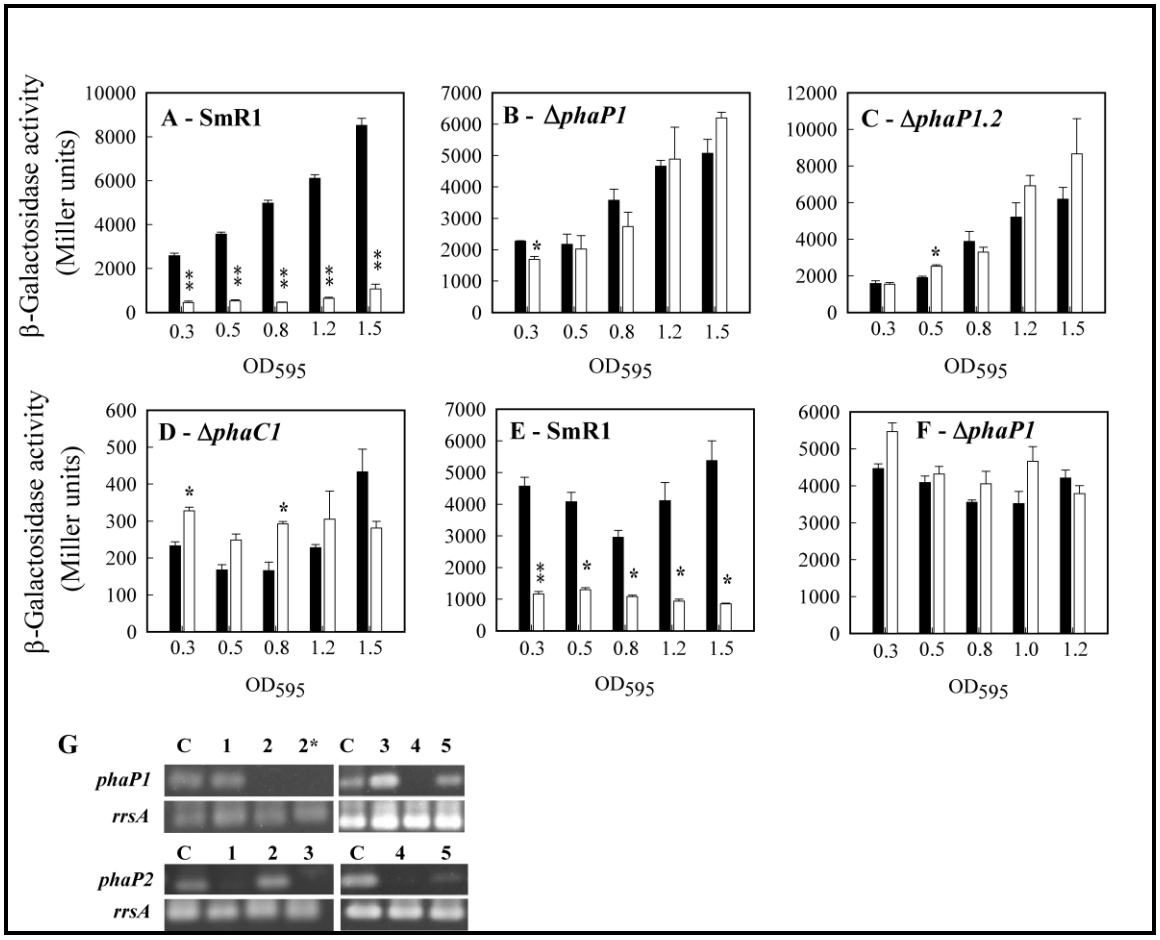

Figure 4. Transcriptional analyses of phaP1 and phaP2 expression in H. seropedicae.. (A - D) Cells harboring P*phaP*1-*lacZ* (black bars) or P*phaP2-lacZ* (white bars) were

grown in liquid NFb medium containing 37 mM DL-malate and 20 mM NH_4Cl at 30°C. (E and F) Cells harboring P*phaP*1-*lacZ* (black bars) or P*phaP*2-*lacZ* (white bars) were grown in liquid cultures of NFb containing 25 mM D-glucose and 20 mM NH_4Cl at 30°C.When the OD_{600} of the cultures reached the indicated values, 100 μL of culture was removed to determine β-galactosidase activity. *H. seropedicae* strains harboring pMP220 (promoterless *lacZ* plasmid) gave an average activity of 50 Miller units in all growth phases analyzed. Experiments were performed in biological triplicates. The means of β-galactosidase activity were tested in pairs for each sample point. Where appropriate, statistical significance is shown (* - p-value ≤ 0.05, ** - p-value ≤ 0.01, independent two-sample t-test). (G) RT-PCR analysis. The RNA from the strains SmR1 (lane 1), Δ*phaP1* (lane 2 and 2* for *phaP1*, lane 2 for *phaP2*), Δ*phaP2* (lane 3), Δ*phaP1.2* (lane 4) and Δ*phaC1* (lane 5) was purified and submitted to direct RT-PCR amplification of *phaP1* and *phaP2* gene as described in Methods. The lane C is a PCR product amplified from gDNA from *H. seropedicae* SmR1 used as positive control of the reaction. The 16S rRNA (*rrsA*) was used as an endogenous expression control. A representative gel from tree independent RNA extractions is showed.

3.6 Complementation with *phaP1* fully restores PHB accumulation, while *phaP2* expression has a partial effect

To determine if both phasins were able to restore PHB accumulation, the *phaP* genes were cloned under control of their native promoter into a medium copy-number plasmid (pBBR1MCS-3) and expressed into the *phaP* mutants. The expression of *phaP1* in the Δ*phaP1*, Δ*phaP2* and Δ*phaP1.2* mutants restored PHB accumulation to native levels (Figure 5A). The expression of a plasmid-borne *phaP1* copy restored PHB granules formation in Δ*phaP1.2* (Figure 5B). Complementation by expression of *phaP2* significantly reduced PHB accumulation, regardless the strain tested. Δ*phaP1* complemented with *phaP2* presented 45 % of reduction in PHB as compared to complementation with *phaP1*. Similarly, 51 % less PHB was observed when Δ*phaP1.2* was complemented with *phaP2*. Once again, these results show that PhaP1 is more effective in controlling PHB accumulation than its homologue PhaP2. As demonstrated before in this work, deletion of *phaP1* or both phasin genes generated mutant strains with growth penalty in minimal medium containing glucose as sole carbon source (Figure 1C). Therefore, to verify if complementation would restore normal growth in NFb-glucose, the strains were complemented with *phaP1* and their growth curves were determined (Figure 5C and D). The strains Δ*phaP1* and Δ*phaP1.2*, which exhibited reduced growth on glucose, grew as the parental strain when complemented with *phaP1*. In conclusion, the expression of *phaP1* recovered PHB accumulation in the

deficient strains, consequently normalizing their metabolic status and turning them able to grow in glucose.

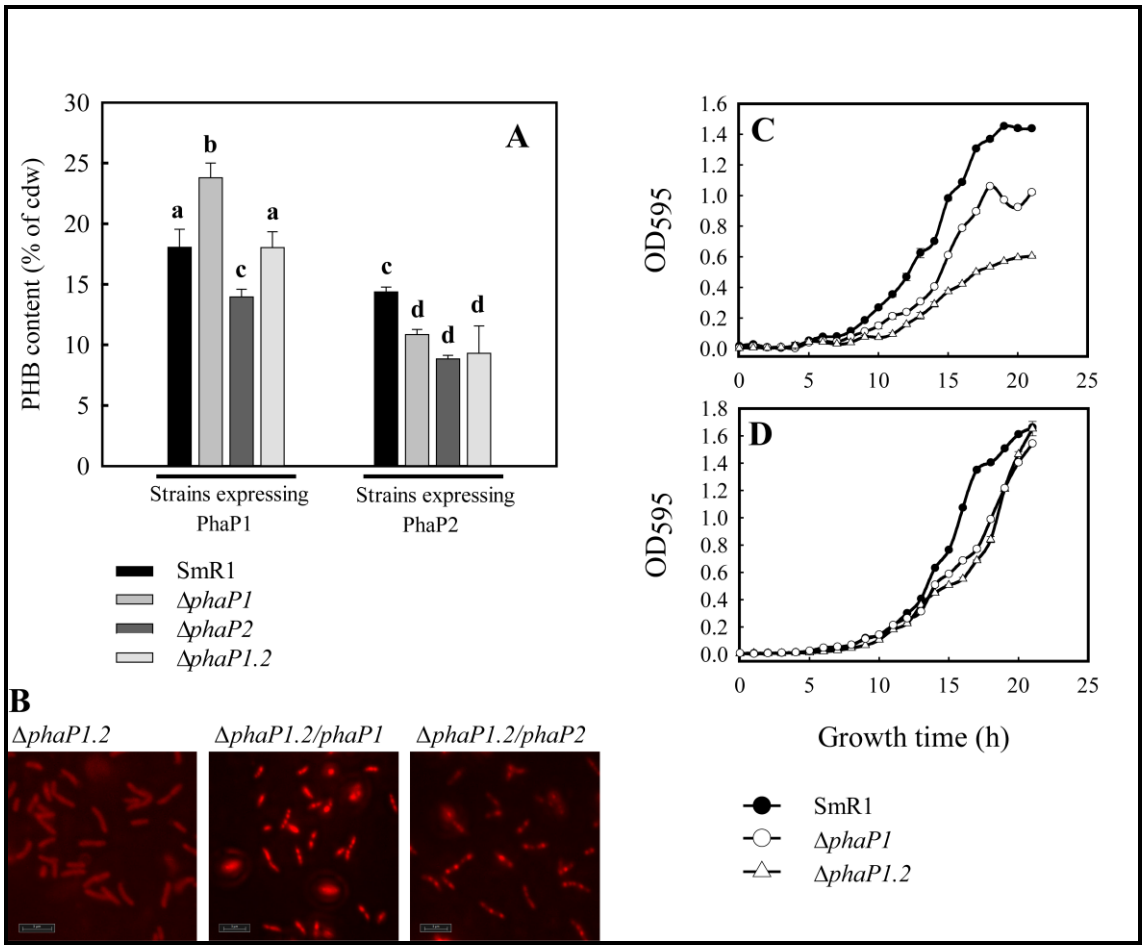

Figure 5. PHB contents of phaP mutants complemented with plasmids harboring *phaP1* and *phaP2*. (A) *H. seropedicae* strains conjugated with pLPA01 were complemented with *phaP*1 *in trans* under control of its native promoter. The strains conjugated with pLPA02 were complemented with *phaP*2 *in trans* under control of its native promoter. Cells harboring pLPA01 or pLPA02 were grown in liquid NFb containing 37 mM DL-malate and 20 mM NH_4Cl at 30 °C. When the OD_{600} of the cultures reached 1.0, 10 mL was removed to determine the PHB content by gas chromatography as described in Materials and Methods. The bars marked with different letters indicate means significantly different (independent two-sample t-test, $p<0.05$). Experiments were performed in quadruplicate. (B) Cells of Δ*phaP1.2* mutant complemented with pLPA01 were stained with Nile Red and visualized by excitation with 543 nm light. Strains harboring pBBR1MCS-3 (C) or pLPA01 (D) were grown in NFb medium with 20 mM of ammonium chloride and 25 mM (w/v) D-glucose at 30°C (orbital agitation at 120 rpm). OD_{595} growth data were obtained from three independent cultures.

4 Discussion

To analyze the role of phasins in *H. seropedicae*, we correlated their presence with PHB production and expression of *phaP1* and *phaP2*. Deletion of *phaP1* reduced PHB accumulation on 50 %, showing that PhaP1 is the key phasin controlling synthesis and stability of PHB granules. Deletion of *phaP2* had little effect, reinforcing the idea that when PhaP1 is expressed, PHB granules are well formed and stocked (Figure 2 and 3). The suggestion that *H. seropedicae* has other phasin-like proteins seems unlikely, since deletion of both genes completely suppressed PHB granules formation. A similar dependence of phasins on accumulation of PHB was also found in *Sinorhizobium meliloti* Rm1021 (Wang et al., 2007), in which deletion of *phaP1,* the main phasin in this bacterium, reduced PHB accumulation by 20 % and increased the doubling time by 2.3 h (Wang et al., 2007). Similarly, deletion of *phaP2* did not significantly affect growth and PHB production of *S. meliloti*, however deletion of both phasins increased the doubling time by 3.6 h and fully abrogated PHB accumulation (Wang et al., 2007). In the insect gut symbiont *Burkholderia* sp. RPE75, the *phaP* deletion reduced PHB accumulation by 2.8-fold and the distribution of PHB granules was heterogeneous among the cells of the Δ*phaP* mutant (Kim et al., 2013). A total of six *phaP* genes were identified within the genome of *Burkholderia* sp. RPE75, therefore it was suggested that the redundancy among phasins could maintain PHB production in the Δ*phaP* mutant and render it with less impact on bacterial-insect symbiosis (Kim et al., 2013). In fact, from our results it is likely that the expression of a backup phasin ensures some level of PHB accumulation and, hence, reduces the impact on bacterial fitness. Possibly, the same mechanism might be occurring in the Δ*phaP* strain of *Burkholderia* sp. RPE75. Recently, Hauf and co-workers reported the deletion of the *ssl2501* expressing a phasin in *Synechocystis* sp. PCC 6803 (Hauf et al., 2015). The Δ*ssl2501* mutant presented a reduction in the number of PHB granules per cell and an increase in the mean PHB granule size, however, the PHB content was only slightly lower in the mutant. Once again, as the deletion of *ssl2501* alone has not abolished PHB accumulation, it was also suggested that possibly in *Synechocystis*, other phasin-like proteins might be expressed (Hauf et al., 2015).

These data contrast with the situation in the well-studied model *R. eutropha* H16, in which deletion of the four phasin genes did not completely impair PHB accumulation (Kuchta et al., 2007). Besides these four phasins, three other proteins have been recently

reported in *R. eutropha* H16, namely PhaP5, PhaP6 and PhaP7 (Pfeiffer and Jendrossek, 2011; 2012). The deletion of these additional phasin genes alone did not affect PHB granules formation and the content accumulated in *R. eutropha* (Pfeiffer and Jendrossek, 2011; 2012). To the best of our knowledge, a mutant of *R. eutropha* with all phasin genes deleted (*phaP1* to *phaP7*) has not been constructed so far. This mutant could reveal whether all phasins are relevant to PHB granule biogenesis and accumulation in *R. eutropha* and, if the functional redundancy among phasins (including PhaP5-7) supports PHB accumulation in the multiple deletion mutants, as related to Δ*phaP1234* strain (Kuchta et al., 2007).

A well accepted model for transcriptional regulation of *phaP* genes in *R. eutropha* H16 and *Paracoccus denitrificans* (Maehara et al., 2001; Maehara et al., 2002; Pötter et al., 2002; York et al., 2002; Yamada et al., 2007; Yamada et al., 2013) suggested that the transcriptional repressor PhaR binds to the regulatory region upstream of *phaP* genes, blocking gene transcription. At the onset of PHB synthesis, the PhaR repressor is sequestered from DNA by PHB and transcription is initiated. PhaR thus couples PHB synthesis with phasin expression (York et al., 2002). In *H. seropedicae* SmR1, we have shown that PhaR (previously named PhbF) also functions as a repressor of transcription (Kadowaki et al., 2011) and here we have extended these observations to include repression of *phaP1* and *phaP2* expression (in *E. coli*) (Table 3). Furthermore, the pattern of *phaP1* and *phaP2* expression in different backgrounds of *H. seropedicae* demonstrated a backup regulation, whereas the genes are dissimilarly expressed (Figure 4). In other words, the simultaneously expression of both phasins seems to be unnecessary, however upon mutation of *phaP1*, expression of *phaP2* is reprogrammed to achieve a similar level compared to expression of *phaP1* in the wild type. This mode of regulation is named responsive backup circuit (RBC) and has important consequences controlling expression of functional redundant proteins to increase the robustness of organisms when facing stressful conditions (Kafri et al., 2006). Clearly, our results demonstrated that the expression of *phaP2* in the Δ*phaP1* reduced the negative impact on PHB accumulation and growth in glucose, rendering a more fit phenotype than the Δ*phaP1.2* strain.

The existence of two highly homologous genes encoding proteins with the same predicted function raises the question of whether the phasins of *H. seropedicae* are genuinely redundant. It is unlikely that *phaP1* and *phaP2* represent truly redundant

genes, as deletion of *phaP1* clearly resulted in a less fit phenotype. Therefore, it is possible that due to its greater efficiency, PhaP1 has been selected as the main phasin and, as consequence, the expression of *phaP2* was attenuated, converting PhaP2 in a backup phasin. This assumption raises two important points: (i) it guarantees proper levels of phasin expression, avoiding perturbations on PHB synthesis and granule formation and (ii) it saves the cells of wasting unnecessary metabolic costs with superfluous gene expression. Other β-Proteobacteria including species of the *Herbaspirillum* genus, *Collimonas fungivorans, Herminiimonas arsenicoxydans* and *Janthinobacterium* sp. possess orthologous to *phaP1* and *phaP2* of *H. seropedicae*, indicating that the backup expression of *phaP2* may be conserved among species phylogenetically related to *H. seropedicae*. In the genome of *Azoarcus* sp. BH72, a plant-associated and PHB-producing β-proteobacterium, four genes expressing putative phasins were found (Krause et al., 2006), suggesting that the backup expression of phasins may be an important mechanism to maintain PHB production also in other species less phylogenetically close to *Herbaspirillum*. Interestingly, in other well characterized plant-associated and PHB-producing bacteria as *A. brasilense* Sp245 and *A. lipoferum* 4B, the genome sequencings revealed only one probable gene encoding a phasin for each organism, AZOBR_p110146 and AZOLI_1409, respectively (Wisniewski-Dye et al., 2011). On the other hand, in *A. brasilense* FP2 (a mutant strain from *A. brasilense* Sp7 resistant to nalidixic acid and streptomycin) were found four phasin genes, which are expressed when the bacteria were epiphytically colonizing roots of *Triticum aestivum* (Camilios-Neto et al., 2014). To the best of our knowledge mutants defective in phasin expression were not constructed to *Azospirillum* species so far. Therefore, these findings pave the way to investigate the impact of phasin gene deletions in other relevant plant-associated bacteria, as those from *Azoarcus* and *Azospirillum* genus.

5 Acknowledgments

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7 Supplementary Material

Backup expression of the PhaP2 phasin compensates for *phaP1* deletion in *Herbaspirillum seropedicae*, maintaining fitness and PHB accumulation

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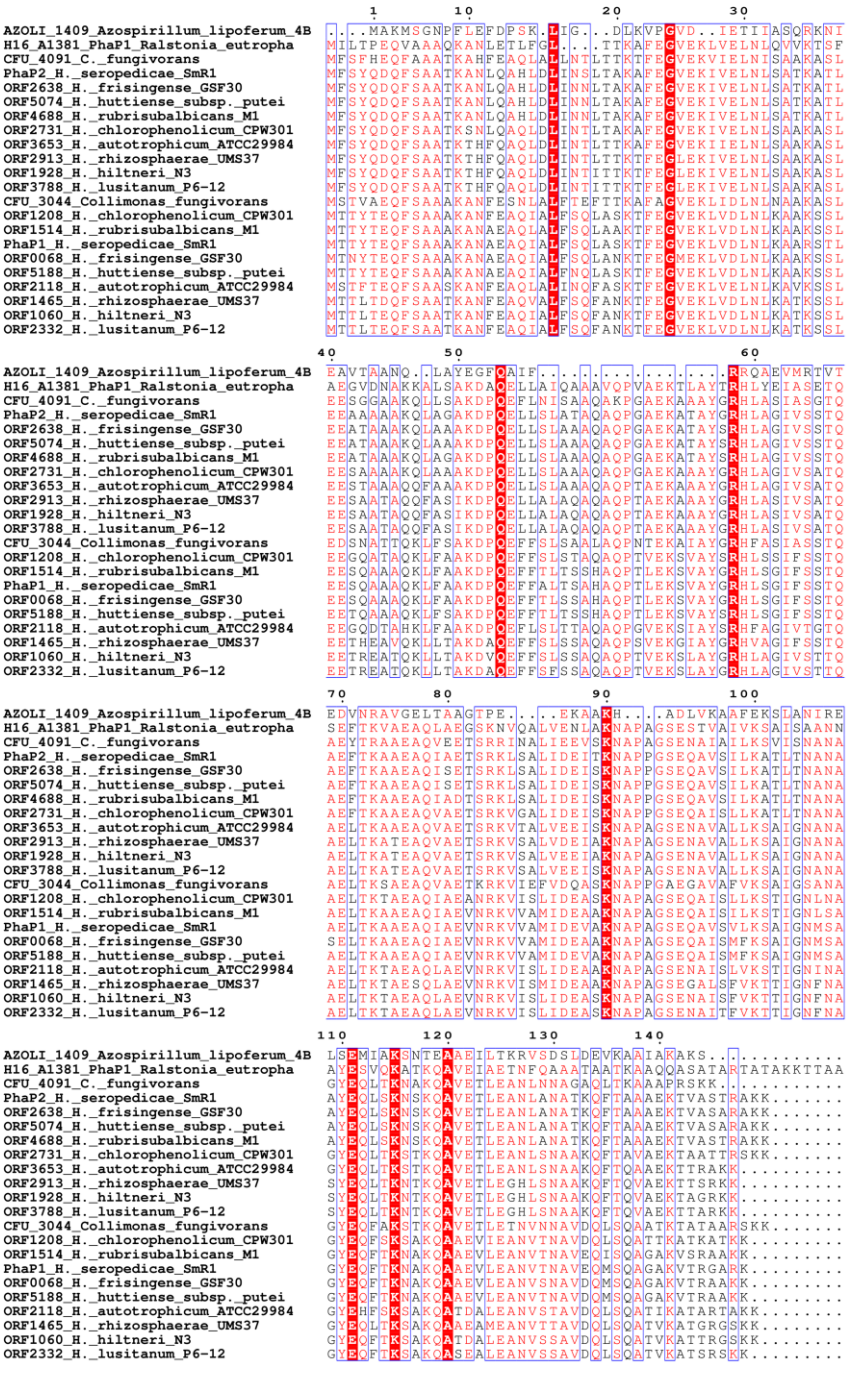
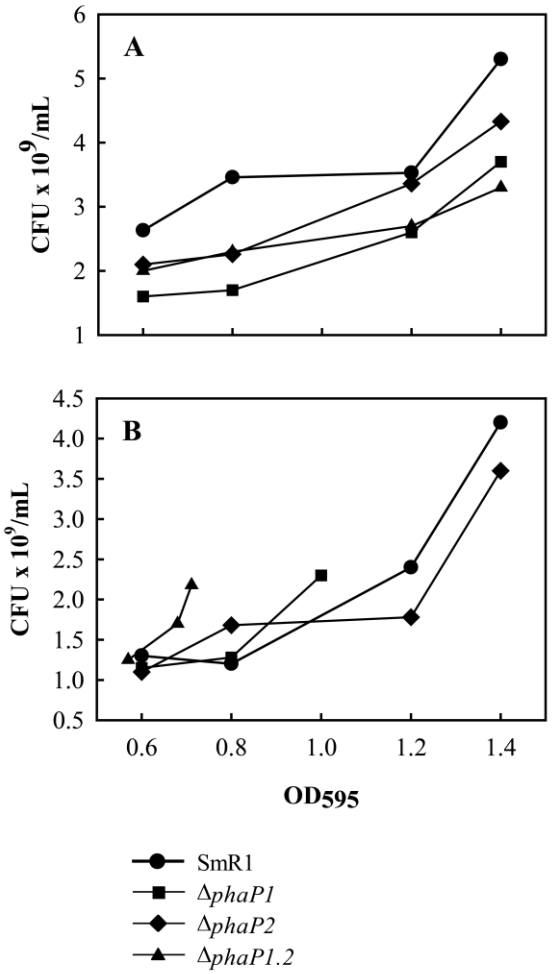
Supplementary Figure 1



Supplementary Figure 1. Protein sequence alignment of phasins from other species of *Herbaspirillum*, *Collimonas fungivorans*, *Ralstonia eutropha* and *Azospirillum lipoferum* used to the phylogenetic analysis. Multiple alignment of phasins was carried out using Espript 3.0 and the default parameters (Robert and Gouet, 2014). The red shaded letters indicate identical residues, while red letters represent conserved residues. The phasin PhaP from *A. lipoferum* 4B was applied as an outgroup.

8 Supplementary Figure 2



Supplementary Figure 2. Growth of H. seropedicae SmR1 (parental strain) and the mutants *ΔphaP1, ΔphaP2 and ΔphaP1.2*. Strains were grown in NFb medium with 20 mM of ammonium chloride and 37 mM DL-malate (A) or 25 mM (w/v) D-glucose (B) at 30°C (orbital agitation at 120 rpm). CFU counts were obtained from three independent cultures though serial dilution and plating in NFb-malate agar with 20 mM of ammonium chloride. Due the growth defect of the Δ*phaP1* and Δ*phaP1.2* strains in glucose, their growths were monitored until the OD_{595} of the cultures stopped increasing.

Supplementary Table 1. Primers used in this work

Primer	Sequence (5' → 3')	Restriction site	Purpose
Fw_*phaP2*_UP	GGATCCCGCAAAGCCAG	BamHI	*phaP2* upstream region amplification
Rev_*phaP2*_UP	GGTACCGGCGGAAAACT	KpnI	
Fw_*phaP2*_DOWN	GGTACCTTCACCGCTGC	KpnI	*phaP2* downstream region amplification
Rev_*phaP2*_DOWN	GTCGACGAACACCAAGGG	SalI	
Fw_prom_*phaP1*	AGATCTCACACCACACTCTCG	BglII	Amplification of *phaP1* upstream region (transcriptional fusion with *lacZ*)
Rev_prom_*phaP1*	CTGCAGCGGAAAATTGCTC	PstI	
Fw_prom_*phaP2*	GTTAGATCTTCGTGGTCCATGACCTGCCC	BglII	Amplification of *phaP2* upstream region (transcriptional fusion with *lacZ*)
Rev_prom_*phaP2*	ATCCTGCAGTCTTGGTACGAGAACATGGTC	PstI	
Fw_*phaP1*_RT	TACACCGAGCAATTTTCCGC		*phaP1* RT-PCR
Rev_*phaP1*_RT	GCTGGTCAGTGCGAAGAATT		
Fw_*phaP2*_RT	CCAGGCTGAATTCACCAAGG		*phaP2* RT-PCR
Rev_*phaP2*_RT	GGCTTGCTTGGAGTTCTTCG		
Fw_*rrsA*_RT	TGGTAGTCCACGCCCTAAAC		*rrsA* RT-PCR
Rev_*rrsA*_RT	TCGAGCACTCCCAAATCTCT		
Fw_Pro_*phaP1*	CTCGAGTGCAACAAAACTGCC	XhoI	*phaP1* cloning for complementation
Rev_Gen_*phaP1*	TCTAGAAGCGCCTGAAACGGC	XbaI	
Fw_Pro_*phaP2*	CTCGAGCGGGTAAAAGAACCG	XhoI	*phaP2* cloning for complementation
Rev_Gen_*phaP2*	TCTAGACGCAGTCCTGATGGC	XbaI	

CAPÍTULO III

Manuscrito não submetido

The role of poly-3-hydroxybutyrate (PHB) in anti-stress response in *Herbaspirillum seropedicae* SmR1

The role of polyhydroxybutyrate (PHB) in the heat shock in *Herbaspirillum seropedicae* SmR1

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Abstract

The accumulation of PHB has been reported as a protective factor against stress factors in bacteria. Several studies with non-synthesizing or non-PHB-degrading mutants showed lower survival under carbon fasting conditions and other stresses such as thermal, osmotic or oxidative shock. However, it is still unclear whether mobilisation of the PHB stock is integrated into the stress sensing by bacteria. In this work, we demonstrated that in the bacterium *Herbaspirillum seropedicae* SmR1 the mobilisation of PHB was activated in the condition of thermal shock at 45°C. This activation led to the release of 3-hydroxybutyrate (3HB) monomers that could be monitored in situ by nuclear magnetic resonance. Mutants that did not produce or degrade PHB died abruptly as soon as the heat shock was established. Also, we determined that the RpoS sigma factor that is linked to the general stress response in bacteria participates actively in the process of PHB mobilisation in *H. seropedicae*. The data presented in this paper reinforce recent data that PHB mobilisation is activated under abiotic stress conditions, in addition to well know carbon starvation condition. These findings bring new discussions to the role of PHB in the mechanism of bacterial adaptation.

1. Introduction

Bacteria are continually adapting to survive when facing adverse environmental conditions (Brooks et al., 2011; Shimizu, 2016). For instance, stressful conditions such as heat and osmotic shock can lead to protein denaturation and oxidation of cellular components, resulting in metabolic impairment and cell death (Yura et al., 1993). Several mechanisms have been evolved to make bacteria proficient in environment sensing and adaptation (Krell et al., 2010). Transcriptional regulatory mechanisms jointly with metabolic fluctuations of protector molecules help bacteria to cope with adverse conditions (Mosier et al., 2013; Pavlov and Ehrenberg, 2013).

The synthesis of intracellular polymers is one of the implemented strategies. For instance, the synthesis of glycogen (Damrow et al., 2016), polyphosphate (Gray and Jakob, 2015) and polyhydroxyalkanoates (Kim et al., 2013) have been described as key for bacteria survive in non-supportive conditions, such as starvation and several kinds of abiotic stress.

Polyhydroxyalkanoates (PHA) are polyesters synthesised by condensation of 3-hydroxyalkanoic acids (Madison and Huisman, 1999). These polymers are water-insoluble and accumulated in the cytoplasm in the form of insoluble granules (Jendrossek, 2009). They are produced and stored when the bacteria face a unbalance growth condition, a high amount of carbon source with a low amount of another essential nutrient such as phosphorous or nitrogen (Anderson and Dawes, 1990). Hence PHA are recognised mainly as means of carbon and energy reserve. However, recent data are correlating PHA synthesis and mobilisation to additional roles in anti-stress response in some species of bacteria (Obruca et al., 2016a; Obruca et al., 2017; Sacomboio et al., 2017).

The most common PHA produced by bacteria is the poly-3-hydroxybutyrate (PHB). The PHB synthesis begins with condensation of two molecules of acetyl-CoA into acetoacetyl-CoA by the enzyme beta-ketothiolase (PhaA). After this, the enzyme acetoacetyl-CoA reductase (PhaB) reduces the acetoacetyl-CoA into 3-hydroxybutyryl-CoA. The PHA synthase (PhaC) polymerises the monomers into polyhydroxybutyrate (PHB) (Madison and Huisman, 1999). The PHB granules are covered by several proteins (Jendrossek, 2009; Tirapelle et al., 2013) The phasins are the most abundant,

but other proteins are also associated to the granules: PhaR, the transcriptional regulator of phasin expression; PhaC, the PHA synthase and PhaZ, the PHA depolymerase, the enzyme responsible for hydrolysing PHB, releasing 3-hydroxybutyrate.

The conversion of PHB insoluble granules in soluble units of 3-hydroxybutyrate is called PHB mobilisation. The mobilisation of PHB granules has been studied in the model bacterium *Ralstonia eutropha* H16 and other species. In *R. eutropha* H16, the authors demonstrated that this bacterium mobilises PHB granules in the absence of an exogenous carbon source, but in the presence of a nitrogen source (NH_4Cl) (Handrick et al., 2000). Since a nitrogen source is required for *R. eutropha* mobilises PHB, it seems that this process is actively coupled to protein synthesis. *R. eutropha* H16 can only grow two cell divisions, metabolising the products generating by PHB mobilisation (Handrick et al., 2000). In *Legionella pneumophila*, it was also demonstrated that PHB mobilisation maintains the bacteria viable for at least 600 days in low-nutrient tap water environment (James et al., 1999). Interestingly, besides carbon starvation, PHB synthesis has been correlated with resistance against other stressful conditions such as heat, cold, oxidative and osmotic shock (Kadouri et al., 2003a; Zhao et al., 2007; Obruca et al., 2016a). However, it is not clear if the synthesis of PHB *per se* confers the resistance or in fact its mobilisation. Recently, Koskimäki et al. demonstrated that the degradation of PHB in the endophytic bacteria *Methylobacterium extorquens* generates oligomers of 3-hydroxybutyric acid that act as an antioxidant defence during the infection of roots of Pinus sylvestris (Koskimaki et al., 2016).

Endophytic bacteria are attractive models to study microbial environmental adaptation since they are subjected continuously to changes, as they leave the soil to associate with the rhizosphere or colonise internal plant tissues (Hardoim et al., 2015). Several endophytic bacteria produce high amounts of PHB (Sessitsch et al., 2012). However, few studies have investigated the impact of PHB metabolism in plant-bacteria interaction. *Herbaspirillum seropedicae* SmR1 is an endophytic and nitrogen-fixing β-proteobacteria, accumulating high amounts of PHB *in vitro* and *in planta* (Catalan et al., 2007; Pedrosa et al., 2011). PHB accumulation in *H. seropedicae* was also reported even under unfavourable conditions such as low carbon-to-nitrogen ratio (Sacomboio et al., 2017). This finding indicates that PHB synthesis in *H. seropedicae* SmR1 seems to be essential for its proper metabolic function and possibly is involved in anti-stress response and adaptation during plant colonisation.

Transcriptomic analyses of corn and wheat roots colonised by *Herbaspirillum seropedicae* SmR1 indicate that both the synthesis and the mobilisation of PHB must be occurring during colonisation since *phaC* and *phaZ* genes were up-regulated (Balsanelli et al., 2015; Pankievicz et al., 2016). Also, the PHB cycle (consisting of the balance between PHB synthesis and degradation) has been suggested as an important factor in rhizobia-leguminous plants (Trainer and Charles, 2006).

To investigate whether the PHB cycle protects *H. seropedicae* SmR1 against abiotic stress, we determined the survival of the parental strain and several mutants defective in PHB synthesis and degradation. The heat shock was the most challenging condition for PHB defective mutants. The results showed that PHB mobilisation is activated during heat shock and enhanced survival of *H. seropedicae* SmR1.

2. Materials and methods

2.1 Bacterial strains, plasmids and growth conditions

Strains and plasmids used in this work are listed in Table 1. *Escherichia coli* strain Top10 (Thermo Fisher Scientific Inc., Waltham, MA, USA) and S17.1 (Simon et al., 1983) were used for cloning and conjugation procedures, respectively. *E. coli* strains were grown at 37°C in LB medium and shaken at 160 rpm. *Herbaspirillum seropedicae* parental SmR1 (Souza et al., 2000) and mutant strains were grown in NFbHP media (Pedrosa and Yates, 1984) with 37 mM DL-malate or 25 mM glucose and 20 mM NH_4Cl at 30°C and shaken at 120 rpm. Bacteria were grown in penicillin glass bottles of 60 mL of capacity filled with 10 mL of medium.

2.2. Bacterial survival assays after heat shock in *H. seropedicae* SmR1 and defective mutant strains.

The parental strain *H. seropedicae* SmR1, the mutants Δ*phaP1,* Δ*phaP2,* Δ*phaP1.2*, Δ*phaC1,* and the mutant Δ*phaP1.2* complemented with pLPA01 or pLPA02*,* were cultured in NFb-malate HPN medium at 30°C and 120 rpm of orbital agitation. When the bacterial cultures reached the OD_{600} of 1.2, two flask cultures (60 mL of capacity) containing 10 mL of medium were transferred to a water bath at 30°C and in parallel to a water bath at 45°. The cultures were incubated up to 30 minutes. During the heat

shock 200µL of culture were collected and serially diluted for colony forming units (CFU) counting.

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	(Simon et al., 1983)
ET8000	Wild-type strain	(MacNeil et al., 1982)
H. seropedicae strains		
SmR1	Parental strain, Nif^+, Sm^R, PHB^+	(Souza et al., 2000)
Δ*phaP1*	Chromosomal deletion of *phaP1*	(Tirapelle et al., 2013)
Δ*phaP2*	Chromosomal deletion of *phaP2*	(Tirapelle et al., 2013)
Δ*phaP1.2*	Chromosomal deletion of *phaP1* and *phaP2*	(Tirapelle et al., 2013)
Δ*phaC*1	Chromosomal deletion of *phaC1*	(Tirapelle et al., 2013)
Δ*rpoS*	Chromosomal deletion of *rpoS*	This work.
Plasmids		
pK18*mobsacB*	Suicide vector; Km^R, *sacB*, mobilizable plasmid	(Schafer et al., 1994)
pK18mobΔ*rpoS*	pK18*mobsacB* harbouring the Δ*rpoS* product of overlapping PCR	This work
pBBR1MCS3	Broad-host-range vector	(Kovach et al., 1995)
pLPA01	pBBR1MCS3 harbouring *phaP1* of *H. seropedicae*. Over-expression of PhaP1	(Alves et al., 2016)
pLPA02	pBBR1MCS3 harbouring the *phaP2* of *H. seropedicae*. Over-expression of PhaP2	(Alves et al., 2016)

Bacteria survival was expressed as a percentage of the number of CFU (colony forming units) relative to time zero (before heat shock), which was assumed to be 100%. We assayed the parental and the Δ*phaC1* mutant in OD_{600} at 0.2 when the strains do not produce PHB. Alternatively, it was also assayed a condition leading to high PHB

accumulation (35% PHB/mg dcw) with 25 mM of glucose and 5 mM of NH_4Cl. The procedures for heat shock and CFU counts were made as described above.

2.3. PHB quantification by gas chromatography and flow cytometry

PHB was quantified by methanolysis and GC-FID (gas chromatography coupled to a flame-ionisation detector) analyses as described previously (Braunegg et al., 1978) with 5 to 10 mg of lyophilised bacteria. Amounts of PHB in each sample were normalised to the cell dry weight (cdw; weight of the lyophilised bacterial pellet) and expressed as % of PHB/cdw. Alternatively, PHB was quantified by flow cytometry sampling 100 µL of cultures heat shocked, following the protocol previously described (Alves et al., 2017). Briefly, 100 µl of culture was withdrawn for time zero, and the culture flasks were transferred to a 45°C in water bath for 30 minutes. Samples of 100 µl of culture were collected in different points, centrifuged for 1 min at 10,000 x *g*, resuspended in 1 ml of PBS buffer and then stained with Nile Red (10 µM in DMSO) for 5 minutes in darkness. Flow cytometry quantifications were performed in a BD Accuri C5® Flow Cytometer equipped with a 488-nm laser for fluorescence excitation. For each sample, 100,000 events were acquired, and the median fluorescence intensities were obtained from histograms of FL2-H 585/40 nm channel. Flow cytometry calibration was performed using spherothech 8-peak beads (BD Accuri™) according to the manufacturer's recommendations and instructions.

2.4. Fluorescent microscopy

To visualise PHB granules in *H. seropedicae* SmR1 and mutant strains, fluorescence microscopy was performed after staining with the fluorescent probe Nile Red, which stains neutral lipids (Spiekermann et al., 1999). Bacterial cultures (1 mL) were harvested by centrifugation for 60 seconds at 10,000 x *g*. The pellets were resuspended in 30 % (v/v) ethanol in PBS (Na_2HPO_4 10 mM, KH_2PO_4 1.8 mM, NaCl 130 mM) and 3 µL of 1.6 mM Nile Red (λ_{ex} 586 – 579 nm; λ_{em} 637 – 597 nm) dissolved in DMSO was added and incubated in the dark for 5 min. Then, the samples were centrifuged again for 60 s at 10,000 x *g*, resuspended in PBS and viewed under a fluorescent microscope. The images were obtained using an Axio Imager Z2 microscope (Carl Zeiss Microscopy GmbH, Jena, Germany), equipped with 4 Metafer automated capture

software (Metasystems GmbH, Altlussheim, Germany) and a CoolCube 1 camera **(**with 100 x magnification).

2.5. Monitoring PHB mobilisation by ^{1}H-Nuclear Magnetic Resonance (NMR) spectroscopy

To observe PHB mobilisation during heat shock, ^{1}H-NMR was applied to monitor the releasing of 3-hydroxybutyric acid (3HB) monomers. Thus, the SmR1 parental and mutant strains were grown in NFb-malate medium with 37 mM DL-malate and 5 mM ammonium chloride. When the cultures reached OD_{600} of 1.2, 1 mL was centrifuged at room temperature for 30 s at 10,000 x *g* and the pellet resuspended in 1 ml of MilliQ™ water. The cell suspension was centrifuged again for 30 s at 10,000 x *g* and then resuspended in 1 ml of D_2O. The samples were transferred to NMR tubes of 4.2 mm of internal diameter and 178 mm of length. ^{1}H-NMR spectra were acquired for each sample at 600 MHz (Ascend™ 600, Bruker). Spectrometer equipped with a 5 mm QXI inverse probe and a Sample Case autosampler. The temperature was controlled at 30°C during 5 min; then the temperature was shifted up to 45°C and keep throughout the experiment. Standard ^{1}H-NMR pulse sequence with water pre-saturation (Bruker pulse program zgpr) was applied to each sample. A total of 64 transient free induction decays (FID) were collected for each experiment with a spectral width of 20 ppm. The relaxation delay was set to 1s. The 90° pulse length was automatically calibrated for each sample at around 11 μs. The total acquisition time of each sample was 1 min 27 sec. The metabolite concentrations were measured based on the height of the peaks in phosphate buffer pH 7.0: 3-hydroxybutyrate at δ 2.376 ($-CH_2$ multiplet). A standard of 3-hydroxybutyrate was prepared at 5 mM in 25 mM of phosphate buffer pH 7.0 and applied to validate the results.

2.6. Construction of Δ*rpoS* mutant of *H. seropedicae* SmR1

The in-frame marker-less deletion of *rpoS* (Hsero_2959, GenBank: ADJ64447.1) was obtained by cloning upstream and downstream fragments of the gene into the non-replicating plasmid pK18*mobsacB*, which carries a kanamycin resistance cassette along with *sacB* that confers sucrose sensitivity (Schafer et al., 1994). Briefly, 500 bp fragments to either flank of *rpoS* were amplified by PCR with primers Fw_*rpoS*_UP

(5'GTGAATTCCCGGTGGGCAGCAACAAGAC3', EcoRI site underlined) and Rev_*rpoS*_UP (5'AGATCTCTTAGGTCGTCTCTATGGCAAGTGATCGCGGCGAT 3', the underlined site was used for overlapping PCR) for the upstream region, whereas Fw_*rpoS*_DOWN (5' TAGAGACGACCTAAGAGATCTGAGCGTGTGCGTCAGGT GCAA3', the underlined site was used for overlapping PCR) and Rev_*rpoS*_DOWN (5'GGTTAAGCTTAGGTCGGGCCATAGATGGGAC3', HindIII site underlined) were used for the downstream region. The PCR products were fused by overlapping PCR and cloned into pBlueScript II KS. The entire construction was digested with EcoRI and HindIII to ligate the Δ*rpoS* fragment into pK18*mobsacB* digested with the same enzymes, yielding pK18Δ*rpoS*. *E. coli* S17-1 was transformed with pK18Δ*rpoS* and the plasmid conjugated to *H. seropedicae* SmR1 using bi-parental mating. Single-recombinants were selected on NFb-malate agar containing streptomycin 80 $\mu g.mL^{-1}$, nalidixic acid 5 $\mu g.mL^{-1}$ and kanamycin 500 $\mu g.mL^{-1}$. A single-recombinant colony was collected in 3 mL NFb-malate and cultivated overnight without antibiotics. The culture was serially diluted and plated on NFb-malate agar containing 10 % (w/v) sucrose. Colonies that grew on sucrose were screened for deletion by PCR using the primers Fw_*rpoS*_UP and Rev_*rpoS*_DOWN.

3. Results

3.1. The production and storage of PHB support *H. seropedicae* survival during heat shock

To verify the best temperature to perform the experiments involving heat shock, we screen different temperatures using parental SmR1 strain (PHB^+) and Δ*phaC1* mutant (PHB^-) **(Figure 1A)**. Thus, we measured bacterial survival after 30 min of incubation in usual temperature for growth of *H. seropedicae* at 30°C and increasing 5°C up to 45°C. The large difference of survival was measured at 45°C since the parental strain maintained 80% of survival whereas the Δ*phaC1* only 20%. Then, the heat shock at 45°C was chosen as the experimental condition to the other experiments presented in this work. To verify if the amount of accumulated PHB was directly linked to survival, we measured the survival of mutants with known difference in the amount of PHB (Alves et al., 2016). The data on **Figure 1B** shows that the parental strain SmR1 and the Δ*phaP2* mutant which accumulate high PHB contents had a decrease of only 20% of survival until 15 min, while the survival decreased to 40% after 30 min of incubation. The Δ*phaP1* mutant (which accumulates around 50% less PHB than the parental strain) survived less than the parental strain, as in 15 min its survival decreased by 40% and after 30 min only 10% of the initial population was still alive. Interestingly, the mutant strains unable to synthesise PHB (Δ*phaC1*) or unable to store it in the form of granules (Δ*phaP1.2*) struggled more during the heat shock. A fast decay in the survival of these strains was observed even short as 5 min of incubation and, reaching 15 min, only 20% of the initial population was still viable. As reported previously, the complementation of the Δ*phaP1.2* mutant with a native copy of *phaP1* is enough to restore the parental level of PHB accumulation (Alves et al., 2016). Following this indication, the Δ*phaP1.2* expressing a plasmid-borne copy of *phaP1* or *phaP2* was challenged at 45°C. The expression of *phaP1* or *phaP2* restored the homeostatic heat shock resistance, suggesting that indeed the accumulation of PHB granules is a key factor assisting the heat shock response (**Figure 1C**).

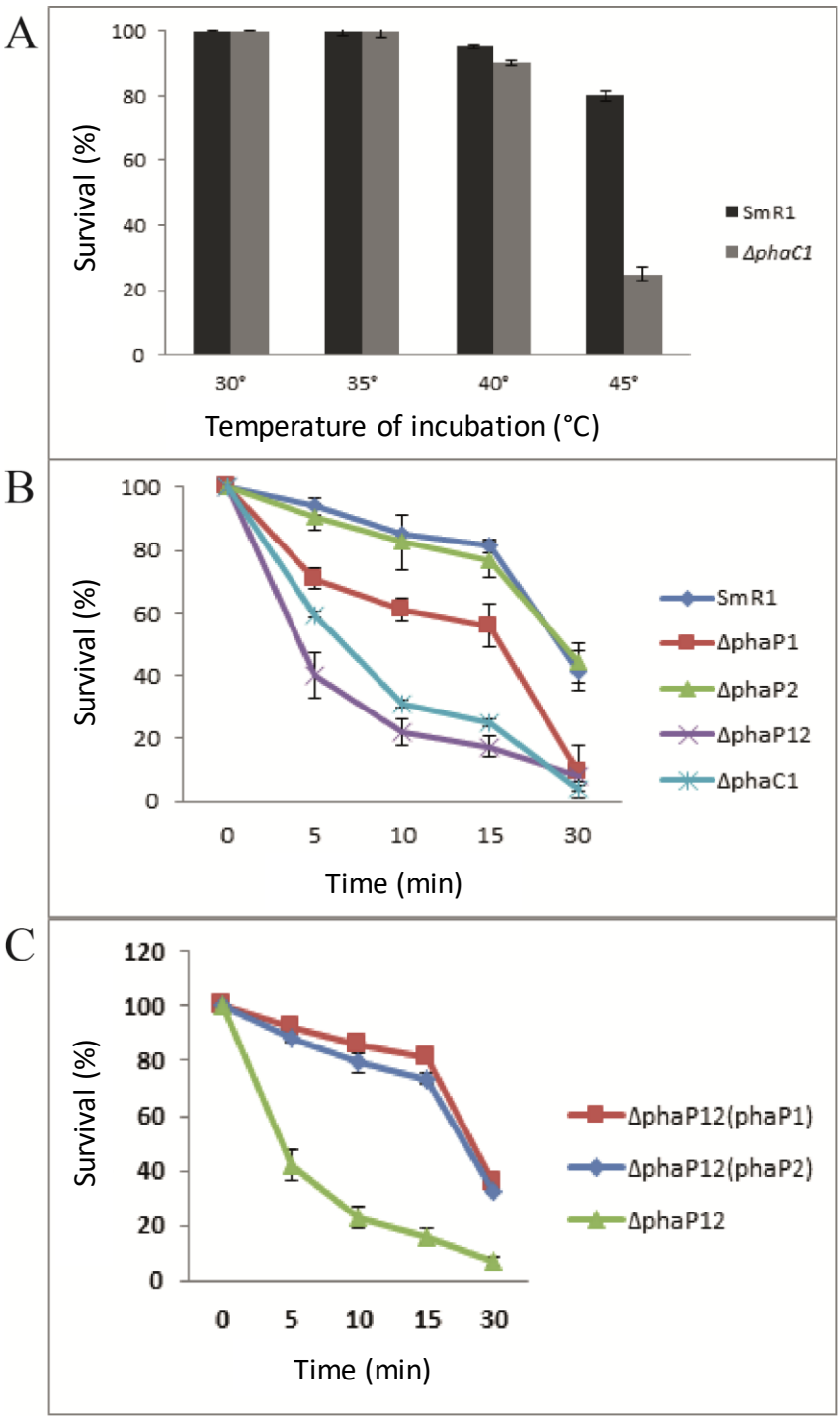

Figure. 1 The synthesis of PHB supports the survival of *H. seropedicae* under heat shock.

1A. Survival percentages of parental strain SmR1 and the Δ*phaC1* mutant (PHB$^-$) were calculated by CFU counting at 0 and 30 minutes after heat shock. The heat shock was performed in a water bath at different temperatures. The bacteria were grown in NFb medium with 37 mM DL-malate and 20 mM NH_4Cl, in penicillin glass bottles of 60 mL, containing 10 mL of medium. The bacterial cultures were collected for the heat shock at OD_{600} around 1.2. The culture flasks were transferred to a heated water bath, starting the heat shock and kept for 30 min. **1B**. Survival percentages were calculated by counting CFU at 0, 5, 10, 15 and 30 minutes after heat shock at 45°C. The experimental procedure was the same as

described in 1A. **1C**. The Δ*phaP1.2* mutant (unable to store PHB) was complemented with a *phaP1* or *phaP2* plasmid-borne copy. The experimental procedure was the same as described in 1A.

In order to verify whether the content of PHB produced was significant to *H. seropedicae* resist the heat shock, we challenged cells of the parental strain SmR1 cultivated in a low-PHB storage condition (OD_{600} 0.2; 37mM of DL-malate and 20 mM of NH_4Cl) (**Figure 2A**) and in a high-PHB storage condition (OD_{600} 1.0; 25mM of glucose and 5 mM of NH_4Cl) (**Figure 2B**). In the low-PHB condition, the content of PHB/cdw was only 1%, while in the high-PHB condition the content was 30% of PHB/cdw. The survival of the SmR1 culture with high PHB content was reduced 49.5% ± 9.2 after 30 min of incubation. The population of SmR1 with low-PHB amount was notably sensitive to heat shock since its survival decayed abruptly only with 5 min of incubation at 45°C (**Figure 2A**). In conclusion, the cells accumulating more PHB were more resilient to the heat shock at 45°C. Taken together, these findings indicate that PHB accumulation contributes to *H. seropedicae* resists against the deleterious effects of the heat shock.

3.2. PHB mobilisation towards PHA depolymerases is essential to *H. seropedicae* resistance to the heat shock

PHA mobilisation has been reported as protective against heat shock and other stress in bacteria like *Methylobacterium extorquens* (Ruiz et al., 2001; Koskimaki et al., 2016). To verify whether the depolymerisation of the PHB is also relevant to protect the *H, seropedicae* against the heat shock, we measured the survival of the double mutant Δ*phaZ1.2* of *H. seropedicae* SmR1 defective in the expression of the PHA depolymerases PhaZ1 and PhaZ2. This mutant did not present a significant PHB depolymerisation in a carbon-starved condition (data not shown). When shifted from 30°C to 45°C, the Δ*phaZ1.2* mutant presented a decrease in its survival by 96.5% ± 5 after 15 min of incubation (**Figure 3A**). This level of survival was similar to the Δ*phaC1* and Δ*phaP1.2* mutants which do not accumulate PHB. Therefore, only PHB storage is not enough to protect against heat shock, but indeed its degradation is necessary. Moreover, we measured the amount of PHB in the parental SmR1 and in the Δ*phaZ1.2* mutant to test whether PHB mobilisation is active during the heat shock. For this, we challenge both strains at 30°C and 45°C and measured the amount of PHB by gas chromatography (**Figure 3B**) and flow cytometry (**Figure 3C**).

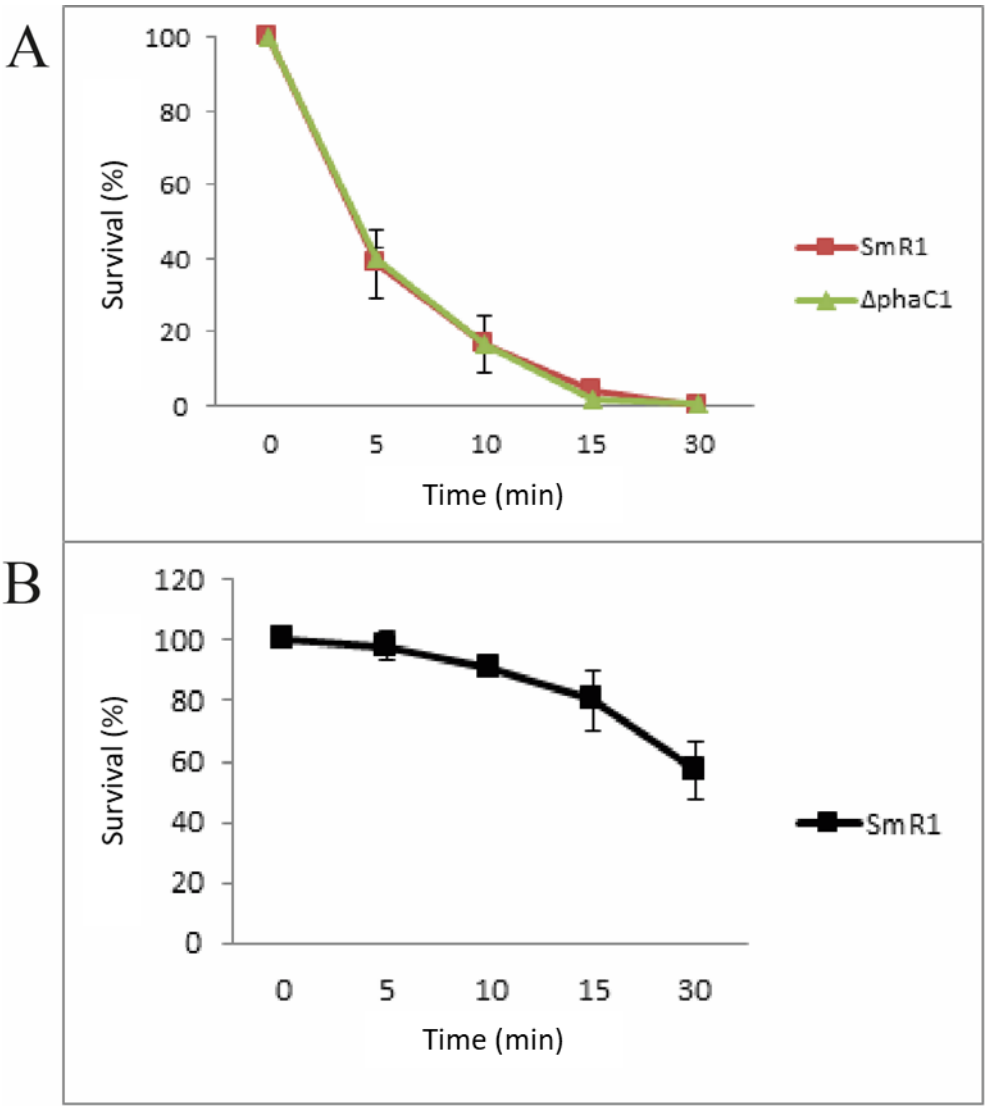

Figure. 2 The amount of accumulated PHB is linked to the survival of *H. seropedicae* under heat shock.

2A. Survival percentages of parental strain SmR1 and the Δ*phaC1* mutant (PHB^-) were calculated by CFU counting at 0, 5, 10, 15 and 30 minutes after heat shock. The bacteria were grown in 10 mL of NFb medium with 37 mM DL-malate and 20 mM NH_4Cl at 30°C in penicillin glass bottles of 60 mL. The heat shock was established transfering the bacterial culture flasks to a heated water bath at 45°C when the cultures reached OD_{600} around 0.2. **2B**. The same experimental procedure as 1A was applied, though SmR1 was grown in NFb medium with 25 mM D-glucose and 5 mM NH_4Cl (high PHB storage condition). Since Δ*phaC1* mutant does not grow using D-glucose as carbon source (Alves et al., 2016), this condition was tested only to the parental strain.

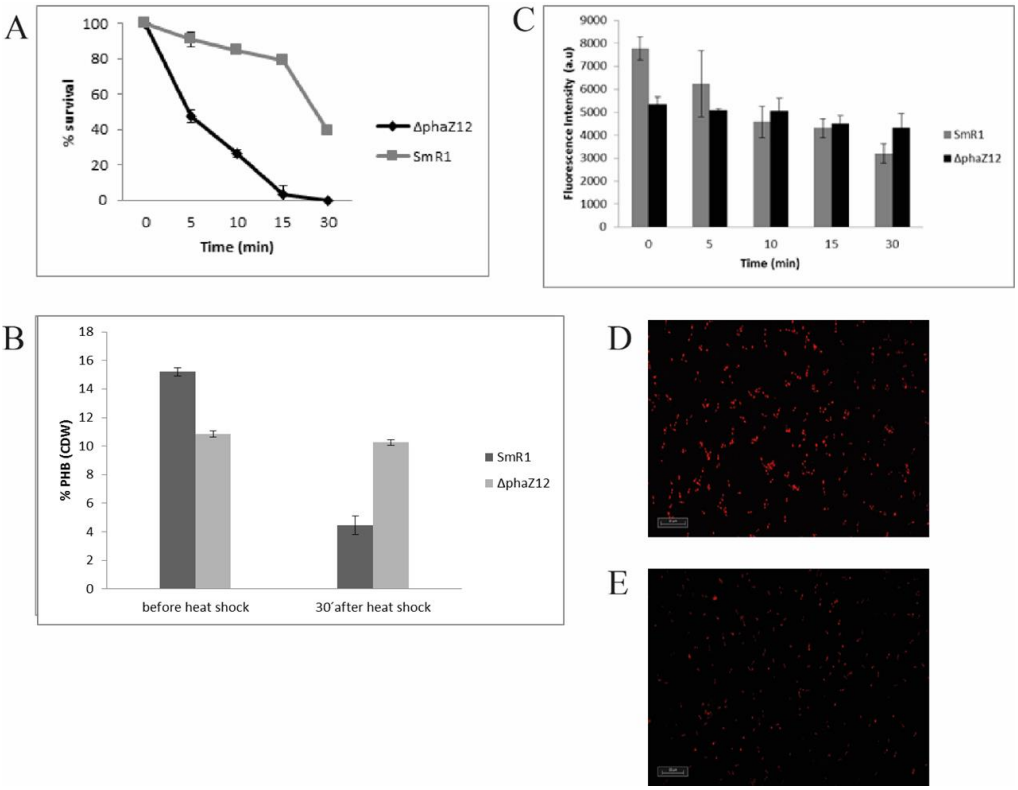

Figure. 3 The PHB mobilisation is activated in H. seropedicae during the heat shock.

3A. Survival percentages of parental strain SmR1 and the Δ*phaZ1.2* double mutant (unable to mobilise PHB) were calculated by CFU counting at 0, 5, 10, 15 and 30 minutes after heat shock. The bacteria were grown in 10 mL of NFb medium with 37 mM DL-malate and 20 mM NH_4Cl at 30°C in penicillin glass bottles of 60 mL. The heat shock was established transfering the bacterial culture flasks to a heated water bath at 45°C when the cultures reached OD_{600} around 1.2. **3B**. The PHB content was determined by methanolysis of lyophilised cells and gas chromatography. The PHB content was normalized by the cell dry weight of the sample. **3C**. The PHB mobilization kinetics was followed by Nile Red staining and flow cytometry measurements. The values of fluorescence in arbitrary units (a.u.) are the median fluorescent intensity already normalised by the number of counts. The same experimental procedure in 3A was applied. **3D** and **E,** fluorescent microscopy of SmR1 strain before and after 30 min of heat shock at 45°C, respectively.

For the parental strain after the heat shock, there was a 30% reduction in the amount of PHB/cdw, while for the Δ*phaZ1.2* mutant the amount of PHB/cdw remained.the same. It was also verified a reduction in the fluorescence of the SmR1 cells stained with Nile Red, indicating that the PHB was continuously degraded at the moment the heat shock started (**Figure 3C**). On the contrary, the Δ*phaZ1.2* mutant maintained the same level of fluorescence even after the beginning of the heat shock. The fluorescence microscopy of the parental strain before and after 30 min at 45°C showed the difference in PHB content (**Figure 3D and E**), corroborating the chromatography and flow cytometry data.

3.3. The PHB depolymerisation is activated by heat shock releasing 3-hydroxybutyrate

To observe the extent of PHB mobilisation during heat shock, we monitored the products released by ^{1}H-NMR of whole cells in solution. For this, the bacteria strains evaluated were grown in NFb medium with 37 mM DL-malate as a carbon source and 5mM NH_4Cl as a nitrogen source. This condition favours the high production of PHB in *H. seropedicae* (Sacomboio et al., 2017). As expected the parental and Δ*phaP2* strains, which accumulate high amount of PHB, can mobilise the polymer releasing 3HB (3-hydroxybutyrate) during the heat shock (**Figure 4**). Interestingly, the Δ*phaZ1.2* strain accumulates the same amount of PHB as the parental SmR1 and Δ*phaP2* strains. However, a low releasing of 3HB was measured for this strain since it is defective in the expression of PHA depolymerases PhaZ1 and PhaZ2. In the same way, the non-PHB-accumulating mutants Δ*phaC1* and Δ*phaP1.2* had no increase in the 3HB releasing at any time of the heat shock.

3.4. The alternative sigma factor RpoS is involved in PHB mobilisation in *H. seropedicae* SmR1

The RpoS sigma factor has been related to the protection of bacteria against different types of stress (Battesti et al., 2011). To verify whether the RpoS is involved in PHB mobilisation in *H. seropedicae* SmR1, a Δ*rpoS* isogenic strain was constructed and evaluated for survival and PHB mobilisation under heat shock at 45°C. The *rpoS* deletion caused a continuous loss of survival, leading to complete killing of the bacterial population after 30 minutes of heat shock (**Figure 5A**). Interestingly, the level of PHB accumulated by the Δ*rpoS* mutant remained the same, comparing the time zero and 30 minutes of heat shock (**Figure 5B**). These data demonstrate that RpoS is involved with PHB mobilisation in *H. seropedicae*, once since RpoS has an extensive regulon this action may be indirect.

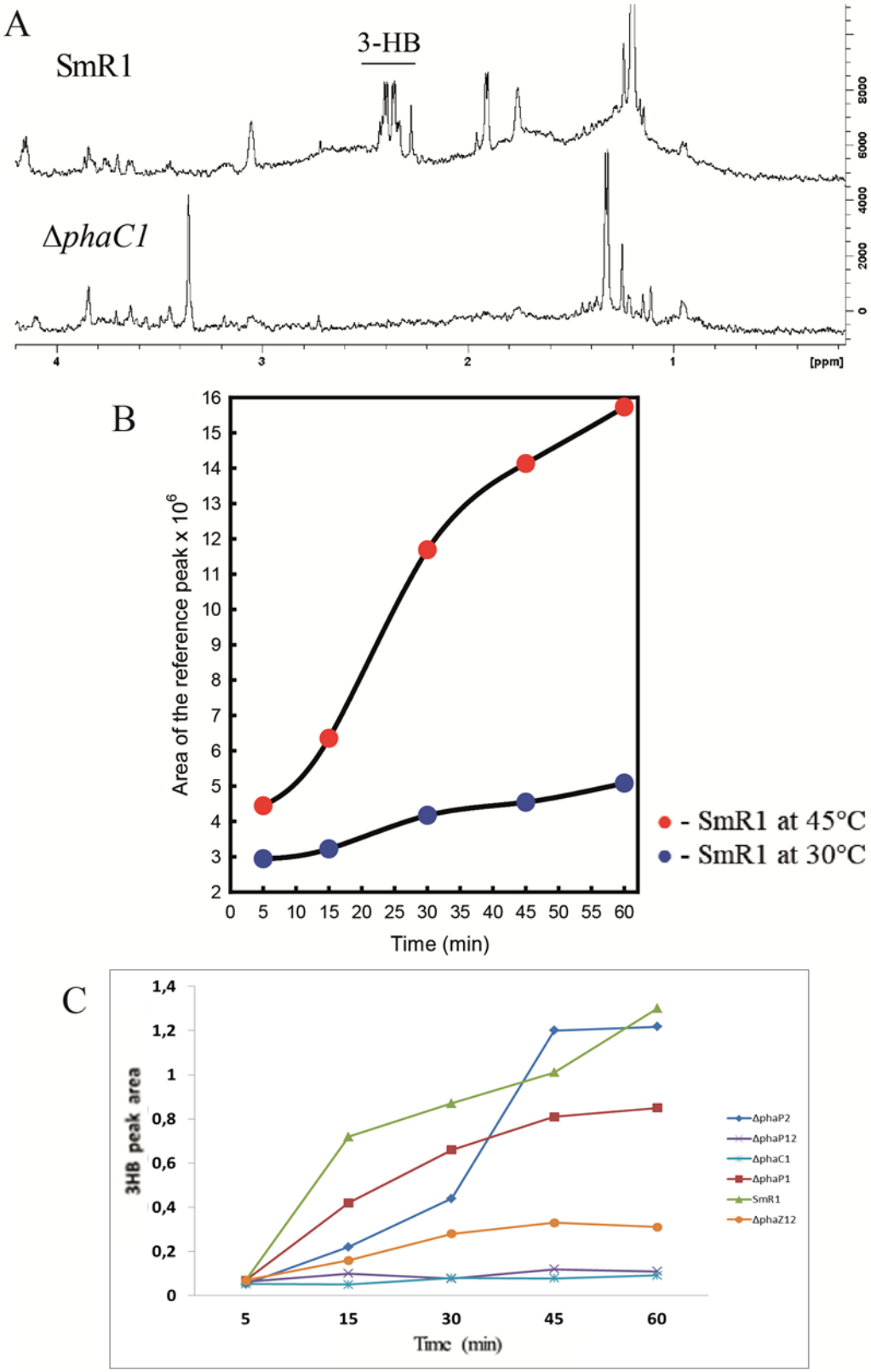

Figure. 4 PHB mobilisation under heat shock was monitored by in situ 1H-nuclear magnetic resonance.

4A. ^{1}H-NMR representative spectrum of SmR1 (upper line) and Δ*phaC1* (lower line) cells in D_2O after 60 min at 45°C. **4B**. The areas of the 3HB peak (2.36 ppm) during the heat shock at 45°C and control condition at 30°C. **4C**. The strains were incubated for 5 min at 30°C, then shifted to 45°C. The whole

analysis from heat shock to spectra acquisiton was performed inside the NMR equipment, The 3HB peak areas after the heat shock start were normalized to the area of the last spectra acquired at 30°C (at 5 min of analysis).

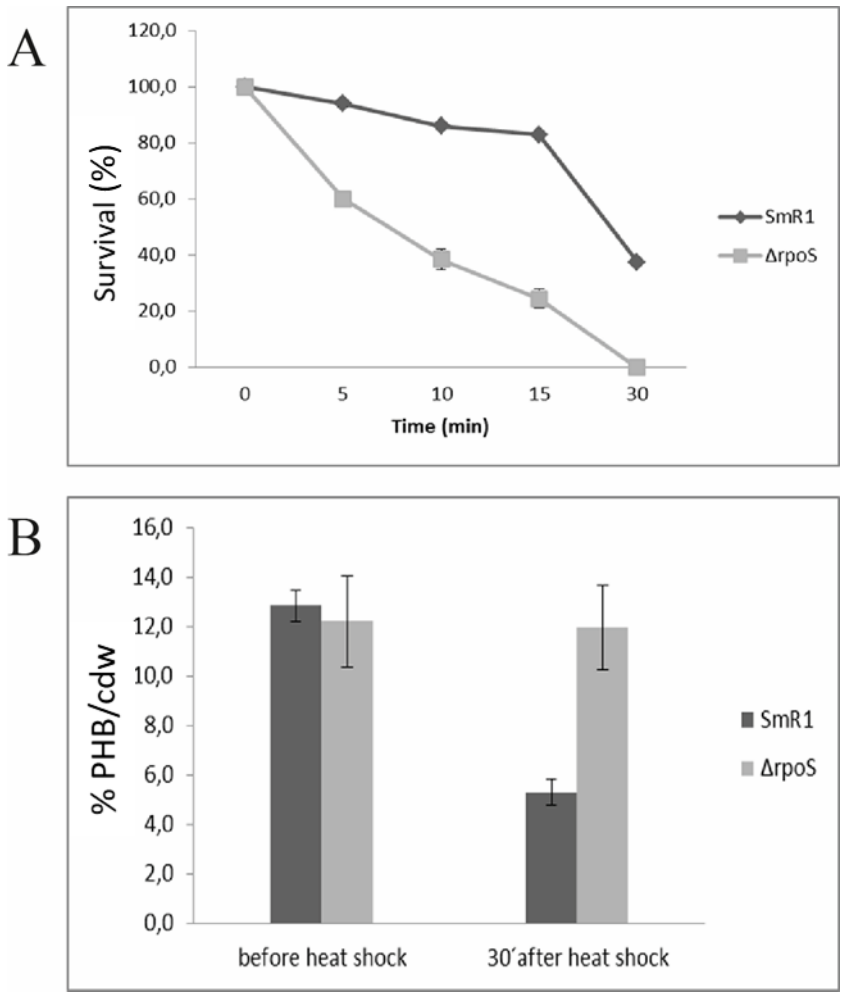

Figure. 5 RpoS is involved in PHB mobilisation in *H. seropedicae* SmR1.

5A. Survival percentages of parental strain SmR1 and the Δ*rpoS* were calculated by CFU counting at 0, 5, 10, 15 and 30 minutes after heat shock. The bacteria were grown in 10 mL of NFb medium with 37 mM DL-malate and 20 mM NH_4Cl at 30°C in penicillin glass bottles of 60 mL. The heat shock was established transfering the bacterial culture flasks to a heated water bath at 45°C when the cultures reached OD_{600} around 1.2. **5B**. The PHB content was determined by methanolysis of lyophilised cells and gas chromatography. The PHB content was normalized by the cell dry weight of the sample.

4. Discussion

PHB has been suggested as a protective factor against stress in bacteria, especially under conditions of carbon starvation (James et al., 1999; Handrick et al., 2000; Kadouri et al., 2003b). In this particular case, PHB mobilisation is responsible for providing carbon, maintaining bacteria viable for a few generations. However, it is known that PHB can also defend bacteria against oxidative, osmotic and thermal stress. Although, it is still not clear whether the synthesis and accumulation of the polymer contributed to alleviate stress, or the polymer has to be mobilized to support bacterial survival.

Our results comparing different defective *H. seropedicae* mutants in the synthesis and mobilisation of PHB indicate that both the lack of accumulated PHB and the inability to mobilise it affect the survival of the bacterium against thermal shock. The data presented here corroborate a recent study showing that in addition to carbon starvation other stresses lead to PHB mobilisation.

Koskimäki et al.(2016) showed that in *Methylobacterium extorquens* DSM13060 the expression of the *phaZ1* and *phaZ2* genes increased when the bacterium was treated with hydroxyl radicals (•OH) or colonising the interior of *Pinus sylvestris* roots (Koskimaki et al., 2016). The expression of PHA depolymerases resulted in the mobilisation of PHB and excretion of methylated 3HB dimers and trimers. These oligomers showed high antioxidative activity against hydroxyl radicals. Such mechanism may be relevant for the adaptation of bacteria inside plants, since the oxidative attack is reported as one of the response of plants to bacterial infection, even in cases of beneficial infection (Liu et al., 2007). However, to the best of our knowledge, to date, the authors have not investigated the colonisation profile or stress resistance of *M. extorquens* mutants defective in the expression of *phaZ1* and *phaZ2*. Such experiment could be useful to determine the effect of PHB mobilisation to *M. extorquens* colonising plants.

The mobilisation of PHB has already been suggested as a protective factor against abiotic stress in *A. brasilense* Sp7, another plant-associated bacterium (Kadouri et al., 2003b). The mutant *phaZ1* of *A. brasilense* Sp7 showed lower survival against heat shock at 50°C, ultraviolet irradiation and osmotic shock (Kadouri et al., 2003b). However, the authors did not verify whether in fact during the stress condition *A.*

brasilense mobilised its PHB reserve. In *Pseudomonas oleovorans* GPo1 it was demonstrated that the mobilisation of mcl-PHA (medium chain length PHA) occurred concomitantly with an increase in the intracellular concentration of the nucleotide ppGpp (Ruiz et al., 2001). The increase in ppGpp concentration usually occurs as a consequence of the low concentration of amino acids that leads to the block of protein synthesis, or by environmental stressors, such as heat shock (Hauryliuk et al., 2015). The ppGpp leads to increased translation of the *rpoS* mRNA, and RpoS remodels gene expression of the bacteria to cope adequately with the stress situation.

Our results showed that the deletion of *rpoS* impairs PHB mobilisation. Possibly, RpoS could be indirectly involved in regulating the expression of the *phaZ1* and *phaZ2* genes, or it regulates other genes leading to the acceleration of PHB degradation.

In an *E. coli* strain engineered to produce and mobilise PHB, the protective effect of PHB mobilisation during heat shock was also observed (Wang et al., 2009). Therefore, it may be that the 3HB generated is responsible for the protective effect against stress. Furthermore, the 3HB action could be universal, even in organisms that do not accumulate PHB naturally. Recent studies have suggested 3HB as a chemical chaperone, serving as a cytoplasmic solute and protecting proteins of the thermal denaturation (Soto et al., 2012; Obruca et al., 2016b).

In this work, we show for the first time that the synthesis of PHB associated with its degradation is in fact protects bacteria from thermal stress. In addition, we have shown by NMR analyses that the release of 3HB occurs as soon as the stress is established. Therefore, it remains to be clarified what role 3HB plays, whether it serves mostly as a protective solute or whether the bacterium metabolizes it for energy production. The demonstration that PHB cycle mutants have lower fitness under stress conditions makes them a valuable tool to further plant-bacterial interaction studies.

5. References

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CAPÍTULO IV

Manuscrito não submetido

Effects of poly-3-hydroxybutyrate (PHB) metabolism in plant colonizing performance of *Herbaspirillum seropedicae*

Effects of poly-3-hydroxybutyrate (PHB) metabolism in plant colonizing performance of *Herbaspirillum seropedicae*

Abstract

Herbaspirillum seropedicae is an endophytic bacteria that can establish associations with some plants such as rice, sorghum, maize and sugar cane. This bacteria has biotenology potential to produce a polymer, which name is polyhydroxybutyrate (PHB), it is stored in cells in the form of insoluble granules . Little information is available about possible PHB role in bacterial root colonization or plant growth promotion. Altough, a number of PGPR strains are known to produce high amounts of PHB. In this work we investigated whether PHB production and mobilisation are relevant to the ability of *H. seropedicae* to colonize plant roots. First of all, we inoculated the parental *H. seropedicae* SmR1 strain and several mutants defective in PHB production *(*Δ*phaP1,* Δ*phaP12,* Δ*phaC1,* Δ*phaR*) or mobilization *(*Δ*phaZ1.2*) to colonize the roots of *Setaria viridis* A10.1. Results showed that strains producing the high amounts of PHB may colonize the roots, thus increasing the root area and number of lateral roots significantly compared with the strains PHB negative, after 25 days post inoculation. Experiments involved low oxygen reveals that only parental strain and Δ*phaP2,* can grow under low oxygen regime. However the other mutants affected PHB metabolism, presented a growth penalty in low oxygen. To investigated the PHB cycle is active or not, we tracked GFP expression under the control the promoters of sinthase and depolimerases genes of *H. seropedicae*. Both genes are acting during the interaction plant-bacteria, but in diferents days. All these results showed in this paper the PHB is relevant to interaction and these findings can be important to future research about the role of polymer for bacteria and the development of some plants.

1. Introduction

Several bacteria produce polymers, such as polyhydroxyalkanoates (PHA), which can serve as a source of energy and carbon storage, especially under limited nutrient conditions. Further, of a carbon reserve, PHA play physiological roles like protection

against exogenous stress factors and maintenance of photosynthetic carbon fixation. In the case of biological nitrogen fixation, the presence of PHA has been shown to prolong nitrogen fixation during the overnight (Bergersen 1991) and also to maintain the redox potential for nitrogen fixing by energy production and NADH oxidation (Encarnación 2002; Koller, 2016). The main proteins involved in metabolism of PHB are PHA synthase (PhaC - synthesis of polymer), Phasins (PhaP – control the size of granules), transcriptional regulator of phasin (PhaR) and PHA depolymerases (PhaZ – PHB mobilization) (Saegusa et al., 2001; Wieczorek et al., 1995; Maehara et al., 1999; Maehara et al., 2001; Pötter et al., 2002; Stubbe & Tian, 2003). The most commom PHA produced by gram-negative bacteria belonging to the β-proteobacteria class (e.g *Herbaspirillum seropedicae)* is polyhydroxybutyrate (PHB) (Steinbüchel, A., 1990; Anderson & Dawes, 1990).

Herbaspirillum seropedicae, is an endophytic, nitrogen-fixing bacteria that can establish beneficial associations with several agricultural relevant grasses, such as rice, sorghum, maize and sugar cane (Pimentel *et al*., 1991; Olivares *et al*., 1996; Olivares *et al*., 1997; James *et al*., 1997; James *et al*., 2002). Its genome was sequenced by Pedrosa and co-workers (2011), which revealed the presence of at least 13 genes involved in the metabolism of polyhydroxybutyrate (PHB).

This polymer is formed by the enzymatic condensation of 3-hydroxyacyl-CoA, and it is stored in cells in the form of insoluble granules (Anderson & Dawes, 1990). Although the biochemical pathways leading to the formation of PHB and PHA granules are well described in bacteria (Jendrossek and Pfeiffer, 2014; Rehm et al. 2010), little information is available about its possible role in bacterial root colonization or plant growth promotion. Interestingly, a high number of plant growth promotion strains are known to produce high amounts of PHB.

Therefore, we investigated whether PHB production and degradation are important for the ability of *H. seropedicae* to colonize plant roots. Specifically, we examined the ability of the parental *H. seropedicae* SmR1 strain and several mutants defective in PHB production (Δ*phaP1,* Δ*phaP12,* Δ*phaC1,* Δ*phaR*) or mobilization (Δ*phaZ1.2*) to colonize the roots of the model grass *Setaria viridis* A10.1, which showed a strong growth response to bacterial inoculation (Pankievicz et al., 2015).

2. Materials and methods

2.1. Bacterial strains and growth conditions

Herbaspirillum seropedicae SmR1 parental (Souza et al., 2000) and mutant strains were cultivated in NFbHP-malate medium containing 37mM of DL-malic acid, 20 mM of phosphate and 20 mM of NH_4Cl (Pedrosa and Yates, 1984) and streptomycin (80 μg/mL). To grow the bacteria under a low-oxygen regime, we employed glass flasks of 30 mL of capacity filled with 25 mL of medium. After inoculation, the flasks were closed with rubber cap and incubate in an orbital shaker at 100 rpm and 30°C. The samples were taken each 2 hours and the last samples at 24 hours for OD_{600} measurement. Several *H. seropedicae* mutants relevant to in the PHB metabolism were employed for this work. The Δ*phaP2*, which is defective in the expression the alternative phasin PhaP2, produces high amounts of PHB similar to the parental strain (Alves et al., 2016). The Δ*phaP1*, which is defective in the expression of the main phasin PhaP1, produces ~50% of the PHB levels found in the parental strain (Alves et al., 2016). The double mutant in both phasins Δ*phaP1.2* and the Δ*phaC1* which is defective in the expression of PHA synthase PhaC1 do not produce PHB (Alves et al., 2016). The Δ*phaR* which is defective in the expression of the transcriptional regulator PhaR produces ~4% of the PHB levels found in the parental strain. The double mutant Δ*phaZ1.2* is defective in the expression of both PHA depolymerases (PhaZ1 and PhaZ2) and produces the same amount of PHB as the parental strain, however, does not mobilize it(Manuscript Chapter III). The Δ*fliA* mutant is defective in the expression of sigma 28 (or FliA) sigma factor involved in the expression of genes encoding proteins of the flagellum. This mutant was applied as a negative control of the endophytic colonization. *Escherichia coli* EC100 and S.17 were used for cloning and conjugation procedures. They were grown in LB at 37°C at 160 rpm. All the strains used in this work are described in Table 1.

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

Strain or plasmid	Relevant characteristics	Reference/source
E. coli strains		
EC100™	Cloning strain	Epicentre
S17-1	Conjugation strain	(Simon et al., 1983)

H. seropedicae strains		
SmR1	Parental strain, Nif^+, Sm^R, PHB^+	(Souza et al., 2000)
Δ*phaP1*	Chromosomal deletion of *phaP1*	(Tirapelle et al., 2013)
Δ*phaP2*	Chromosomal deletion of *phaP2*	(Tirapelle et al., 2013)
Δ*phaP1.2*	Chromosomal deletion of *phaP1* and *phaP2*	(Alves et al., 2016)
Δ*phaC*1	Chromosomal deletion of *phaC1*	(Tirapelle et al., 2013)
Δ*phaZ1*	Chromosomal deletion of *phaZ1*	(Alves et al., manuscript in preparation)
Δ*phaZ2*	Chromosomal deletion of *phaZ2*	(Alves et al., manuscript in preparation)
Δ*phaZ1.2*	Chromosomal deletion of *phaZ1* and *phaZ2*	(Alves et al., manuscript in preparation)
Δ*phaR*	Chromosomal deletion of *phaR*	(Teixeira, 2015)
Δ*fliA*	Chromosomal deletion of *fliA*	This work
Plasmids		
pK18*mobsacB*	Suicide vector; Km^R, *sacB*, mobilizable plasmid	(Schafer et al., 1994)
pK18mobΔ*fliA*	pK18*mobsacB* harboring the Δ*fliA* product of overlapping PCR	This work
pGWB-4 (e-GFP)	Construction of transcriptional fusions with the eGFP as gene reporter	(Nakagawa et al., 2007)
pPHAC1GFP	eGFP under the control fo the *phaC1* promoter of *H. seropedicae* SmR1	This work
pPHAZ1GFP	eGFP under the control of the *phaZ1* promoter of *H. seropedicae* SmR1	This work
pPHAZ2GFP	eGFP under the control of the *phaZ2* promoter of *H. seropedicae* SmR1	This work

2.2. Construction of Δ*fliA* mutant of *H. seropedicae* SmR1

The in-frame marker-less deletion of *fliA* (Hsero_2029, GenBank: ADJ63529) was obtained by cloning upstream and downstream fragments of the gene into the non-replicating plasmid pK18*mobsacB*, which carries a kanamycin resistance cassette along with *sacB* that confers sucrose sensitivity (Schafer et al., 1994). Briefly, 500 bp

fragments to either flank of *fliA* were amplified by PCR with primers Fw_*fliA*_UP (5' GTGAATTCGGCCAACTGGCGGAGATCTT 3', EcoRI site underlined) and Rev_*fliA*_UP (5' GGATCCCTTAGGTCGTCTCTACTTGCCTTTTTTTCCCTTGAC 3', the underlined site was used for overlapping PCR) for the upstream region, whereas Fw_*fliA*_DOWN (5' TAGAGACGACCTAAGGGATCCCTGCGCGAGCATTCCTG GAGCG 3', the underlined site was used for overlapping PCR) and Rev_*fliA*_DOWN (5' GGTTAAGCTTGTCGAAAAACTGCGCCAGGA 3', HindIII site underlined) were used for the downstream region. Overlapping PCR fused the PCR products, and the resulting Δ*fliA* product was cloned into pBlueScript II KS. The entire construction was digested with EcoRI and HindIII to ligate the Δ*fliA* fragment into pK18*mobsacB* digested with the same enzymes, yielding pK18Δ*fliA*. *E. coli* S17-1 was transformed with pK18Δ*fliA* and the plasmid conjugated to *H. seropedicae* SmR1 using bi-parental mating. Single-recombinants were selected on NFb-malate agar containing streptomycin 80 $\mu g.mL^{-1}$, nalidixic acid 5 $\mu g.mL^{-1}$ and kanamycin 500 $\mu g.mL^{-1}$. A single-recombinant colony was collected in 3 mL NFb-malate and cultivated overnight without antibiotics. The culture was serially diluted and plated on NFb-malate agar containing 10 % (w/v) sucrose. Colonies that grew on sucrose were screened for deletion by PCR using the primers Fw_*fliA*_UP and Rev_*fliA*_DOWN.

2.3. Sterilization and germination of *Setaria viridis* A10.1 seeds

Seeds of *S. viridis* were surface sterilized with a 1% sodium hypochlorite plus 0.1 % (v/v) Tween20 solution for 3 min, followed by three rinses with distilled sterile water. Sterilized seeds were plated onto modified Hoagland's nutrient solution (Hoagland and Arnon, 1950) containing 1% (v/v) phytagel. The solution was autoclaved for 30 min 121 °C. Plates were placed horizontally in the dark for 1 day and 2 days in the light at 30°C.

2.4. Inoculation and growth of *Setaria viridis* A10.1

Three-day-old seedlings of *S. viridis* were inoculated with *H. seropedicae* SmR1 or one of the mutant strains to be evaluated. The bacterial cultures were grown in NFbHP-malate medium as described above, until OD_{600} of 1.0 (10^8 cells ml^{-1}). The culture was washed twice with sterile 0.9% saline solution and diluted to $1x10^7$ cells ml^{-1} before

inoculation. The seedlings were inoculated with 1 ml of this bacterial suspension per plantlet and then transferred to pots containing a mixture of sterile Turface and vermiculite in a proportion of 3:1 (w/w), respectively. Plants were grown in the greenhouse at 30°C with a 16 h light/8 h darkness cycle by 25 days. Plants were watered twice a week with Hoagland's solution supplemented with 0.5 mM of potassium nitrate (KNO_3).

2.5. Quantification of bacterial colonization

To verify the ability of *H. seropedicae* SmR1 and mutant strains to colonize *S. viridis* roots, a colonization assay was performed using plants harvested 25 days post inoculation. To quantify the epiphytic colonization, roots were vortexed in 1 ml of saline solution (0.9% sodium chloride) for 1 min. The bacterial suspension was diluted, and the CFU number counted as described below. To quantify the endophytic colonization, roots were surface sterilized with 70% ethanol for 1 min followed by 1% sodium hypochlorite for 1 min and then washed thrice using sterilized distilled water. The sterile roots were then ground in 1 mL of saline solution. The root extract was then ten-fold serial diluted, and 10 µL of each inoculation were pipetted onto NFbHPN – malate medium containing 80 µg ml^{-1} of streptomycin. The CFU number was counted after 2 days of incubation at 30°C.

2.6. Plant growth promotion analysis

S. viridis plants, 25 days post-inoculation, were harvested by carefully removing the soil and washing the roots briefly with distilled water before growth parameter measurements (i.e., root and shoot length, lateral root number, root and shoot fresh and dry weight). Root length and the number of lateral roots were analyzed using WinRHIZO 2002c software (Regent Instrument Inc., Quebec City, Canada), shoot length were measured using ruler, shoot and root weight were measured using a balance.

2.7. Statistical analysis

Statistical analyses were performed by non-parametric *t-test* ($p \leq 0.05$) in the 95% confidence level. The number of plants varied in each treatment due to variability in plant growth and survival.

2.8. Expression of P*phaC1*- and P*phaZ1-gfp* transcriptional fusions in *H. seropedicae* SmR1

The promoter region upstream of the *phaC1* (P*phaC1*, locus tag Hsero_2999), *phaZ1* (P*phaZ1*, Hsero_1622) and *phaZ2* (P*phaZ2*, Hsero_0639) genes were amplified from *H. seropedicae* SmR1 genomic DNA by PCR and cloned into Gateway pDORN^{TM}/ZEO. The clones were obtained by BP clonase *in vitro* recombination overnight followed by proteinase K treatment for 10 min at 37°C, transformed in *E. coli* EC100, then the colonies were growing and did a PCR to check and confirm the clones. The validity of each cloned region was confirmed by sequencing. LR cloning was performed as previously described to BP clonase, though using LR clonase in a recombination reaction. Following confirmation, each construct was transformed into *E. coli* S-17.1 before mating with *H. seropedicae* SmR1.

2.9. Analysis of bacterial PHB genes expression on *S. viridis* roots by confocal microscopy

To determine the expression of PHB genes during *S. viridis* root colonization, three-day-old seedlings were inoculated with *H. seropedicae* SmR1 harboring the transcriptional fusions PHB gene promoter-GFP. Dissected root fragments from control and inoculated plants were placed on a slide in a drop of water and covered with a glass coverslip to be observed under a fluorescence microscope (Olympus IX70 inverted microscope, USA). For confocal microscopy, the roots were prepared as described above and observed using a Zeiss LSM 510 META laser scanning confocal microscope (Germany) equipped with 488 nm Ar and 543 nm He–Ne lasers to detect green fluorescence emitted by GFP-tagged *H. seropedicae* (excitation at 488 nm and detection at 500–550 nm).

Roots were observed within 1, 4, 7, 10 and 15 days after inoculation of *H. seropedicae* SmR1 carrying the PHB gene promoter-GFP fusions. The green fluorescence images from GFP-tagged bacteria and the transmitted images (brightfield mode) of the identical image were overlaid. All composite images were produced using the LSM Image Browser 4.0 software (Carl Zeiss Microimaging). Additional images were obtained using a Nikon Eclipse Ti inverted laser scanning confocal microscope (USA) equipped with an ion laser to detect green fluorescence.

3. Results

3.1. The deletion of PHB gene impacts plant growth promotion in *H. seropedicae* SmR1

Previous research demonstrated a significant impact on fresh root weight, lateral root number and total root area when *S. viridis* plants were inoculated with *H. seropedicae* and *Azospirillum brasilense* (Pankievicz et al., 2015). To examine whether PHB metabolism is important during the plant-bacteria interaction, we inoculated *S. viridis* A10.1 plants with *H. seropedicae* SmR1 and various mutants defective in genes involved in PHB metabolism (Alves, 2016). Consistent with our previous findings, a significant increase in total root area was seen upon inoculation with *H. seropedicae* SmR1, and this same increase was seen in plants inoculated with Δ*phaP2* mutant strain, which produces similar amounts of PHB than the SmR1 parental strain (Fig. 1). Increases in lateral root number were also seen upon bacterial inoculation (Fig. 2). However, plants inoculated with the mutant Δ*phaC1*, which does not produce PHB, had a significant reduction in lateral root number compared to SmR1 and Δ*phaP2* strains. The same was true for plants inoculated with the Δ*phaR* mutant, which produces only 4% of PHB/cell dry weight when compared to 16% of PHB/cdw in the SmR1 (Gavídia, 2017). Plants inoculated with mutants that produce 50% or less PHB than the SmR1 (Δ*phaP1* and Δ*phaR*) showed a decrease in root dry weight (Fig. 3). Similar results were obtained when plants were inoculated with a double mutant, lacking both PhaP1 and PhaP2 (Δ*phaP12*) (Fig.1 and 2). Our results suggest that PHB metabolism is required for the ability of *H. seropedicae* to promote the growth of *S. viridis*. It is interesting to note that not all of the phenotypes measured were equally impacted, although the general trends were consistent.

3.2. Disruption of PHB degradation also decreases bacterial plant growth promotion

PHB mobilization has been correlated with the ability of bacteria to survive under high temperature, osmotic stress, UV radiation and oxidative stress (Ruiz et al., 2001; Kadouri et al., 2003, 2005; Koskimaki et al., 2016). *H. seropedicae* has two PHA depolymerases (PhaZ1 and PhaZ2), which at least the PhaZ1 was already located on the surface of the polymer (Tirapelle et al., 2013), in a position consistent with their role in

degrading the insoluble polymer. Given that gene deletions that affect PHB biosynthesis also affected plant growth promotion (Figure 1), we verified whether the ability to mobilize the PHB reserve might also be essential. Therefore, we inoculated *S. viridis* roots with an *H. seropedicae* double mutant Δ*phaZ1.2*, lacking both PhaZ1 and PhaZ2. Plant growth promotion by this mutant strains was severely impacted as measured by significant decreases in total root area (Fig. 1), lateral root number (Fig. 2) and root dry weight (Fig. 3).

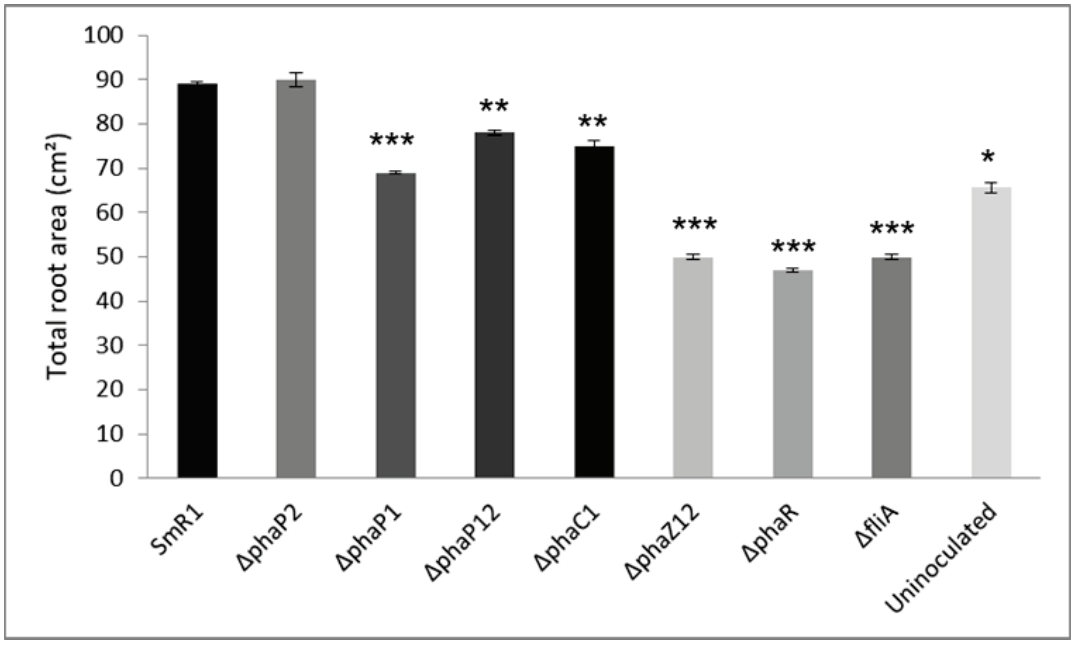

Figure 1. Analysis of total root area of *Setaria viridis* A10.1

Plants were inoculated with *Herbaspirillum seropedicae* SmR1 (parental strain) and mutants as indicated below each bar in the graph. The plants were analyzed 25 days after inoculation. Bars are mean values ± SE (standard error). Asterisk indicates statistical significance across the treatments with * - *P*-value ≤ 0.05; ** - *P*-value ≤ 0.01; *** - *P*-value ≤ 0.001.

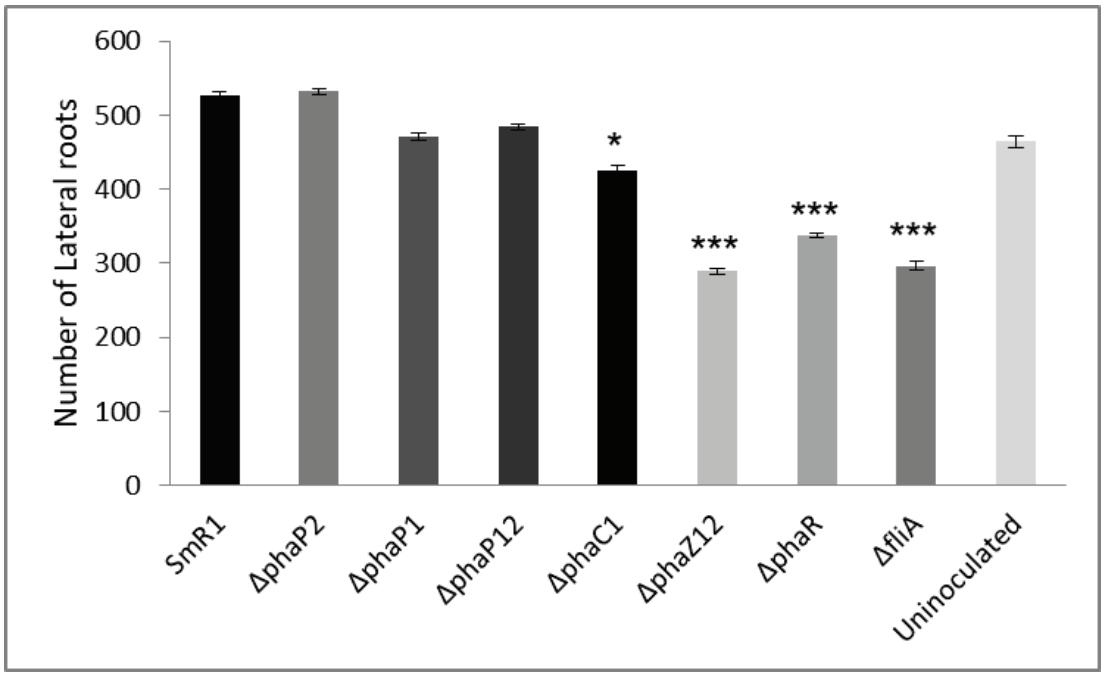

Figure 2. Impact of bacterial inoculation on stimulation of lateral root formation.

Setaria viridis A.10 was inoculated with *Herbaspirillum seropedicae* SmR1 (parental strain) and mutants as indicated below each bar in the graph. The plants grow up in soil with Turface and vermiculite. After 25 days post-inoculation the plants were harvested, their roots washed and analyzed applying WinRHIZO pro software root scanning and quantification (Regent Instruments, USA). All bars are mean values ± SE (standard error). The P-values show statistical significance of the inoculation with the different strains employed in the experiment: * - *P*-value ≤ 0.05; ** - *P*-value ≤ 0.01; ***- *P*-value ≤ 0.001.

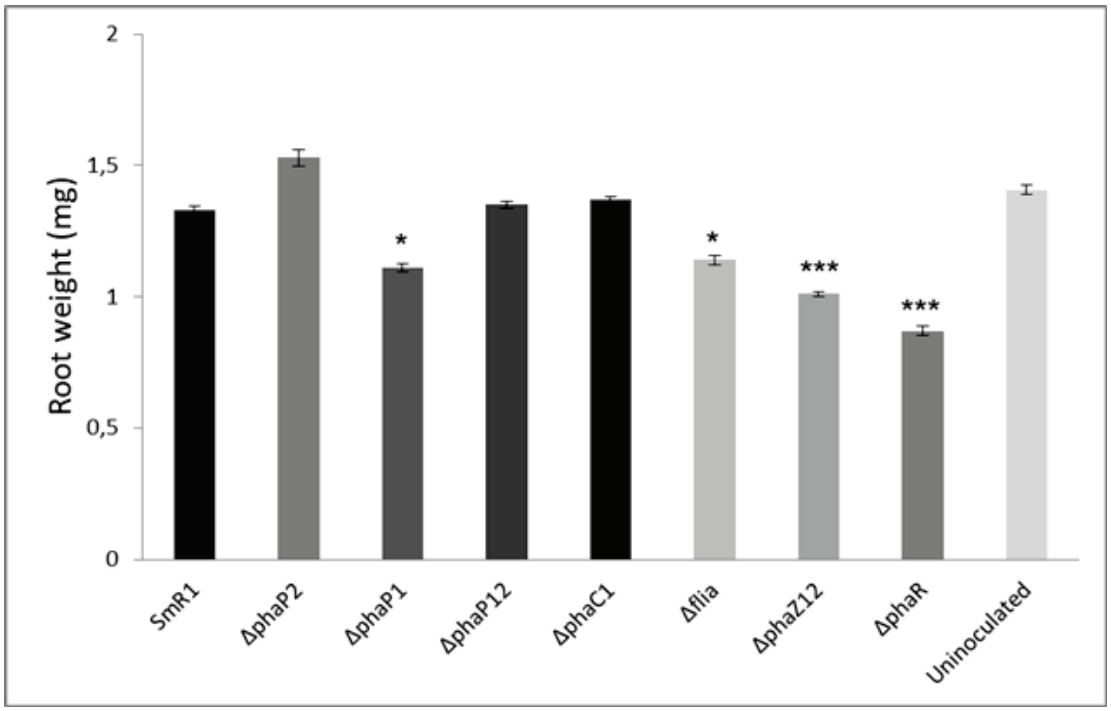

Figure 3. Impact of bacterial inoculation on plant root weight

Root weight was measured 25 days after plant inoculation. All bars are mean values ±. SE (standard error). The P-values show statistical significance of the inoculation with the different strains employed in the experiment: * - *P*-value ≤ 0.05; ** - *P*-value ≤ 0.01; ***- *P*-value ≤ 0.001.

3.3. Low oxygen affects the growth of PHB mutants

The level of oxygen affects synthesis of PHB as related previously for bacteroids that produce PHB and lipids inside legume root nodules, a low-oxygen environment (Senior et al., 1972; Mandon et al., 1998). Based on this, we tested whether low oxygen condition may affect the growth of mutants defective in PHB synthesis or degradation. Thus, to simulate a low-oxygen condition such as the infection of *Setaria viridis* A.10 roots faced by *Herbaspirillum seropedicae*, we grew the SmR1 and mutant strains under a low-oxygen regime as described in Material and Methods. The strains accumulating high amounts of PHB such as SmR1 and Δ*phaP2* had better growth, on the other hand, the mutants that produce less or no PHB presented a slow growth rate.

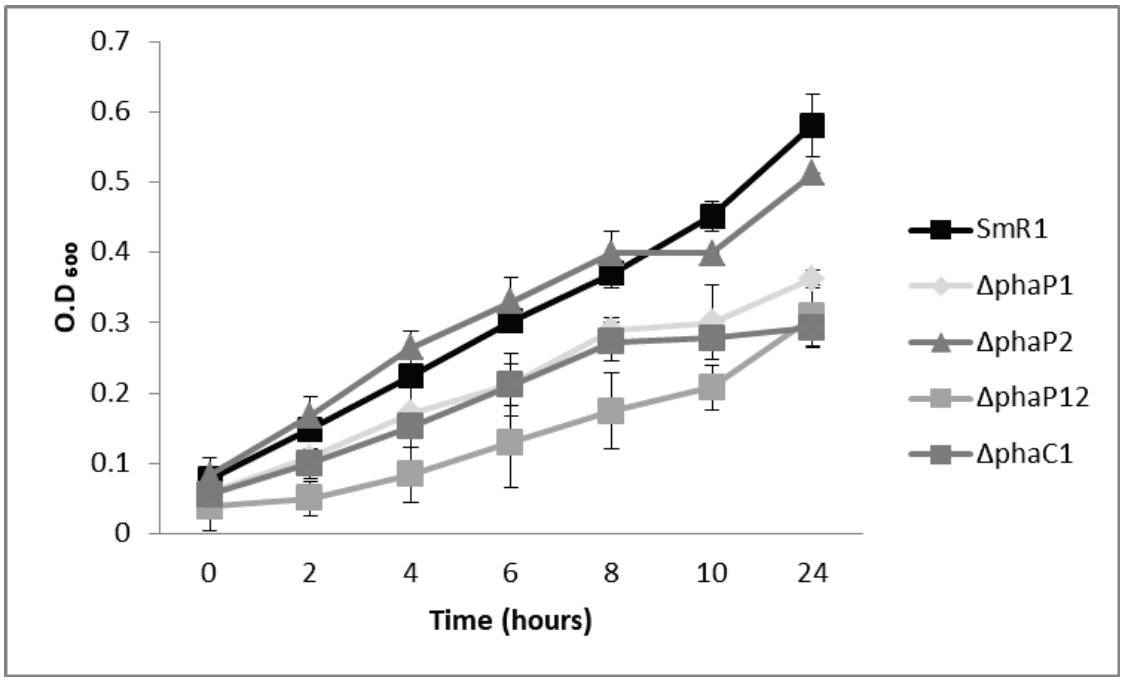

Figure 4. Growth profiles of *H. seropedicae* SmR1 (parental strain) and the mutants Δ*phaP1*, Δ*phaP2*, Δ*phaP1.2,* and Δ*phaC1* under the low-oxygen regime.

Strains were grown in NFb medium with 20 mM of ammonium chloride and 37 mM DL-malate, at 30°C (orbital agitation at 100 rpm) in glass flasks wholly filled with medium and closed with rubber caps.

3.4. Unlike bacterial plant growth promotion, the level of bacterial colonization is not significantly affected in mutants defective in PHB metabolism

The lack of effective plant growth promotion by *H. seropedicae* strains defective in PHB metabolism could be due to direct effects on bacterial colonization. To examine this possibility *S. viridis* A10.1 plants were inoculated with *H. seropedicae* SmR1 and mutant strains. Both epiphytic and endophytic colonization were measured by plate counting. Regardless of which strain was inoculated, there were no significant differences in root colonization (epiphytic or endophytic) when the bacteria were recovered from roots of *Setaria viridis* after 25 days of the inoculation (Fig. 5 and 6). These results are consistent with previous studies comparing root colonization in maize and wheat by *A. brasilense* Sp7 wild-type and PHB defective mutant *phbC* when the authors described no significant differences in root colonization of both strains (Kadouri et al., 2003).

Similar results were reported by Balsanelli et al. (2015), who assayed colonization of maize roots and no significant differences were determined three days after inoculation with *H seropedicae* SmR1 and Δ*phaC1*.

As a control in our experiments, we also inoculated plants with the *H. seropedicae* Δ*fliA* mutant, which is a flagellum deficient and nonmotile strain. This strain was used since it is unable to endophytically colonize plants, which can be seen in Figure 6. It is worth noting that the Δ*fliA* mutant was also defective in plant growth promotion (Figures 1 and 2), but it is unclear whether this can be directly attributed to the lack of endophytic colonization by this strain.

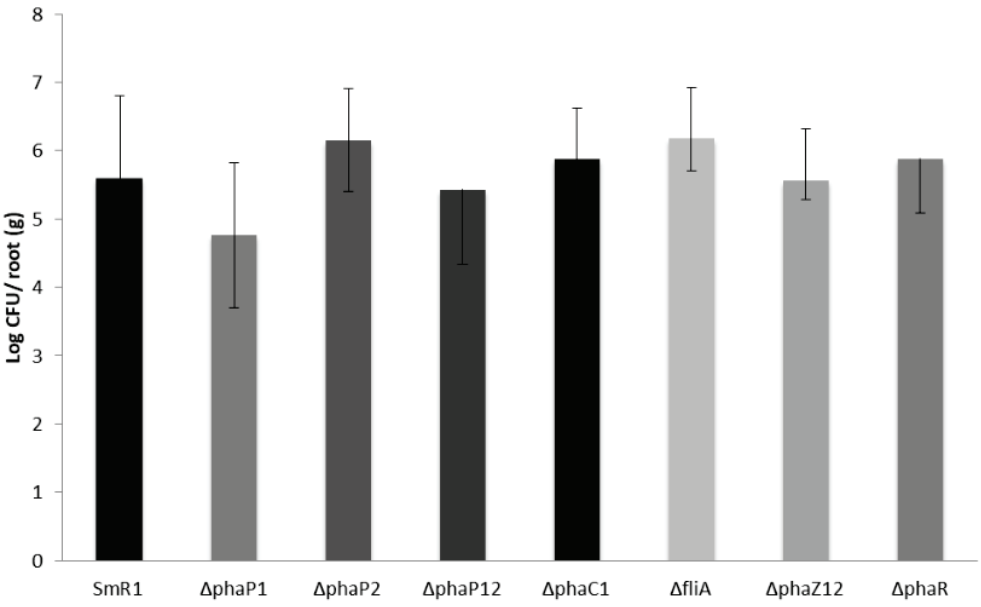

Figure 5. Epiphytic colonization in Setaria viridis A.10 roots inoculated with *Herbaspirillum seropedicae* SmR1 and PHB metabolism mutants.

Note that there was no significant difference in colonization for the various strains tested. All bars are mean values ±. SE (standard error).

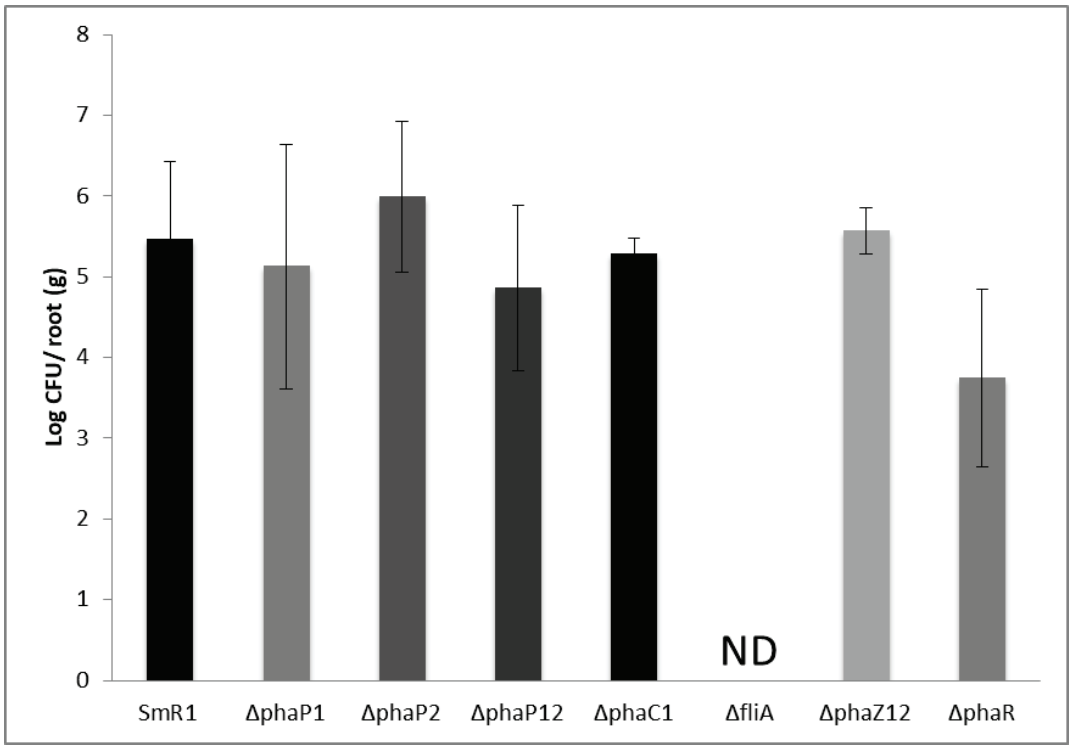

Figure 6. Endophytic colonization in *Setaria viridis* A.10 inoculated with *Herbaspirillum seropedicae* SmR1 and PHB metabolism mutants.

Note that there was no significant difference in colonization for the various strains tested, except for Δ*fliA*, which is unable to colonize the roots endophytically. All bars are mean values ±. SE (standard error).

3.5. Monitoring PHB genes expression in *H. seropedicae* SmR1 colonizing *Setaria viridis* roots

The data above suggests that PHB production by *H. seropedicae* associated with the plant roots is important for their ability to promote plant growth, although it does not significantly impact total root colonization. However, this conclusion assumes that PHB is actually produced by bacteria either in the rhizosphere or on the rhizoplane. To demonstrate such production, as well as the capacity to utilize PHB in association with plant roots, we constructed *H. seropedicae* strains expressing GFP as a reporter under the control of the endogenous promoters of the genes *phaC1, phaZ1* and *phaZ2*. *S. viridis* roots were inoculated with each of these strains, and GFP expression monitored over time (i.e., 1 to 15 days post-inoculation).

As shown in Figure 7, expression of GFP in *H. seropedicae* cells colonizing *S. viridis* roots could be seen with the P*phaC1*-GFP fusion 4 days after inoculation. The images show bacteria forming microcolonies localized in the intercellular spaces of *S.*

viridis root tissue. On the other hand, significant expression by the strains expressing the P*phaZ1*-GFP or P*phaZ2*-GFP fusion was only detectable 10 days after inoculation (Fig. 8 and 9).

Interestingly, GFP expression by the *phaC1*-GFP fusion was not detectable beyond 4 days after inoculation, while GFP expression by the *phaZ1-GFP* and *phaZ2-GFP* fusions was only detectable 10 days after inoculation. The most straightforward explanation for these results is that PHB is synthesized early during bacterial colonization and, under the plant growth conditions used in our study, being mobilized much later, presumably when the bacteria are under stress. However, of primary importance to the current study is that the gene fusion results support the notion that PHB is indeed made by *H. seropedicae* colonizing *S. viridis* roots, consistent with a key role of this polymer in the ability of the bacteria to colonize plants and promote their growth. Uninoculated *S. viridis* roots showed low green fluorescence intensity as can be observed in the Supplementary Figure 1.

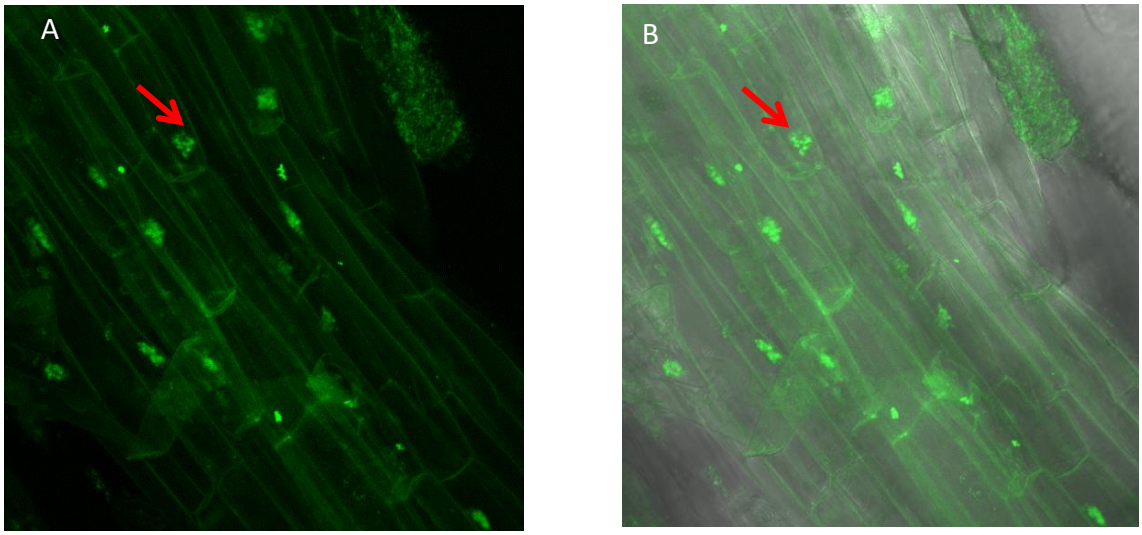

Figure 7. GFP expression from a phaC1-GFP fusion in *H. seropedicae* SmR1, colonizing the *S. viridis* root surface.

Roots of 4-days-old plants were removed, washed and processed to the confocal microscopy analysis. Arrows indicate colonies of *H. seropedicae*. A- GFP fluorescence image; B- Merged images.

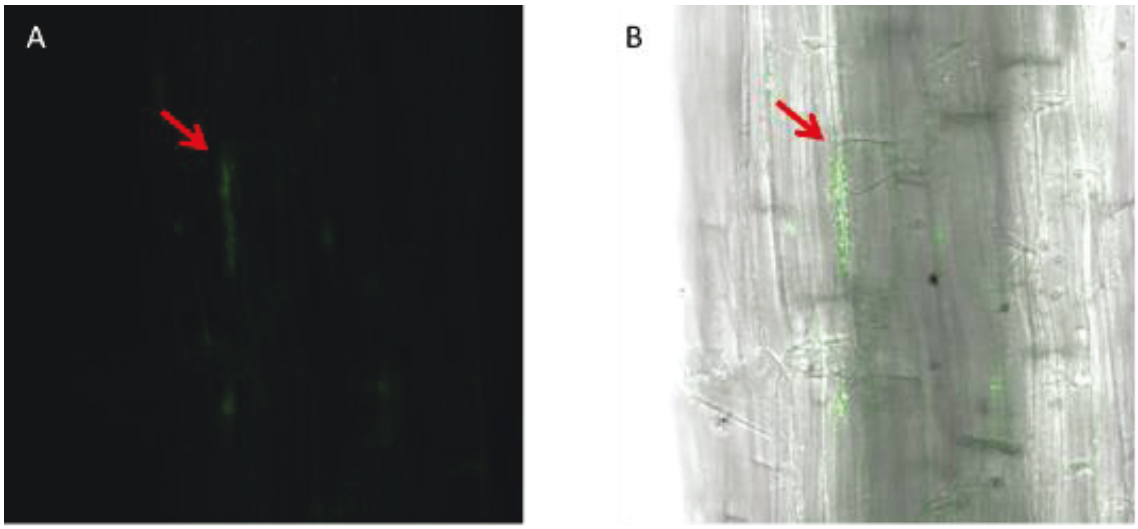

Figure 8. GFP expression from a *phaZ1*-GFP fusion in *H. seropedicae* SmR1, colonizing the *S. viridis* root surface.

Roots of 10-days-old plants were removed, washed and processed to the confocal microscopy analysis. Arrows indicate colonies of *H. seropedicae*. A- GFP fluorescence image; B- Merged images.

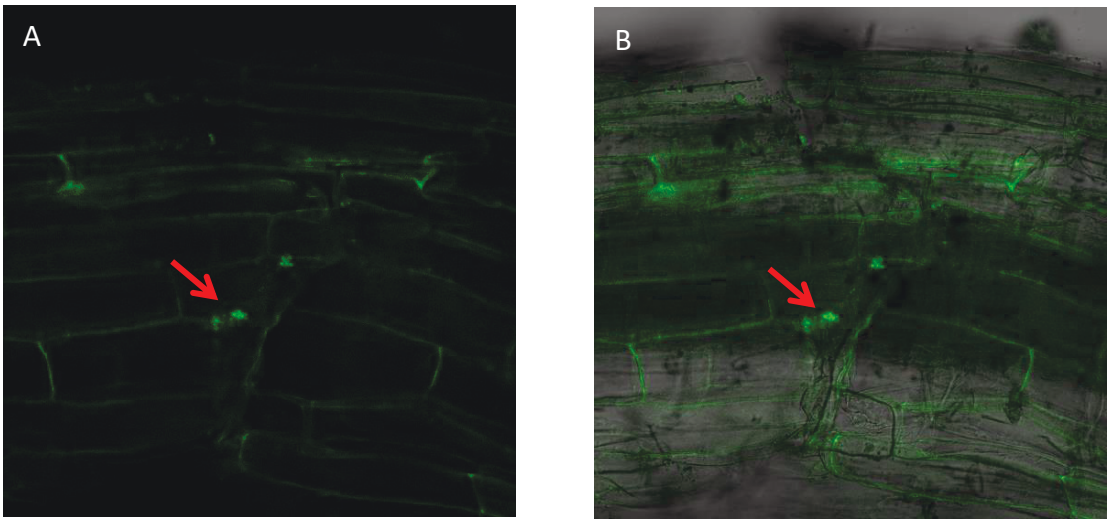

Figure 9. GFP expression from a *phaZ2*-GFP fusion in *H. seropedicae* SmR1, colonizing the *S. viridis* root surface.

Roots of 10-days-old plants were removed, washed and processed to the confocal microscopy analysis. Arrows indicate colonies of *H. seropedicae*. A- GFP fluorescence image; B- Merged images.

Discussion

Previous works demonstrated that *Herbaspirillum seropedicae* could colonize *Setaria viridis* A.10 (Pankievicz et al., 2015). Thus we challenged this bacteria and mutants involved in PHB metabolism to see the effects of this polymer in plant growth promotion in *Setaria viridis* A.10. After 25 days of inoculation, we analyzed the phenotypes of plant growth for the different strains employed in the experiment. Some strains producing the high amounts of PHB may colonize the roots adequately, thus increasing the root area and number of lateral roots significantly compared with the strains PHB negative. These findings are according with other studies relating that many PHB-producing bacteria may colonize the roots and promote plant growth such as *Azospirillum brasilense,* which is used in field experiments with maize and wheat in South America (Dobbelaere et al., 2001; Helman et al., 2011). Likewise, the bacteria that produce and store PHB might improve the cell division, survival and the competition against stress factors or competitors, due to energy release through PHB reserve mobilization when required (Okon & Itzigsohn, 1992).

To verify the influence of PHB mobilization during the colonization, we tested mutant Δ*phaZ1.2* in *S. viridis* A.10. Alves (2018) showed this mutant did not survive 30 min of heat shock at 45°C, due to lacking PHA depolymerases and consequently no PHB mobilization. Probably, this mutant is unable to find an energy source to resist against the thermal stress. Similar results were observed by Ruiz et al. (2001) challenging the *Pseudomonas oleovorans* wild-type and a *phaZ* mutant at 47°C. Therefore, we did not observe a better root development after Δ*phaZ1.2* inoculation in *S. viridis*. This result corroborates some previous works suggesting that PHB mobilization is a relevant feature to increase the root surface area and then uptake of minerals from soil (Okon & Kapulnik, 1986; Fallik et al. 1996; Okon & Vanderleyden 1997; Burdman et al. 2000; Steenhoudt & Vanderleyden 2000).

Additionally, to address whether PHB synthesis and mobilization are active during *S. viridis* colonization, we tracked GFP expression under the control of *phaC1*, *phaZ1* and *phaZ2* promoter in roots of *S. viridis*. Both processes are potentially taking place in plant roots, but there was a temporal separation, whereas *phaC1* expression was highly expressed until the 4th day after inoculation and *phaZ1* and *phaZ2* started to be expressed after the 10^{th} of inoculation.

Our results are according to Pankievicz et al. (2015), which showed a 1.9-fold increase of *phaC1* mRNA levels in *H. seropedicae* SmR1 colonizing wheat roots. Nevertheless, we observed that the genes were expressed in different days; probably at the beginning of colonization *H. seropedicae* found a condition favoring PHB synthesis and accumulation. At long times after inoculation, the starvation of nutrients (e.g., a metabolizable carbon source) or stress factors such as osmotic or oxidative shock led to PHB mobilization and persistence of the bacteria inside the roots. This result agrees with Lodwig et al., (2005), which reported that the accumulation of PHB by the bacteria prepared it for plant infection and increased bacterial differentiation during colonization. Furthermore, Koskimäki et al. (2016) demonstrated that *Methylobacterium extorquens* produced 3-hydroxybutyrate oligomers during the colonization of *Pinus sylvestris* roots. The oligomers were products of PHB mobilization and had a potent antioxidant activity against hydroxyl radicals. Such mechanism of protection may also be present in *H. seropedicae* since in the mutant Δ*phaC1* was already reported a higher ROS level (Teixeira, 2015).

Other works showed that low oxygen levels jointly with high carbon source availability induced lipogenesis and PHB synthesis. For instance, *Azotobacter beijerinckii* produces PHB to adapt to the low-oxygen environment on the legume root nodule (Senior & Dawes et al., 1972). In our results, only parental strain and Δ*phaP2,* which have a significant amount of PHB granules inside the cell, can grow under low oxygen regime. In contrast, the other mutants affected somehow in PHB metabolism presented a growth penalty in low oxygen regime. These results corroborate with the root development data, which it was clear that strains that fail to accumulate or degrade the polymer are unable to assist in the development of the root. In conclusion, the PHB cycle (referring to the synthesis and mobilization of PHB) is critical for *H. seropedicae* to colonize and increase the root area, which largely contributes to nutrient uptake and plant development.

4 REFERENCES

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5 Supplementary Material

5.1 Supplementary Figure 1.

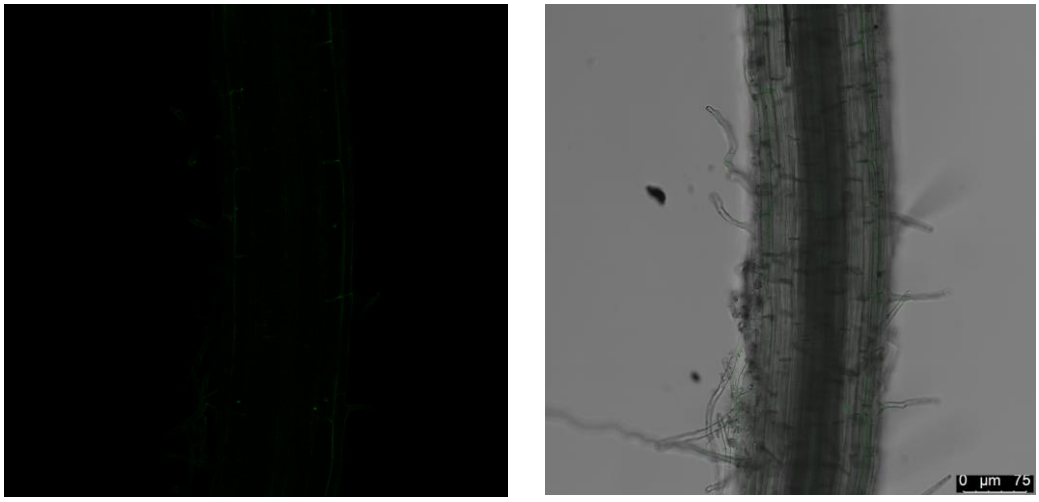

Figure 10. Negative control. Uninoculated S. viridis roots showed low green fluorescence intensity.

CONSIDERAÇÕES FINAIS

Os resultados deste trabalho de doutorado permitiram que se conhecesse ainda mais sobre metabolismo de PHB em *Herbaspirillum seropedicae.* A influência da produção e da mobilização do polímero durante estresse térmico e na sobrevivência da bactéria. A atuação de cada uma das fasinas na síntese do grânulo de *H. seropedicae* e a maneira como age o regulador transcricional das fasinas, PhaR, bloqueando a transcrição dos genes destas proteínas.

Neste trabalho foi realizado ainda a otimização de um protocolo para quantificação de PHB usando o citometro de fluxo. Desta forma, isto contribui significantivamente nos trabalhos que necessitam de uma rápida e eficiente determinação da quantidade de PHB em amostras de culturas de bactérias. Além disto, nesta parte deste trabalho foi possível mensurar PHB a partir de células epifíticas ligadas as raízes de arroz.

A última parte deste trabalho, a qual refere-se ao capítulo IV, foi realizada na Universidade do Missouri nos Estados Unidos da América. Neste capítulo foi possível explorar a importância do metabolismo de PHB na interação planta-bactéria. Os resultados mostram uma significância no aumento do desenvolvimento da raiz em plantas que foram inoculadas com bactérias produtoras de PHB. Isto nos leva a crer que o metabolismo de PHB está ativo durante a interação com a planta e que o polímero tem relevância não somente para a bactéria como também para a planta.

De maneira geral, todos os resultados apresentados nesta tese nos levam a acreditar que o polihidroxibutirato tem influência direta na fisiologia da bactéria e também com a interação em plantas. Portanto, neste trabalho conseguimos ampliar e aprofundar ainda mais nossos conhecimentos sobre o assunto, permitindo, assim, que futuros trabalhos sobre a investigação do metabolismo do PHB possam ser explorados ainda mais.

CONCLUSÕES

Capítulo I

- Quantificação rápida e confiável de PHB, através do uso da técnica de citometria de fluxo em cultura de células de *H. seropedicae* e *A. brasilense* ou isoladas de raízes de plantas.

- Produção de PHB pode ser quantificada com o uso da sonda Nile Red.

Capítulo II

- 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.

Capítulo III

- Estirpes que produzem maior quantidade do polímero possuem uma sobrevivência maior durante o choque térmico.
- PHA depolimerases são importantes para a sobrevivência de *H. seropedicae,* pois permitem a mobilização do grânulo durante o estresse.
- Síntese e mobilização do PHB protegem a bactéria do choque térmico.
- Fator sigma S (RpoS) pode estar envolvido na regulação dos genes *phaZ1* e *phaZ2*.
- Liberação de 3-hidroxibutirato é importante para *H. seropedicae* mitigar o estresse.

Capítulo VI

- Desenvolvimento da raiz de *S. viridis* A.10 aumenta quando colonizada por estirpes que produzem PHB em grande quantidade.
- Mobilização do PHB é relevante para aumentar a área da raiz.
- Em condições com baixos níveis de oxigênio, bacterias produtoras de PHB conseguem se adaptar melhor.
- O início da colonização favorece a síntese de PHB.
- Em estágios mais tardios de colonização há a mobilização do grânulo.

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