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CONSTRUÇÃO DE MUTANTES DE *Pseudomonas* ABRIGANDO DIFERENTES PHA SINTASES EM SEU GENOMA, PARA PRODUÇÃO DE 3HB-*co*-3HA$_{MCL}$

Dissertação apresentada ao Programa de Pós-Graduação em Microbiologia do Instituto de Ciências Biomédicas da Universidade de São Paulo, para obtenção do Título de Mestre em Ciências.

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RESUMO

OLIVEIRA, E. R. **Construção de mutantes de *Pseudomonas* abrigando diferentes PHA sintases em seu genoma, para produção de 3HB-*co*-3HA$_{MCL}$.** 2016. 114 f. Dissertação (Mestrado em Microbiologia) – Instituto de Ciências Biomédicas, Universidade de São Paulo, São Paulo, 2016.

Polihidroxialcanoatos (PHA) são biopolímeros naturalmente produzidos e acumulados por diversos organismos, como bactérias, archaeas e alguns eucariontes, como fungos e leveduras. São materiais termoplásticos, biodegradáveis, biocompatíveis e podem ser produzidos a partir de fontes renováveis, exibindo grande potencial para substituir plásticos produzidos a partir de recursos não renováveis. Copolímeros híbridos de PHA, que podem ser formados por monômeros de cadeia curta e média, como P(3HA$_{SCL}$-*co*-3HA$_{MCL}$), apresentam características físico-químicas diferenciadas, semelhantes às dos plásticos derivados de petróleo, sendo por isso interessantes para a indústria de materiais. A PHA sintase é considerada a enzima chave na síntese de PHA, responsável por catalisar a polimerização de diferentes monômeros de (*R*)-hidroxiacil-CoA, influenciando a composição monomérica do polímero formado. Sistemas de recombinação baseados em transposons bacterianos são explorados como ferramentas moleculares para inserção de sequências gênicas no cromossomo de bactérias Gram-negativas. Por exemplo, elementos mini-Tn*7* podem ser prontamente transferidos para a construção de cepas recombinantes. No presente trabalho, é apresentada a construção de diferentes linhagens recombinantes a partir de *Pseudomonas* sp. LFM046 e LFM461, abrigando em seus cromossomos genes de PHA sintase de *Ralstonia eutropha*, *Aeromonas hydrophila* ou *Aeromonas* sp. TSM 81. Clones candidatos foram triados quanto a inserção das sequências de interesse em seu cromossomo, sendo os positivos avaliados em relação à capacidade de produção de PHA em ensaios em agitador rotativo, com glicose como única fonte de carbono. Um dos recombinantes obtidos se mostrou produtor do copolímero P(3HB-*co*-3HHx-*co*-3HD), acumulando aproximadamente 2% de sua massa seca celular na forma de PHA.

Palavras-chave: *Pseudomonas* sp. PHA sintase. Polihidroxialcanoatos. Biopolímeros. Mini-Tn*7*.

ABSTRACT

OLIVEIRA, E. R. **Construction of recombinant *Pseudomonas* strains harboring different PHA synthases in its genome to produce 3HB-*co*-3HA$_{MCL}$.** 2016. 114 p. Masters thesis (Microbiology) – Instituto de Ciências Biomédicas, Universidade de São Paulo, São Paulo, 2016.

Polyhydroxyalkanoates (PHA) are biopolymers naturally produced and accumulated by many organisms such as bacteria, archaeas and some eukaryotes, such as fungi and yeasts. As thermoplastics, biodegradable, biocompatible and possibly made from renewable resources, they exhibit great potential to replace oil-derived plastics. Hybrid PHA copolymers can be formed by short and medium-chain monomers, P(3HA$_{SCL}$-*co*-3HA$_{MCL}$), and present industry desired physicochemical properties, becoming similar to conventional oil-based plastics. PHA synthase is the key enzyme in PHA biosynthesis, responsible for catalyzing the polymerization of (*R*)-hydroxyacyl-CoA molecules, influencing polymer monomeric composition. Tn*7*-based recombination strategies represent powerful molecular tolls designed for gene delivery in Gram-negative bacteria, as mini-Tn*7* elements can be readily transferred to recombinants production. In this work, its presented the constructions of recombinant *Pseudomonas* strains harboring PHA synthase genes from *Ralstonia eutropha* and *Aeromonas* strains in specific sites of its chromosome, and the production of P(3HB-*co*-3HHx-*co*-3HD). Obtained clones were screened to confirm chromosomic insertion of the *phaC* sequences. Positive clones PHA production and composition were evaluated in shaken-flasks assays using glucose as the only carbon source. One of the constructed recombinants accumulated P(3HB-*co*-3HHx-*co*-3HD), corresponding about to 2% of its Cell Dry Weight as PHA.

Keywords: *Pseudomonas* sp. PHA synthase. Polyhydroxyalkanoates. Biopolymers. Mini-Tn*7*.

1 INTRODUÇÃO

A sociedade moderna é dependente do uso de materiais plásticos em grandes quantidades. Plásticos são usados em embalagens e diversos produtos descartáveis, assim como em produtos de aplicações nobres, como fios de sutura, próteses e outros insumos cirúrgicos, por exemplo. Devido a seu uso em grandes quantidades, a sua difícil degradação no meio ambiente e dificuldades em seu processo de reciclagem, esse material está se acumulando, poluindo rios, praias e outros ambientes. Como alternativa ao uso de plásticos convencionais, derivados do petróleo, diversas famílias de bioplásticos vêm sendo estudadas, como as poliaminas, policaprolactonas, polilactato, guta-percha e polímeros da família dos polihidroxialcanoatos (PHA).

Neste contexto, os PHA, produtos naturais, produzidos e acumulados por diversas espécies bacterianas, dentre outros organismos, como reserva de energia, na forma de grânulos intracelulares, se destacam. Esses biopolímeros são biocompatíveis, naturalmente degradados por organismos no meio ambiente quando em condições favoráveis (pH, umidade, temperatura adequados, entre outros), e podem ser produzidos a partir de fontes renováveis, como resíduos agroindustriais e urbanos.

A síntese dos PHA é influenciada por três fatores principais: vias metabólicas presentes nas bactérias produtoras, tipo de PHA sintase, considerada a enzima chave no metabolismo de PHA, e da fonte de carbono fornecida durante a fase de acúmulo. De acordo com o substrato fornecido, a bactéria dará origem a diferentes moléculas de 3-hidroxiacil-CoA, o que depende das vias metabólicas que apresenta. A incorporação ou não desses monômeros ao polímero nascente depende da especificidade da enzima PhaC que a bactéria apresenta.

A partir disso, dois principais grupos de monômeros de PHA foram definidos: monômeros de cadeia curta, formados por 3-hidroxiacil-CoA contendo de 3 a 5 átomos de carbono (HA_{SCL}), sendo o poli-3-hidroxibutirato P(3HB), seu principal representante, e monômeros de cadeia média, formados por 3-hidroxiacil-CoA contendo de 6 a 14 átomos de carbono em sua composição (HA_{MCL}). Polímeros compostos apenas por monômeros de cadeia curta são pouco elásticos, enquanto os compostos apenas por monômeros de cadeia média são elastoméricos. Apesar do P(3HB) ser o PHA mais bem estudado, é um material muito quebradiço, fator que limita muito suas aplicações. Copolímeros híbridos de PHA, contendo em sua composição polimérica monômeros de cadeia curta e média, possuem características físicas e mecânicas intermediárias, consideradas semelhantes a certos plásticos petroquímicos, como polietileno de baixa

densidade, por exemplo. Assim, a diversificação de sua composição pode aumentar a sua gama de aplicações na indústria de materiais, sendo o desenvolvimento desses copolímeros alvo de diversas patentes recentes.

Algumas linhagens bacterianas são capazes de produzir naturalmente copolímeros híbridos, mas ainda é necessário desenvolver bactérias recombinantes capazes de produzi-los de forma mais eficiente ou a partir de apenas uma fonte de carbono ou até mesmo a partir de resíduos.

Pseudomonas sp. LFM046, linhagem que foi isolada em solo de canavial no estado de São Paulo, é considerada boa produtora de PHA contendo apenas monômeros de cadeia média a partir de glicose e frutose. Essa linhagem, que teve seu genoma recentemente sequenciado e analisado, sendo considerada não patogênica, é alvo de diversos estudos relacionados à biossíntese de PHA e análise de fluxos metabólicos. A partir dela, já foram gerados diversos mutantes, entre eles LFM461, deficiente no acúmulo de PHA a partir de glicose e octanoato.

Para possibilitar a produção de copolímeros híbridos a partir de glicose, estratégias envolvendo a inserção de genes de PHA sintase no cromossomo de *Pseudomonas* LFM046 e LFM461 foram adotadas. Genes de PHA sintase de classe I, proveniente de *Ralstonia eutropha* (linhagem modelo na produção de PHA) e de duas espécies de *Aeromonas*, capazes de produzir P(3HB-co-3HHx) a partir de óleos vegetais (substratos mais caros que açúcares), porém com baixo rendimento, foram usados. A inserção no cromossomo resultou em linhagens estáveis, com apenas uma cópia do gene de interesse e dispensa a necessidade de seleção contínua por antibióticos, características de grande interesse industrial.

6. CONCLUSÕES E PERSPECTIVAS FUTURAS

6.1 Conclusões

a. O sistema de recombinação sítio específica por meio do transposon mini-Tn*7* se mostrou eficiente nas linhagens *Pseudomonas* sp. LFM046 e LFM461. Nesta etapa, foi estabelecido no Laboratório de Bioprodutos metodologia de inserção de sequências gênicas no DNA cromossômico de *Pseudomonas*;
b. A inserção de gene de PHA sintase de *Ralstonia eutropha* em LFM461 reestabeleceu a capacidade de acúmulo de PHA, mas não resultou no acúmulo dos copolímeros mistos desejados;
c. A inserção cromossômica do gene de PHA sintase de *Aeromonas* sp. TSM81 não reestabeleceu a capacidade de acúmulo de LFM461. Fatores relacionados à baixa expressão das sequências gênicas inseridas podem explicar o resultado observado;
d. Foram produzidos copolímeros mistos quando o gene de PHA sintase de *A. hydrophila* ATCC 7966 foi inserido em LFM046. O mesmo foi observado em LFM461, sendo sua capacidade de acúmulo reestabelecida, porém a quantidade de polímero acumulada ainda é muito baixa quando comparada coma linhagem selvagem LFM046 (aproximadamente 4,5% da capacidade de acúmulo de LFM046).

6.2 Perspectivas futuras

6.1.1 Avaliação das capacidades de acúmulo das linhagens recombinantes obtidas via mini-Tn5

As linhagens obtidas a partir de *Pseudomonas* sp. LFM461 já tiveram confirmada a inserção do gene *phaC* de *Ralstonia eutropha* em seu cromossomo. Ensaios de coloração com Sudan Black B serão realizados no Laboratório de Bioprodutos, de modo a selecionar os clones capazes de acumular PHA.

6.1.2 Construção de outras linhagens recombinantes de Pseudomonas sp. LFM461 por meio do sistema mini-Tn5

Sendo confirmadas como positivas as análises de quantidade e composição de PHA obtidas com a linhagem construída usando o sistema mini-Tn*5*, esse sistema (agora disponível no Laboratório de Bioprodutos nos vetores pBAMD) se mostra promissor para futuros estudos de inserção das demais sequências de PHA sintase estudadas neste trabalho (*Aeromonas hydophila* e *Aeromonas* sp. TSM81), assim como outros genes relacionados ao metabolismo de PHA.

6.1.3 Publicações em andamento

Com os dados obtidos neste projeto está sendo elaborado um artigo científico, cujo título será "**Production of hybrid PHA copolymers by a recombinant *Pseudomonas* strain harboring an *Aeromonas hydrophila* PHA synthase gene in its chromosome**", sendo os autores Edmar Ramos de Oliveira Filho, Linda P. Guamán, Thatiane Teixeira Mendonça, Marilda Keico Taciro, José Gregório Cabrera Gomez e Luiziana Ferreira da Silva.

O levantamento de literatura bem como a atualização bibliográfica realizados para o desenvolvimento deste projeto gerou um artigo de revisão sobre PHA sintases, que está em fase final de correção para ser enviado ao periódico *Biotechnology Advances*. O título da publicação será "**PHA synthase as a key-feature in biotechnological advances and future prospects to develop new PHA as bioproducts**", sendo os autores Edmar Ramos de Oliveira Filho, Thatiane Teixeira Mendonça, Gabriela Cazonato Lozano-Sakalauskas, Aline Carolina da Costa Lemos, José Gregório Cabrera Gomez e Luiziana Ferreira da Silva.

As folhas de rosto dos artigos estão disponíveis no Apêndice C.

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