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Systems Biology of the *Ralstonia eutropha* Polyhydroxyalkanoate Granule

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Systems biology examines organisms and processes by a holistic approach instead of the traditional reductionist approach. This can be beneficial to researchers and bioengineers as it allows for the formulation of the "big picture" where visualization of the influences and interactions of system components can occur. Significant amounts of data about the system are required, however, for this approach to be successful. Here, the polyhydroxyalkanoate (PHA) homeostasis process in Ralstonia eutropha is examined with a systems biology slant. Copious amounts of data exist about the enzymes, structural proteins and other players that make up the PHA production/mobilization process in R. eutropha. These data are utilized to create an interaction map of the PHA granule (and PHA homeostasis enzymes) that can inform PHA production strain engineering by providing information about key interactions.

Keywords: Ralstonia eutropha, polyhydroxyalkanoate, systems biology, bioplastic, cell growth, granules, enzymes.

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Системная биология полигидроксиалканоатных гранул *Ralstonia eutropha*

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Системная биология изучает организмы и процессы, используя целостный подход вместо традиционного редукционистского. Это может быть полезно для исследователей и биоинженеров, поскольку позволяет создать «общую картину», где может быть визуализировано взаимное влияние и взаимодействие компонентов системы. Однако для этого подхода требуется значительный объем данных о системе. В данной статье рассмотрен процесс гомеостаза полигидроксиалканоатов (ПГА) в Ralstonia eutropha с уклоном в системную биологию. Существует огромное количество данных о ферментах, структурных белках и других соединениях, которые включены в процесс синтеза/мобилизации ПГА в R. eutropha. Эти данные использованы для создания карты взаимодействия гранул ПГА (и ферментов гомеостаза ПГА), которая дает информацию для инженерии штамма, продуцирующего ПГА, обеспечивая данными о ключевых взаимодействиях.

Ключевые слова: Ralstonia eutropha, полигидроксиалканоаты, системная биология, биопластики, рост клеток, гранулы, ферменты.

Introduction

In recent years, many researchers have adopted a systems biology approach to studying key organisms and industrially relevant biocatalysts. Systems biology uses a holistic ("whole cell/organism") approach that focuses on the complex interactions within biological systems (Longo and Montevil, 2017). As a paradigm, systems biology is often thought of as opposite to the traditional reductionist method of biological study. We can consider it a way of "putting the organism back together," as opposed to dissecting it (Noble, 2018).

Nowadays, we can draw upon many different tools for systems biology study. Genomic, transcriptomic, proteomic, and other studies can generate a large amount of data about an organism that will undoubtedly aid in painting a holistic picture of the organism as a complete biological system. The rationale behind the approach is: No single data set can comprehensively define a cellular pathway or metabolic cycle, so consequently we can integrate multiple similar data sets. The endgame is for the integration of the data sets is to provide insight that is not attainable using one type of data set alone (Kumar, 2005). This can be valuable for studying industrial organisms that are frequently used as biocatalysts to produce value-added compounds. For example, there is a wealth of published literature on systems biology of *Saccharomyces cerevisiae* (see (Banos et al., 2017)) and *Escherichia coli* (see (Engstrom and Pfleger, 2017)) and other organisms. The data

presented in the cited works (and similar papers) give us a more complete picture of how each organism synthesizes value-added products and how cell growth and maintenance affects production.

The case for *Ralstonia eutropha*

R. eutropha (also known as *Cupriavidus necator*) is a model organism for polyhydroxyalkanoate (PHA) metabolism. This bio-based, biodegradable polyester is studied as a potential alternative in several applications to synthetic, petroleum-based plastics (Philip et al., 2007). In microorganisms, PHAs are synthesized as a response to environmental stress, namely non-carbon nutrient limitation. Wild-type *R. eutropha* typically produces the PHA homopolymer polyhydroxybutyrate (PHB), but it can be engineered to produce different, and more useful, PHA polymers (Budde et al., 2011; Sudesh et al., 2000). The amenability to genetic manipulation, along with the ability to produce up to 90% of its cell dry weight as PHA (Budde et al., 2011; Steinbüchel, 1991), makes *R. eutropha* a valuable chassis for potential industrial PHA production.

Systems biology study is possible for *R. eutropha*, given the increasing number of publications that provide large amounts of data on many different aspects of PHA homeostasis (i.e., the "PHA cycle"). Microarray analyses have been performed to demonstrate gene expression changes over the course of growth, PHA synthesis and PHA mobilization (Brigham et al., 2012a; Peplinski et al., 2010). Proteomic data from cells grown in various conditions have been analyzed (Schwartz et al., 2009). Also, metabolomic studies have been performed involving PHA production (Fukui et al., 2013). Furthermore, many classic works have been published detailing the interactions of different components of the PHA pathway. The data produced by these studies can be integrated to achieve a more detailed picture of the *R. eutropha* PHA homeostasis system.

Construction of an interaction map of the PHB granule of *R. eutropha* strain H16

In this work, the output of a systems biology approach to PHB homeostasis is an interaction map. Organisms like *R. eutropha* store the intracellular polymer in inclusion bodies known as granules. These granules are covered with different proteins, many of which are enzymes required for PHA homeostasis. The interaction map is type of output that is often used to illustrate signaling pathways (Kumar, 2005). In the case of PHB homeostasis, most of the interactions are not in the form of a signaling cascade, but as interactions that affect the ability of the cell to synthesize and mobilize the polyester, as well as maintain the structure of the granule. A diagram serving as a legend for the interaction map is shown in Fig. 1. Positive interactions can be defined by this example: the PHA synthase (PhaC) would have a positive interaction with PHB because it is the enzyme responsible for polymer synthesis. Negative interactions can then be exemplified by the interactions of the PhaZ1 depolymerase with the PHB polymer. In the PHB granule interaction map, it is important to illustrate known physical interactions between components. Note that, by design, the interaction map generated here does not focus on interactions with precursors and other small molecules.

For a systems biology approach to PHA granules, we enumerate and classify the interactions of the biosynthesis pathway enzymes. *R. eutropha* converts acetyl-CoA to 3-hydroxybutyryl-CoA (3HB-CoA) molecules, which are substrates for polymerization to make PHB. The enzymes involved in this bioconversion are β-ketothiolase (PhaA), acetoacetyl-CoA reductase (PhaB), and the PhaC enzyme. For

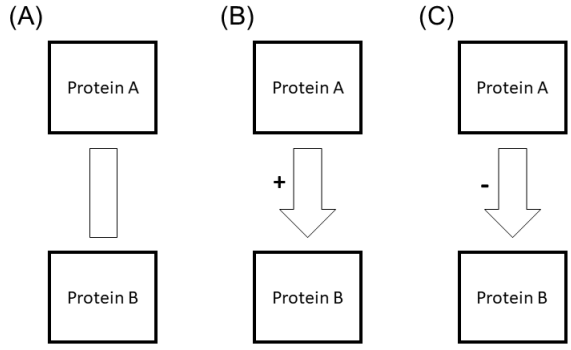

Fig. 1. Interaction key for polyhydroxyalkanoate (PHA) interaction map. Illustrated in (A) is the physical interaction of two proteins. Illustrated in (B) is one factor having a positive influence on another factor. This interaction could be positive gene regulation, activation of activity, or some other interaction that increases quantity or activity of the factor. Illustrated in (C) is one factor having a negative influence on another factor. This interaction could be negative gene regulation, repression of activity, or some other interaction that decreases quantity or activity of the factor. Note that one component of each of these interactions in any case could be a non-protein factor, such as PHA

this interaction map, we assume an interaction between PhaA and PhaB enzymes, as well as between PhaB and PhaC enzymes. Likely, the enzymes must be in close proximity to each other in order to pass products/substrates to the next enzyme in the pathway. It should be noted that, as of this writing, no physical interaction between PhaA, PhaB and PhaC has been characterized in *R. eutropha*. In PHB-containing cells, immunogold localization was used to show that PhaC is localized at the surface of polymer granules (Gerngross et al., 1993). In addition, it was shown that a high molecular weight species was present in purified PhaC enzyme preparations from *R. eutropha* cell extracts, which contained the synthase enzyme, as well as small quantities of PHB. It was determined that the presence of the polymer in the active site of the enzyme in this fraction reduced the lag phase in activity (Cho et al., 2012). Taken together, and considering the fact that PhaC enzymes actively produce the PHB that makes up the bulk of the intracellular granule, PhaC can be classified as a granule-associated protein.

Another key protein group in PHA homeostasis in *R. eutropha* are the granule structural proteins known as phasins, designated PhaP. It was initially shown that relatively large quantities of phasin molecules covered a large portion of the surface of PHB granules (Wieczorek et al., 1995). Following this discovery, a link between PHB accumulation and PhaP accumulation was established. The presence or absence of PhaP was also shown to play a role in PHB accumulation. In *phaP* deletion strains, significantly less intracellular polymer accumulated (York et al., 2001). It was later demonstrated that PhaP expression was affected by the regulatory protein, PhaR, which acts as a repressor of PhaP expression when PHA was not being produced (York et al., 2002). Further studies by Pötter and coworkers showed that the regulatory protein PhaR can bind to PHB granules, acting as a derepression mechanism to allow PhaP expression during PHB synthesis (Pötter et al., 2002). Kinetic analysis was performed, allowing detailed observations of binding of the PhaR protein to both PHB and *phaP* promoter DNA. In the presence of PHB, PhaR will bind irreversibly, relieving repression of the *phaP* gene and allowing for accumulation of PhaP that will coat the bulk of the surface of the PHB granule

(Yamada et al., 2013). Pötter and coworkers discovered multiple phasin homologues in the *R. eutropha* cell. Three homologues (named PhaP2, PhaP3 and PhaP4) were shown to bind to PHB granules. Thus, what was initially "PhaP" is now termed "PhaP1." The phasin PhaP2 was not shown to bind to PHB granules during *in vivo* studies, but an *in vitro* study demonstrated PhaP2's PHB binding capabilities (Pötter et al., 2004). A follow-up work on this study showed that the PhaR regulatory protein would bind to the promoter of the *phaP1* and *phaP3* genes, but not the *phaP2* and *phaP4* genes (Pötter et al., 2005), suggesting PhaR-mediated regulation of PhaP1 and PhaP3 but not the other two phasin molecules. Transcriptome analysis offered confirmation of this finding (Brigham et al., 2012a). Additional phasin proteins have been discovered in *R. eutropha*. Two-hybrid screen experiments helped to characterize the presence and interactions of a new phasin, PhaP5. It was revealed that PhaP5 interacted with several established proteins and enzymes associated with the granule, such as PhaP1-PhaP4, PhaR and the PHA depolymerase enzyme PhaZ1. The same two-hybrid screen demonstrated physical interactions between PhaP2 and PhaP3, as well as PhaP2 and PhaP4 (Pfeiffer and Jendrossek, 2011). Follow-up yellow fluorescent protein (YFP) tagging experiments showed that PhaP5 localized at the PHB granule. It is suggested that PhaP5 plays a role in PHA granule localization in the cell (Pfeiffer et al., 2011). Further exploration of potential phasin homologues in *R. eutropha* revealed the presence of two new putative phasins, PhaP6 and PhaP7. Expression of both of these phasins was shown to increase under PHB-permissive conditions, and YFP fusions of PhaP6 and PhaP7 were shown to colocalize with PHB granules (Pfeiffer and Jendrossek, 2013).

A 27-kDa protein, originally shown to be associated with residual PHB present in a fraction of partially purified, epitope tagged PhaC enzyme preparation from *R. eutropha* (Cho et al., 2012), was further observed to be associated with PHB granules, interacting concomitantly with PHB and with DNA as a segregation mechanism during cell division. Physical interaction of this protein, known as PhaM, with PhaC and PhaP5 was demonstrated using a 2-hybrid screen (Pfeiffer and Jendrossek, 2011). It was later demonstrated in an *in vitro* setting that PhaM is the physiological activator of PhaC enzyme activity, with increasing concentrations of PhaM in PhaC activity assays reducing the length of lag phase in enzyme activity. Crosslinking experiments also confirmed physical interactions between PhaC and PhaM (Pfeiffer and Jendrossek, 2014).

Another key group of proteins involved in PHA homeostasis, and potential interactors on the surface of the granule, are the PHA depolymerases, PhaZ. There are two main types of PhaZ enzymes, those that act on the amorphous (i.e. intracellular) PHA substrate and those that act on the crystalline (i.e., extracellular) PHA substrate (Jendrossek and Handrick, 2002). Early studies provided evidence of the existence of PHB depolymerases (called PhaZ1, PhaZ2, and PhaZ3). Removing PhaZ1 and PhaZ2 from the *R. eutropha* cell nullified the ability to break down intracellular PHB under conditions lacking extracellular carbon (York et al., 2003). A more recent study discovered another intracellular PhaZ enzyme, PhaZ5 (Brigham et al., 2012). Experiments confirmed that both PHB synthesis and degradation occur in granules, and that all machinery needed for both activities are present on the surface of granules (Uchino and Saito, 2006). It was later shown that phasin PhaP1 plays a role in PHB depolymerization. One *in vitro* study showed that PhaZ1 can perform thiolysis of PHB, yielding 3HB-CoA, in the presence of PhaP1 (Uchino et al., 2007). PHB depolymerization by granule-associated PhaZ1 was shown to

require phasin proteins, with PhaP1, PhaP2 and PhaP4 shown to affect PHB depolymerization by PhaZ1. The absence of phasin proteins on granules almost completely eliminated PhaZ1-mediated depolymerization of PHB (Eggers and Steinbuchel, 2013). Sznader et al. have recently examined the protein complement on *R. eutropha* PHA granules. Their data suggest that the only PHA depolymerase protein present in significant amounts on the polymer granule is PhaZ1 (Sznajder et al., 2015). Thus, there is ample evidence of interaction with PhaZ1 interacting not only with the PHA granule but also granule-associated proteins like PhaP1; there appears to be insufficient evidence and perhaps evidence to the contrary that other intracellular PhaZ proteins (PhaZ2, PhaZ3, PhaZ5) interact with the granule.

The complete proteome of *R. eutropha* PHB granules has recently been examined, coupled with an examination of *in vivo* subcellular localization data, revealing the presence of 4 novel granule-associated proteins. Bioinformatic methods show that these proteins are not related to any of the previously-characterized granule-associated proteins. Two of these proteins (locus tags H16_B1632 and H16_A0671) are annotated as potential α/β-hydrolases, another (locus tag H16_A0225) is annotated as a putative phospholipase, and the final protein (locus tag H16_A2001) is annotated as a hypothetical protein. Interestingly the *H16_A2001* gene is present in an operon with two genes encoding previously characterized and potentially PHB-involved proteins, PhaB2 and PhaC2 (Sznajder et al., 2015).

Figure 2 shows the interaction map of the *R. eutropha* PHB granule, based on the data from the previous works discussed here. Some granule-associated proteins are depicted twice (such as PhaP2 and PhaP3) for ease of illustration. The interaction map in Fig. 2 illustrates the complex interactions that take place on the surface of a PHB granule. Future experiments could shed light on the newest of the granule-associated proteins, indicating a heretofore unimagined role for these enzymes in PHB homeostasis.

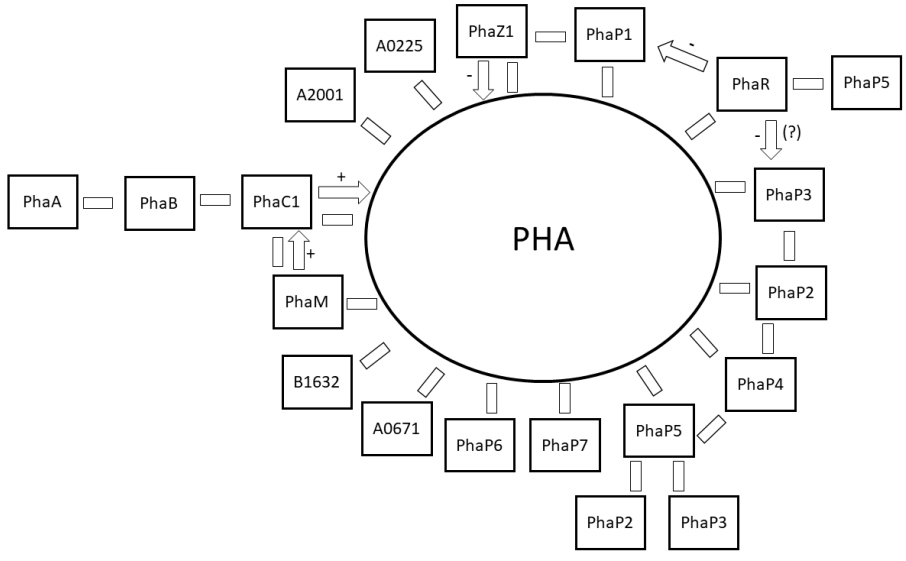

Fig. 2. Interaction map of the *R. eutropha* PHA granule, constructed by a systems biology approach. Interactions among granule-associated proteins and PHA are as described in Fig. 1

Conclusion and Outlook

The PHA cycle in *R. eutropha* has been well studied, so there exist plentiful data with which to model interactions of key components of the cycle. There are, of course, still questions that remain about certain components (enzymes and other proteins) and the nature of their interaction with the PHA granule and other granule-associated proteins. For example, if PHA depolymerases like PhaZ2 do not *de facto* interact with the granule, what is their true localization and role in the cell during polymer synthesis and mobilization? PhaZ2 has been determined biochemically to play a role in PHB mobilization (York et al., 2003; Brigham et al., 2012), but the *in vivo* nature of that role is still unclear. The next steps in elucidating the systems biology of the PHA granule include defining the roles of these and other periphery proteins.

Systems biology of any organism or process helps "paint the big picture" of that particular system. This can be of great help when biotechnologists are seeking to expand the roles of the process or of the biocatalyst. In *R. eutropha*, many research groups have replaced the native PhaC1 enzyme with PHA synthases possessing a greater substrate range (Budde et al., 2011; Bhubalan et al., 2011). This replacement undoubtedly affects many interactions among PHA homeostasis enzymes. With these novel strains, one can examine the key interactions and construct an updated interaction map that not only provides information about the engineered biocatalyst, but also tells the researcher about the molecular interactions in the wild-type system. Another example is the transfer of PHA biosynthesis genes into heterologous host organisms. Many different hosts, from *S. cerevisiae* to switchgrass, have been engineered to produce PHA (Somleva et al., 2008; Zhang et al., 2006). The interaction map can give us a clue as to what genes are needed (and not needed nor wanted) for optimal production of PHA in a heterologous host. In short, a systems biology approach will help the researcher to design better biocatalysts, identify key components of pathways quicker and potentially maximize yields and productivities.

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