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BIOSURFACTANT SYNTHESIS IN THE OIL EATER RHODOCOCCUS ERYTHROPOLIS MK1 STRAIN

Krisztán Laczi, Á. Kis, K.L. Kovács, G. Rákhely and K. Perei

Deparment of Biotechonology, University of Szeged, Közép fasor 52. H-6726 Szeged, Hungary
Mail: perei@brc.hu

ABSTRACT

Oil pollution is a very serious problem in the world. There were numerous oil spills in the last three decades and had great impact on the environment. They caused damages in wildlife as well as in economy by cutting down the agriculture, fishing, and tourism.

Surfactants are useful weapons in the war against oil pollution. They are suitable to clean oil tanks and pipes and they are useful to solublize animal fats in food industrial wastewater. Many bacteria can produce substantial amount of biosurfactants which can emulsify hydrophobic hydrocarbons, so that the native microflora can utilize the pollutants. An additional advantage of the biosurfactants over the synthetic surface active molecules is that these compounds are easily biodegradable.

A special biosurfactant group is composed of mycolic acids which are basically α-alkyl, β-hydroxy fatty acids. Mycolic acids are the most characteristic components of the cell wall of the so called mycolata bacterial group. This group belongs to the Actinomycetales and contains the genera *Mycobacterium*, *Corynebacterium*, *Nocardia*, *Rhodococcus* and others.

We aimed to map the mycolic acid biosynthesis pathway in *Rhodococcus erythropolis* MK1 strain isolated by us from polluted soil. In first step, we sequenced the genome of our strain by SOLiD™ next generation DNA sequencer. The reads were mapped on the *R. erythropolis* PR4 genome in the NCBI database. We searched for rhodococcal homologs of the known mycobacterial and corynebacterial genes involved in mycolic acid biosynthesis. We found conserved regions in the genome which are likely responsible for the biosynthesis of mycolic acids. The ongoing comparative whole genome transcript analysis will reveal the genes really necessary for the anabolism of mycolic acids.

INTRUDUCTION

Marine and terrestrial oil spills occurred all around the world in the last few decades. Oil has a great impact on wildlife. It covers animal's skin, spreads on surface of the water obstructing it from the air. Crude oil contains many toxic compounds that are harmful for human and animal health and plants. Oil pollution influences plant life by inhibiting seed germination, reducing photosynthetic pigments, slowing down nutrient assimilation and shortening roots and other organs (S. Peng et al. 2009).

There are many bacteria that could utilize some compounds of oil. However, the first big problem for them is the insolubility of oil in water. Some microorganisms synthesize surface active molecules so-called biosurfactants. These molecules lower the surface tension of the interface and emulsify oil enhancing its bioavailability for the cells. Biosurfactants can also

enhance desorption of insoluble materials from surfaces. Bioemulsifiers have many advantages compared to the synthetic ones. For example they are biodegradable and consequently environmental friendly. These bioemulsifiers are suitable for many industrial purposes, such as bioremediation or solubilization of hydrophobic (waste) materials. It was shown, that addition of biosurfactants to the polluted soil or body of water could enhance utilization of oil compounds by the local bacterial flora. (E.Z. Ron. et al 2002). Biosurfactants with low critical micelle concentration and high degree of sorption to soil enhance soil washing efficiency (K. Urum et al 2004).

One group of biosurfactants are mycolic acid (MA) containing products. Basically, MAs are α-alkyl, β-hydroxy fatty acids of variable chain length depending on the source. Mycolic acids are attached to the arabinogalactan cell wall or they can be found in free form as trehalose-dimycolates. The biosynthesis of mycolic acids is studied recently in *Mycobacterium spp.* (E. Raffidinarivo et al. 2009, S. Gavalda et al. 2009) and *Corynebacterium spp.*(R. Gande et al. 2004). Some key enzymes and reactions of the biosynthetic pathway of MA are known. The first key step is the synthesis of the C_{20} fatty acid catalyzed by an eukaryotic like fatty acid synthase I (FASI) enzyme. The C_{20} fatty acid chain is further elongated by the fatty acid synthase II (FASII) enzyme system which synthesizes the meroacyl chain. When the appropriate chain length was synthetyzed, the meroacyl chain is attached to a C_{22-26} fatty acid by Claisen type condensation. This synthesized MA is attached to a trehalose headgroup by a mycolil transferase and transported to the periplasmic space. In the periplasmic space further modifications are carried out by the periplasmic mycolil transferases (Takayama et al. 2005).

The goal of this project is to map the mycolic acid biosynthesis pathway in *Rhodococcus erythropolis* MK1 strain.

The *R. erythropolis* is gram positive, mesophilic, ubiquitous bacterium which can be found worldwide from deep seas to the mountains. The rhodococci are closely related to the genera *Mycobacterium* and *Corynebacterium* but not so pathogenic..This bacterium can grow on various hydrocarbons, emulsifying them with trehalo-mycolates. Our strain is able to grow on hexadecane, fuel/heavy oil, refuse oil and animal fat as carbon source. The cells form aggregates at the interface of water and organic phase with cell wall bound mycolic acids.

The first step of this project was to sequence the genome of *R. erythropolis* MK1 with SOLID™ next generation sequencer.

MATERIALS AND METHODES

Bacterium and growth conditions

Rhodococcus erythropolis MK1: This strain was isolated by us from polluted soil.
The culture was grown aerobically in 30 ml Luria-Bertani medium (5g yeast extract, 10g Trypton and 10g NaCl/L) at room temperature with shaking at160 rpm until OD_{600}=0.7.

Purification of genomic DNA

Genomic DNA was purified from 30 ml of *R. erythropolis* MK1 strain cultured in LB medium by phenol-chloroform method as follows. After the culture reached OD_{600}=0.7 1mg/mL Ampicillin was added to the culture medium. The culture was shaked at 160 rpm at room temperature for 2 hours. After incubation. the culture was centrifuged at 13,000 rpm, 4°C for 10 minutes. The pellet was resuspended in 5ml 100µg/mL lyzozime solution. The suspension was aliquoted by 1mL in five 1.5 mL centrifuge tubes and incubated for 2 hours on 37 °C. After incubation, the cells were collected by centrifugation at 13,000 rpm, 4°C for 10 minutes. The

pellet was resuspended in 600µl Genomic I solution (10mM NaCl, 2mM Tris pH=8.0, 1mM EDTA and 0,5 w/v % SDS in each tube. 100 µg/ml proteinase K was added and the suspension was incubated on 50°C overnight. Then, it was phenol-chloroform (1:1) extracted The DNA was precipitataed by ethanol and air dried. The pellet was resuspenden in 50 µl DNA grade water. The quantity and quality of DNA was checked spectroscopically and on 1% agarose gel.

Whole genome sequencing
The whole genome sequencing was carried out by SOLID™ next generation DNA sequencer in the Bay Zoltán Foundation for Applied Research, Institute for Plant Genomics, Human Biotechnology and Bioenergy. The reads were mapped on the genome of *R. erythropolis* PR4 strain (AP008957) from the NCBI database.

BLAST search
We used NCBI BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to identify homologues of mycobacterial and corynebacterial genes involved in mycolic acid synthesis. BLAST searches were carried out using the default options.

RESULTS AND DISCUSSION

During the next generation sequencing of the *R erythropolis* MK1 over 9 million reads were generated. The results of the mapping showed that the reads cover 97.4 % of the reference sequence (*R. erythropolis* PR4). According to the BLAST searches, there are some regions in the *R erythropolis* MK1 genome which are likely responsible for the biosynthesis of mycolic acids.

The FasI system is responsible for the synthesis of C_{20}-S-CoA and $C_{22\text{-}26}$-S-CoA (Fig. 1). The Mycobacterial and Rhodococcal enzymes strongly resemble (~ 75 %) indicating similar biochemical reactions.

Moreover, the mycobacterial genes of FASII system are 70-80% identical to the corresponding rhodococcal genes. However, there is one major difference in the two FASII systems. In the mycobacterial genome there are two type II β-ketoacyl-ACP synthases (*kasA*) and *kasB*) but in the rhodococcal genome we found only one (*fabF*⇔ *kasA*). (Fig.1) This fact can serve as an explanation for the shorter meroacyl chains in *Rhodococcus*. According to the literature *kasB* is responsible for further elongation of long meroacyl chains. Rhodococcal *fabF* is more similar to *kasA* which gene is responsible for elongation of the shorter meroacyl chains.

The next key step of MA synthesis is the Claisen type condensation carried out by the polyketide synthase 13 (Pks13) and acetyl-CoA decarboxylases (AccDs). Two enzyme types are activating the two interacting molecules before the condensation. The FadD32 enzyme is a fatty acid AMP ligase that transfers the meroacyl chain from CoA to AMP. The other enzymes are AccD4 and AccD5 that carboxylate the $C_{22\text{-}26}$-S-CoA at position 2. The *pks13* gene of *Rhodococcus erythropolis* MK1 is 71% identical to the mycobacterial homologue. Similarly to the mycobacterial and corynebacterial gene organization, the *R. erythropolis* MK1 *fadD32* gene is also adjacent to the *pks13* gene which is followed by *accD4* gene (Fig. 2). Moreover, two mycolil transferase genes can be seen downstream of the *faD32* gene. These genes are *fbpA* and *fbpD* in *M. tuberculosis* and *csp1* and RER02160 in *R. erythropolis* MK1.

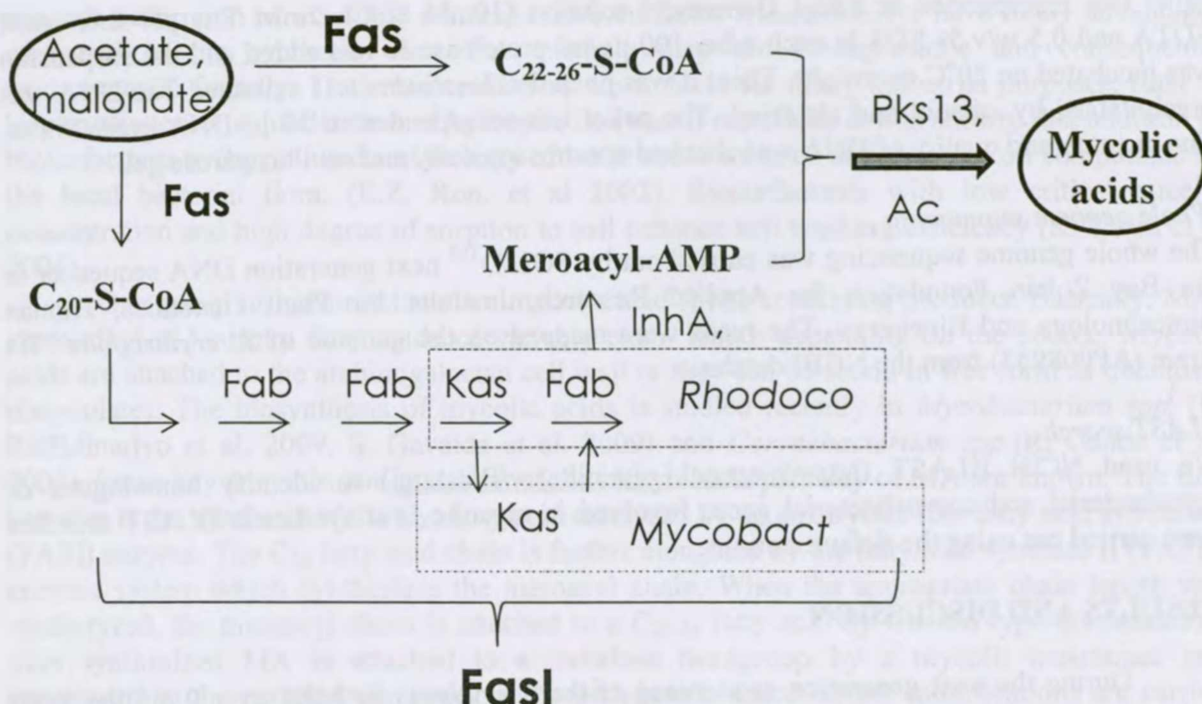

Figure.1 Mycolic acid biosynthesis pathway in mycobacteria and rhodococci

The *cps1* gene is likely homologue to *fbpA* and shows 50% similarity at amino acid level. The product of *fbpA* gene is the part of the so called antigen 85 complex, which is responsible for attaching two trehalose-monomycolates together or to the arabinogalactane cell wall in *M. tuberculosis*.

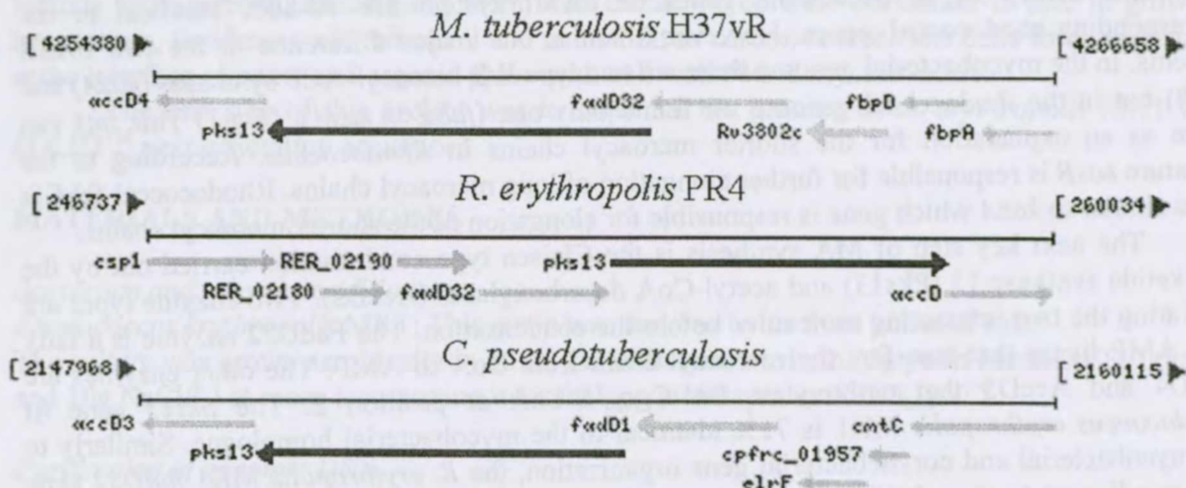

Figure 2. Genes involved in Claisen type condensation and mycolic acid processing (http:/www.ncbi.nlm.nih.gov)

CONCLUSION

Rhodococcus erythropolis is a useful tool for bioremediation of oil pollution and food industry waste waters. This organism synthesizes trehalomycolates, which are surface active agents of small molecular weight. These molecules are responsible for emulsification of oil and/or the cells can attach to the oil droplets via cell wall bound mycolates. According to BLAST results there are genes which are homologous to the known mycobacterial genes involved in MA synthesis. In addtion to the sequence resemblance, the genomic organization of the genes in these species are similar. We suggest that mycolic acid synthesis is carried out similar but not identical way as in *Mycobacterium tuberculosi*, which results in biosurfactant of special physicochemical properties.

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