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Highlights

- The emergence of homologous enzymes with different subcellular localizations.

- Localization switch of homologous enzymes needs multiple evolutionary steps.

- Localization switch requires innovations at genomic, genetic and enzymatic levels.

Understanding the intracellular-to-extracellular localization switch of polyhydroxybutyrate polymerase in *Pseudomonas* backgrounds as a microevolutionary process

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Abstract

After gene duplication, paralogous genes evolve independently, and consequently, the new proteins encoded by these duplicated genes are exposed to changes in their subcellular location. Although there are increasing evidence that phylogenetically related proteins play different functions in different subcellular compartments, the number of evolutionary steps required for the emergence of a novel protein with a novel subcellular localization remains unclear. Regarding this intriguing topic, here we examine in depth our previous reports describing both intracellular and extracellular polyhydroxybutyrate polymerases (PhaC) in the *Pseudomonadales* group. The recapitulation of the intracellular-to-extracellular localization switch of PhaC in these strains shows a gradual evolution from a simple cytosolic PhaC form to a complex extracellular PhaC form specifically secreted via the type 1 secretion system. This gradual evolution includes several adaptive and pre-adaptive changes at the genomic, genetic and enzymatic levels, which are intimately related to the lifestyle of organisms during the evolution of protein localization. We conclude that the protein localization switch can be an extremely complex process in nature.

Introduction

In both eukaryotic and prokaryotic cells, proteins localize in particular subcellular sites. In gram-negative bacteria, such as the model strains *Escherichia coli* DH5α and *Pseudomonas fluorescens* Pf-5, these localizations include the cytoplasm, the inner membrane, the periplasmic space, the outer membrane and the extracellular space. While cytosolic proteins usually play roles related to bacterial cell growth and metabolism, secreted proteins often display functions related to bacterial-environment interaction processes, such as defense, attachment and virulence (Gardy et al., 2003; Green and Mecsas, 2016; Ivankov et al., 2013). In addition, homologous enzymes codified by paralogous genes have been reported to display different functions depending on their subcellular localization. For example, peroxisomal and cytosolic thiolases II are extremely similar enzymes (>85 % of amino acid identity) but display catabolic (e.g. fatty acid β-oxidation) and anabolic (mevalonate biosynthesis) functions, respectively (Fox et al., 2014; Soto et al., 2011). Although it is well known that functional divergence after gene duplication is sometimes related to a switch in the subcellular localization of an enzyme, the molecular mechanisms involved in this switch are unknown.

Probably due to the exceptional relevance of the specific localization for each protein, complex cellular machinery exists to identify and distribute proteins to their correct cell location in the cell. In gram-negative bacteria, the identification and exportation of specific proteins out of the cell by the Type 1 Secretion System (T1SS) is generally associated with a C-terminal secretion sequence within its substrate, which consists of a non-conserved secretion signal of around 60 to 80 amino acids and a variable number of repeats of the consensus sequence GGXGXD (Boyd et al., 2014). The T1SS machinery consists of a continuous channel composed of three proteins: HlyD, HlyB and TolC. In addition, the genes that codify for this transport system and the secreted substrate are often clustered within a large operon (Thanabalu et al., 1998). Similar to other transport systems, T1SS has been extensively characterized, but how T1SS-extracellular proteins have emerged is still completely unknown. Regarding this type of evolutionary transition, here we propose to examine our reports describing both intracellular and extracellular polyhydroxybutyrate (PHB) polymerases (PhaC) (Ayub et al., 2009; Ayub et al., 2004; Ayub et al., 2006; Ayub et al., 2007; Soto et al., 2012; Stritzler et al., 2018) to understand the putative evolutionary steps involved in the intracellular-to-extracellular localization switch of the PhaC enzymes in *Pseudomonas* backgrounds (Fig. 1). Particularly, we propose a gradual mode of evolution of PhaC enzymes (Fig. 2) and discuss how the localization switch of these enzymes could lead to a change in their function.

Methods

The evolution of PhaC proteins in *Pseudomonas* backgrounds (*Azotobacter* sp. FA8, *Pseudomonas* sp. 14-3 and *Pseudomonas fluorescens* FR1) were analyzed as previously described (Ayub et al., 2007; Pascuan et al., 2015). Phylogenetic analysis was conducted using MEGA 7.0. (https://www.megasoftware.net/). Protein sequences were aligned using the ClustalW program. Phylogenetic trees were constructed using the Neighbor-Joining method with genetic distances computed with the p-distance model, bootstrap analysis of 500 resamples, using PhaC from *Ralstonia eutropha* H16 (a non-*Pseudomonas* background) as outgroup. Protein structure of PhaC enzymes from strains FA8, 14-3 and FR1 were predicted by using Phyre 2 public server (Kelley et al., 2015) and the crystal structure of the PhaC from strain H16 (2h5z pdb code PDB from www.pdb.org). Pictures of the PhaC enzymes were produced using VMD software (Humphrey et al., 1996). The transmembrane domains were identified using Phobius software (Kall et al., 2004). Putative catalytic, transmembrane and secretion domains, conserved catalytic sites, cysteine residues for disulfide bonds and GGXDG export signals were identified by Geneious software (https://www.geneious.com/).

Results and discussion

PHB is a biopolymer synthesized by a wide range of microbes as a way to store energy, which, consequently, displays important roles in bacterial survival under starvation conditions (Lopez et al., 1995). Consistent with this intracellular function, PHB is generally produced in the cytoplasm and involves a three-step synthesis, commonly known as the ABC pathway (Fox et al., 2014) (Fig. 2a-b). The first step is catalyzed by β-ketothiolase (PhaA), which condenses two acetyl-CoA into acetoacetyl-CoA. Then, this intermediate is reduced to 3-hydroxybutyryl-CoA by a NADPH-dependent reductase (PhaB). Finally, a PHB polymerase (PhaC) catalyzes the incorporation of the monomer 3-hydroxy-butyryl-CoA to the PHB polymer (Steinbuchel and Hein, 2001). PHB production has been classically and extensively described in the model bacterium *Ralstonia eutropha* H16 (Raberg et al., 2017; Wittenborn et al., 2016), in which the PHB biosynthesis genes are codified within the *phaCAB* operon (Fig. 2a). PhaC from strain H16 is a relatively small (589-amino-acid long) intracellular protein without transmembrane domains, which has one disulfide bond and a C-terminal catalytic domain (Fig. 3). In congruence with previous predictions, the crystal structure of this enzyme confirms that it is a typical intracellular globular protein (Wittenborn et al., 2016).

PHB production is not an ancestral feature of the *Pseudomonadales* order (Kessler and Palleroni, 2000; Palleroni, 2003). However, some strains belonging to this group, such as *Azotobacter* sp. FA8, *Pseudomonas* sp. 14-3, and *Pseudomonas fluorescens* FR1, have acquired this biosynthetic process via the transfer of PHB genes (Ayub et al., 2007; Pettinari et al., 2003; Stritzler et al., 2018). In strain *Azotobacter* sp. FA8, as in other nitrogen-fixing *Pseudomonadales* strains, including *Pseudomonas stutzeri* A1501 (Yan et al., 2008), PHB is synthesized in the cytoplasm via the ABC pathway (Pettinari et al., 2003; Pettinari et al., 2001) (Fig. 2b), and its production is probably related to the non-enzymatic regulation of nitrogen fixation, an energy-costly intracellular process (Pascuan et al., 2015). Although the ABC pathway and the intracellular function of PHB in *Azobacter* sp. FA8 are conserved, its PHB genes are organized in a different arrangement (*phaBAC* operon), where the *phaC* gene is exposed at the end of this novel cluster (Fig. 2b). Importantly, the transfer of PHB genes into *Pseudomonadales* implies that the *phaC* gene is within a genomic background containing the T1SS (Fig. 2b), and is thus susceptible to coevolve with this transport system. PhaC from strain FA8 is also a small (567-amino-acid long) protein without transmembrane domains, but has two disulfide bonds (Fig. 3). These additional two covalent interactions related to PhaC from strain H16 and the switch to a non-globular form (Fig. 2b) could be important prerequisites for PhaC adaptation to extracellular environments.

In the case of the bacterium *Pseudomonas* sp. 14-3, the PHB genes are within a large genomic island (32-kb), which contains several transposons (Ayub et al., 2007). Probably due to the recombination of some of these mobile elements, the *phaC* gene from strain 14-3 undergoes a localization shift away from the *phaBA* genes and close to a T1SS, which specifically secretes a lipoprotein (*lp*) (Fig. 2c). As a consequence of this rearrangement, this *phaC* gene forms a transcriptional unit with the T1SS-*lp*, independent of the *phaBA* operon (Fig. 2c). Impaired PHB production via the ABC pathway in strain 14-3 is due to a deletion in the *phaA* gene (Ayub et al., 2006), giving rise to a truncated *phaBA* operon (Fig. 2c). However, strain 14-3 can accumulate PHB via the degradation of fatty acids (Ayub et al., 2006) (Fig. 2c). In fact, this PHB production is essential for the maintenance of the intracellular redox state of strain 14-3 under cold stress (Ayub et al., 2009). Therefore, strain 14-3 has several genetic changes in the production of PHB related to typical PHB-producing strains (e.g. H16 and FA8) but conserves the classical intracellular role of PHB. PhaC from strain 14-3 has high amino acid similarity (80%), and almost the same size (566 amino acids) and the same two disulfide bonds as the PhaC from strain FA8, but strain 14-3 also has an additional pre-adaptive transmembrane domain (Fig. 3).

Pseudomonas fluorescens FR1 is an endophytic bacterium isolated from Pangola grass adapted to cold conditions. Similar to strain 14-3, strain FR1 lost the ABC pathway and its *phaC* gene is near a mobile element and forms an operon with the T1SS (Stritzler et al., 2018). Contrary to strain 14-3, strain FR1 completely lost the *phaAB* and *lp* genes and its *phaC*-T1SS operon is within a small transposase flanked by inverted repeats (Fig. 2d), showing a strong association between PhaC and T1SS, and an irreversible loss of the ABC pathway. In addition, PhaC from strain FR1 is almost identical (99% amino acid identity) to PhaC from strain 14-3 in its first 566 amino acids, and PhaC protein shows a transmembrane domain and two disulfide bonds (Fig. 3). Importantly, PhaC from strain FR1 is longer than previously described PHB polymerases (671 amino acids) and this increased size is due to an extension within its C-terminal region that contains two evolutionary novelties for its exportation via T1SS: three tandem GGXGXD repeats and a secretion signal (Fig. 2d and Fig. 3) (Stritzler et al., 2018). The last region has 100% amino acid identity to that previously described in the lipoprotein next to T1SS in strain 14-3, suggesting that *phaC* from FR1 derived from a fusion between typical *phaC* and *lp* genes. In concordance with its localization switch, PhaC from strain FR1 has an extracellular function: the protection of the plant host cell against cold stress (Stritzler et al., 2018).

Conclusion

The change in the subcellular localization of proteins during the evolution of species has been proposed since the detection of large sets of paralogous genes within the genomes, but the evolutionary steps involved in this type of localization switch has not yet been analyzed. Here, we showed that the switch from a cytosolic to an extracellular enzyme needs multiple microevolutionary steps, requiring several innovations at genomic, genetic and enzymatic levels. To our knowledge, this is the first report describing the probable evolutionary innovations required to evolve the intracellular-to-extracellular localization switch of an enzyme.
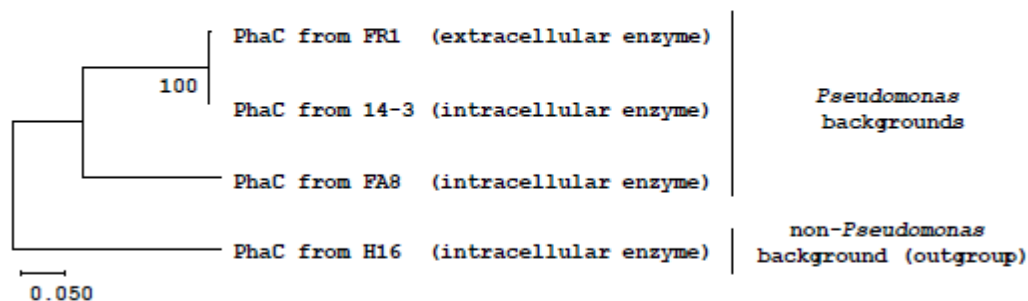
Figure legends

Figure 1

Figure 1. Evolutionary analysis supports the recent emergence of the extracellular variant of PhaC. Phylogenetic tree of PhaC proteins from *Azotobacter* sp. FA8, *Pseudomonas* sp. 14-3 and *Pseudomonas fluorescens* FR1 (*Pseudomonas* backgrounds) based on Neighbor-Joining analysis using intracellular PhaC from *Ralstonia eutropha* H16 (non-*Pseudomonas* backgrounds) as outgroup. The bacterial groups (*Pseudomonas* and non-*Pseudomonas* backgrounds) and the protein localization (intracellular and extracellular) are shown. Bootstrap percentages are indicated at the branch points.
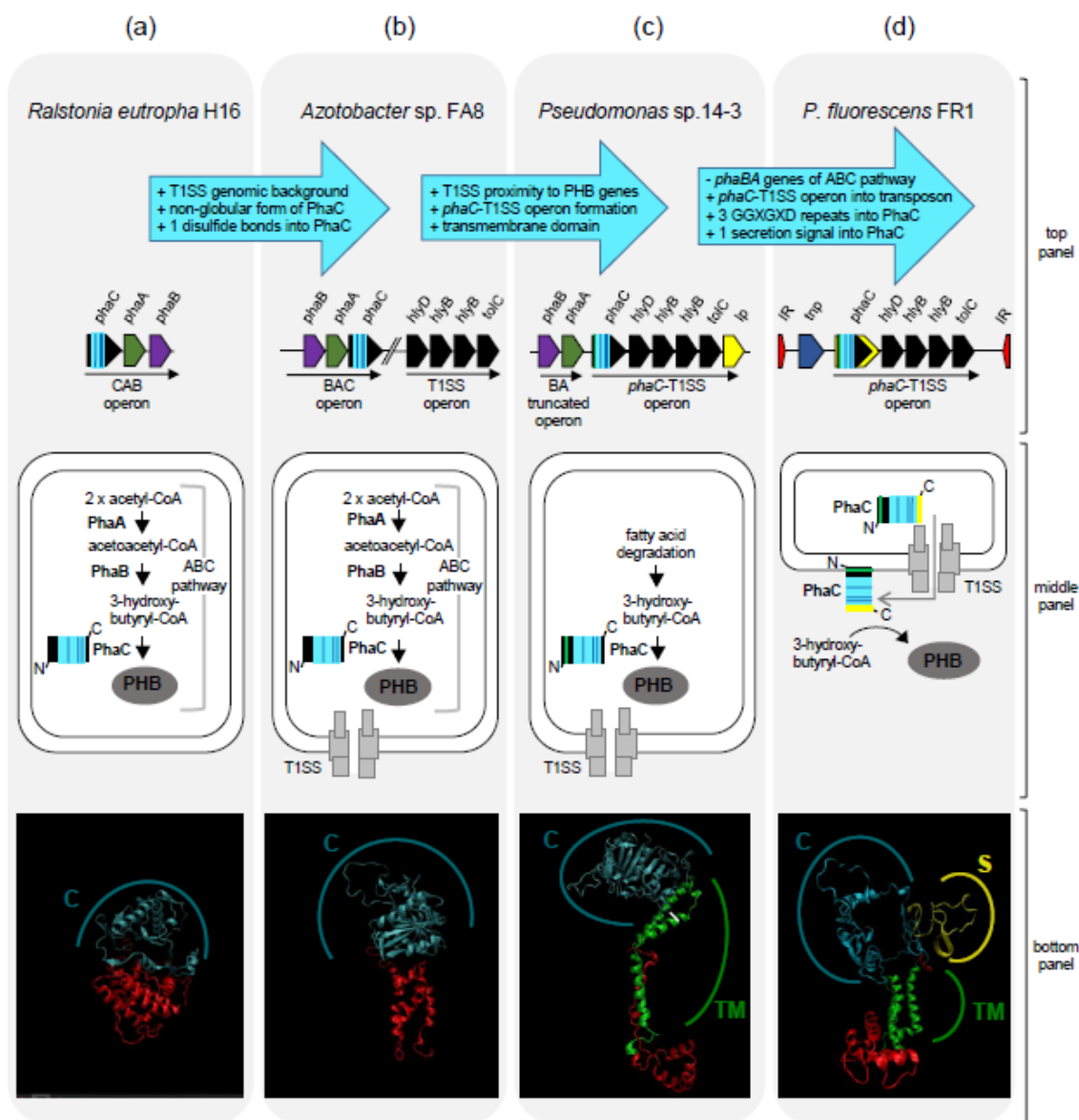
Figure 2. Schematic representation of the evolutionary steps involved in the switch from a cytosolic to a specifically-secreted membrane-bound PhaC enzyme. In an effort to understand the evolutionary steps related to the intracellular-to-extracellular localization transition of the PhaC enzyme, we analyzed genomic (top panel), genetic (middle panel) and enzymatic (bottom panel) features of four PHB-producing strains: *Ralstonia eutropha* H16 (a), *Azotobacter* sp. FA8 (b), *Pseudomonas* sp. 14-3 (c) and *Pseudomonas fluorescens* FR1 (d). In this scheme, we highlight the three most significant evolutionary steps among these strains (light blue arrows), showing that the radical change from a typical cytosolic PhaC enzyme (in strain H16) to an atypical PhaC enzyme secreted via a T1SS (in strain FR1) needs large number

of modifications at genomic, genetic and enzymatic levels. T1SS: Type I Secretion System; C: catalytic domain; TM: transmembrane domain; S: secretion domain.
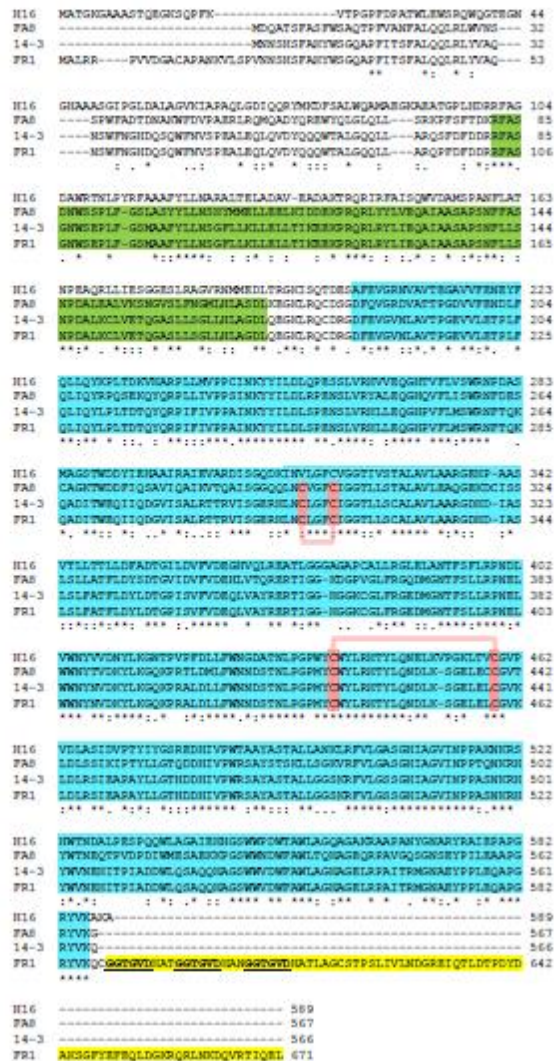
Figure 3

Figure 3. Alignment comparison of amino acids sequence of the PhaC enzymes from *Ralstonia eutropha* H16, *Azotobacter* sp. FA8, *Pseudomonas* sp. 14-3 and *Pseudomonas fluorescens* FR1. Multiple amino acid alignment showing catalytic (light blue), transmembrane (green) and secretion (yellow) domains, highly conserved catalytic sites (blue), cysteine residues for disulfide bonds (boxed in pink) and GGXDG export signals (underlined).

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