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LETTER TO THE EDITOR

Exploring the Ancestral Mechanisms of Regulation of Horizontally Acquired Nitrogenases

Cecilia Pascuan[1,2] · Ana Romina Fox[1,2] · Gabriela Soto[1,2] · Nicolas Daniel Ayub[1,2]

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Abstract The vast majority of *Pseudomonas* species are unable to fix atmospheric nitrogen. Although several studies have demonstrated that some strains belonging to the genus *Pseudomonas* sensu stricto do have the ability to fix nitrogen by the expression of horizontally acquired nitrogenase, little is known about the mechanisms of nitrogenase adaptation to the new bacterial host. Recently, we transferred the nitrogen fixation island from *Pseudomonas stutzeri* A1501 to the non-nitrogen-fixing bacterium *Pseudomonas protegens* Pf-5, and interestingly, the resulting recombinant strain Pf-5 X940 showed an uncommon phenotype of constitutive nitrogenase activity. Here, we integrated evolutionary and functional approaches to elucidate this unusual phenotype. Phylogenetic analysis showed that polyhydroxybutyrate (PHB) biosynthesis genes from natural nitrogen-fixing *Pseudomonas* strains have been acquired by horizontal transfer. Contrary to Pf-5 X940, its derived PHB-producing strain Pf-5 X940-PHB exhibited the inhibition of nitrogenase activity under nitrogen-excess conditions, and displayed the typical switch-on phenotype observed in natural nitrogen-fixing strains after nitrogen deficiency. This indicates a competition between PHB production and nitrogen fixation. Therefore, we propose that horizontal transfer of PHB biosynthesis genes could be an ancestral mechanism of regulation of horizontally acquired nitrogenases in the genus *Pseudomonas*.

Keywords Nitrogenase · Polyhydroxybutyrate · Evolution · Horizontal transfer · Regulation

Introduction

Nitrogen is a critical element for life and a limiting factor in organism growth in almost all natural environments (Galloway et al. 2008). Some bacterial and archaeal strains, named diazotrophs, are able to use atmospheric nitrogen via an endergonic process known as biological nitrogen fixation, which is the conversion of dinitrogen to ammonia (Dixon and Kahn 2004). Diazotrophs contain nitrogenase, a metallo-enzyme complex extremely conserved in free-living and symbiotic nitrogen-fixing strains. The nitrogenase complex is normally coded by large mobile genetic elements incorporated through horizontal transfer events (Dos Santos et al. 2012; Kechris et al. 2006). For example, nitrogen fixation genes are located on rhizobial symbiotic megaplasmids (Finan et al. 2001), which are large extrachromosomal elements able to transfer to other non-fixing rhizosphere bacteria by conjugation (Althabegoiti et al. 2014).

Nitrogen fixation is an energy-costly process and, accordingly, nitrogenase is strongly regulated at a diversity of levels in response to extracellular nitrogen availability (Peters and Szilagyi 2006). However, the transcriptional and post-translational cellular machinery to switch-off or switch-on nitrogen fixation in response to nitrogen changes in the environment could be completely absent or

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✉ Nicolas Daniel Ayub
ayub.nicolas@inta.gob.ar

[1] Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Buenos Aires, Argentina

[2] Instituto de Genética Ewald A. Favret (CICVyA-INTA), De los Reseros S/N, Castelar C25(1712), Buenos Aires, Argentina

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incomplete in the new host cell. Therefore, the presence of alternative simple mechanisms for early adaptation of transferred nitrogenase, such as metabolic competence, is expected. Nitrogen fixation is naturally in competition with other highly endergonic processes present in the microbial cell, such as the biosynthesis of polyhydroxybutyrate (PHB) in the natural nitrogen-fixing strain *Rhizobium etli* (Cevallos et al. 1996). In fact, nitrogen fixation effectiveness of chimeric nitrogenase clusters is maximized when PHB production is completely inhibited in these types of symbiotic microorganisms (Peralta et al. 2004). However, the efficiency of PHB production as a mechanism to regulate transferred nitrogenase still needs to be explored.

The inability of *Pseudomonas* sensu stricto species to fix nitrogen has been classically proposed as an important taxonomic value (Palleroni 2003). However, some particular strains belonging to this genus such as *Azotobacter vinelandii* DJ and *Pseudomonas stutzeri* A1501 have acquired this endergonic process, apparently through the transfer of nitrogenase genes located within large mobile elements (Rediers et al. 2004; Yan et al. 2008). Recently, we transferred the nitrogen fixation island from *Pseudomonas stutzeri* A1501 to the non-nitrogen-fixing rhizosphere bacterium *Pseudomonas protegens* Pf-5 (Setten et al. 2013). The recombinant strain *Pseudomonas protegens* Pf-5 X940 showed an uncommon phenotype of constitutive nitrogenase activity (Setten et al. 2013), confirming the predicted difficulty of nitrogenase to properly adapt to the new *Pseudomonas* host.

Many *Pseudomonas* species have two classII PHA synthases, called PhaC1 and PhaC2, involved in the production of medium-chain-length polyhydroxyalkanoates (mcl-PHAs) (Rehm 2003). In contrast, *Pseudomonas* strains are commonly unable to produce PHB due to the absence of classI PHA synthases, which preferentially use short-chain-length polyhydroxyalkanoates including the PHB monomer (Kessler and Palleroni 2000). However, the natural nitrogen-fixing *Pseudomonas* strains *Azotobacter vinelandii* DJ and *Pseudomonas stutzeri* A1501 possess classI PHA synthases (ACO78551 and ABP78389, respectively) downstream from genes coding for a β-ketothiolase (*phaA*) and an acetoacetyl-coenzyme A reductase (*phaB*), a gene cluster involved in PHB production via the ABC pathway (Fox et al. 2014).

The occurrence of PHB genes within nitrogen-fixing *Pseudomonas* strains suggests a possible linkage between the incorporations of genes involved in PHB production and nitrogen fixation by horizontal transfer. We here integrated evolutionary and functional approaches to understand the relevance of horizontal transfer of PHB biosynthesis genes in the adaptation of heterologous nitrogenases in the genus *Pseudomonas*. The information presented is particularly important, not only for a better understanding of ancestral mechanisms of regulation of horizontally acquired nitrogenases but also for biotechnological applications such as the production of recombinant nitrogen-fixing inoculants for biological nitrogen fixation in non-legume crops.

Methods

The strains used in this study were *Pseudomonas stutzeri* A1501 (Desnoues et al. 2003), *Pseudomonas protegens* Pf-5 X940 (Setten et al. 2013), and its derived strain Pf-5 X940-PHB, constructed by introducing the entire wild-type *phaBAC* gene cluster (Ayub et al. 2006) into pBBR1MCS-3, as previously (Ayub et al. 2009). The evolution of PhaC proteins and the C+G content of *phaC* genes were analyzed previously (Ayub et al. 2007). Sequence search was performed using BLASTP tools (Online Resource 1). Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 5.0. Protein sequences were aligned using the ClustalW program. Phylogenetic trees were constructed using the neighbor-joining (NJ) method with genetic distances computed using p-distance model and bootstrap analysis of 1000 resamples and root on midpoint.

Nitrogenase activity was evaluated as previously reported (Setten et al. 2013) with slight modifications. Bacterial cells from an overnight culture in L medium containing 37 mM glucose were centrifuged and resuspended in a 50-ml flask containing 10 ml N-free L medium supplemented with 100 mM glucose, at an OD 580 nm of 0.1. The suspension was incubated for 8 h at 28 °C with shaking under an argon atmosphere containing 1 % oxygen and 10 % acetylene. Ethylene production was determined by gas chromatography according to Galimand et al. (1989) and protein content was determined by the Bradford method (Bradford 1976). The composition of the PHB polymer and monomer was evaluated as previously described (Soto et al. 2012).

Results

To investigate the origins of PHB polymerase (*phaC*) genes from the nitrogen-fixing γ-*Proteobacteria* strains *Azotobacter vinelandii* DJ ($phaC_{DJ}$) and *Pseudomonas stutzeri* A1501 ($phaC_{A1501}$), we analyzed the evolutionary relationships of the PhaC proteins from well-studied species belonging to the α, β, and δ subclasses of *Proteobacteria* (Fig. 1). Consistent with the functional classification, the phylogenetic analysis showed that classI and classII polymerases form two distinct clusters (Fig. 1). Interestingly, $PhaC_{DJ}$ and $PhaC_{A1501}$ from *Azotobacter vinelandii* DJ and

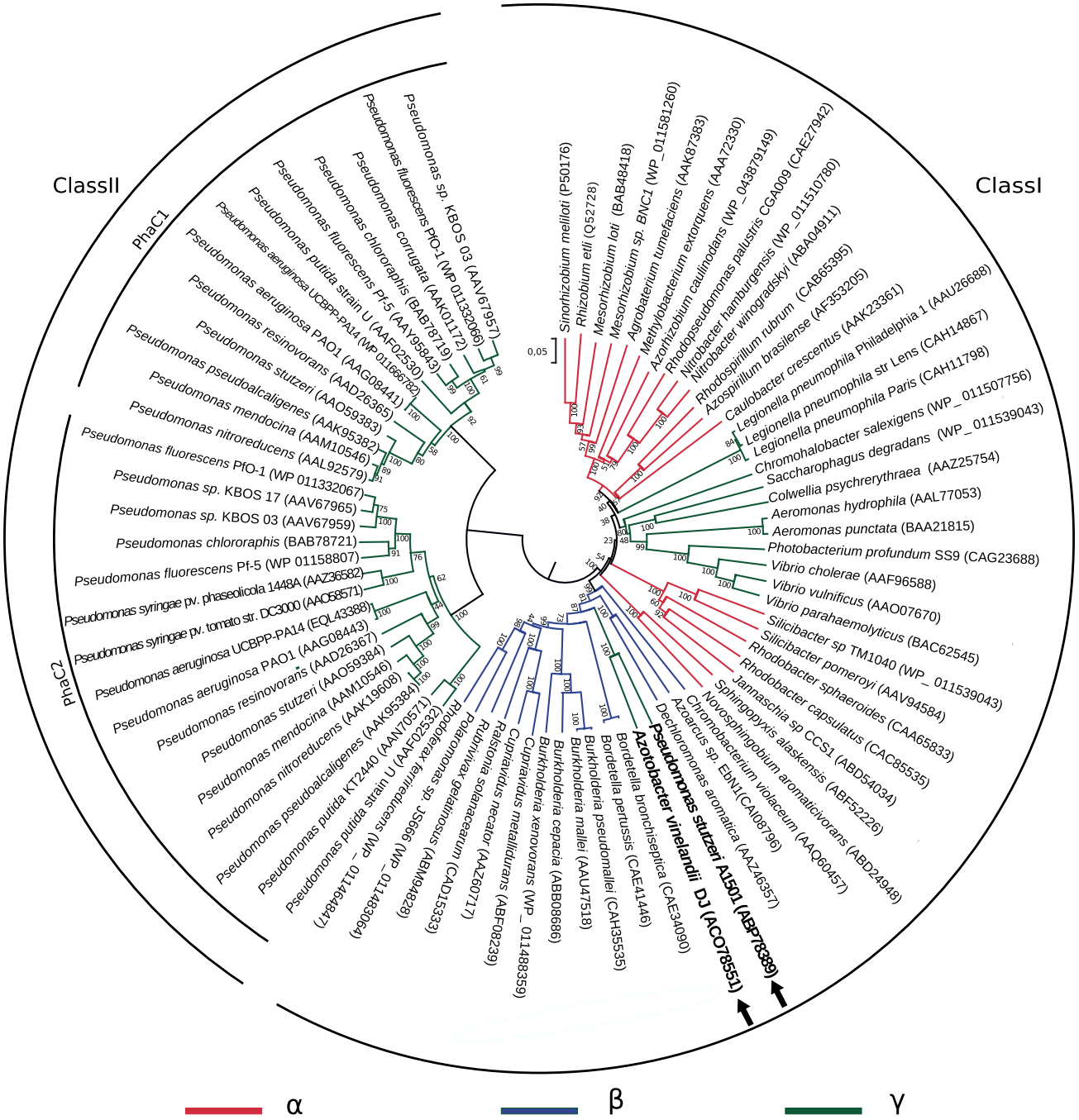

Fig. 1 Phylogenetic tree of PhaC proteins based on neighbor-joining (NJ) analysis. The α, β, and δ subclasses of the phylum *Proteobacteria* and functional classification (classI and classII) of synthases are shown. *Bold* type indicates proteins that fall within groups not corresponding to their 16S rRNA-derived phylogenetic affiliation. Bootstrap percentages are indicated at the branch points

Pseudomonas stutzeri A1501 were not related to the classI polymerases from γ-*Proteobacteria* or classII polymerases from *Pseudomonas* strains, but were found to be associated with polymerases from *Burkholderiales* (β-*Proteobacteria*) with a high bootstrap value (Fig. 1), suggesting that the $phaC_{DJ}$ and $phaC_{A1501}$ genes were acquired by horizontal transfer. To further corroborate the non-*Pseudomonas* origin of the $phaC_{DJ}$ and $phaC_{A1501}$ genes, we analyzed the G+C content of these specific polymerases. The average percent G+C content for both genes (51.3 % for $phaC_{DJ}$ and 58.1 % for $phaC_{A1501}$) was close to that for the *Burkholderiales* (55.8 %), but was lower than that for the *Azotobacter vinelandii* DJ (65.7 %) and *Pseudomonas stutzeri* A1501 genomes (63.9 %).

Table 1 Production of PHB monomer and polymer, and nitrogenase activity by recombinant strains Pf-5 X940 and Pf-5 X940-PHB under nitrogen-excess conditions

Strains	PHB monomer	PHB polymer	Nitrogenase activity
Pf-5 X940	ND	ND	4150 ± 310
Pf-5 X940-PHB	0.3 ± 0.1	11.8 ± 1.4	160 ± 40

Nitrogenase activity is expressed as nanomoles of ethylene/micrograms of protein. PHB monomer and polymer content is expressed as a percentage of total cell dry weight (wt%). Values are the mean of four replicates ± SD. ND <0.1 wt%

To investigate whether horizontally acquired PHB production can affect Pf-5 X940 nitrogen fixation activity, we performed microaerobic cultures of Pf-5 X940 and its derived PHB-producing strain Pf-5 X940-PHB in L medium or nitrogen-free L medium. In nitrogen-excess conditions, and contrary to Pf-5 X940, Pf-5 X940-PHB exhibited high PHB polymer production and low nitrogenase activity (Table 1). PHB monomer production in Pf-5 X940-PHB was almost undetectable, suggesting that monomer molecules are at least predominantly in the polymer form (Table 1). In nitrogen-deficient conditions, and in opposition to Pf-5 X940, Pf-5 X940-PHB displayed a typical derepression phenotype observed in the natural nitrogen-fixing strain *Pseudomonas stutzeri* A1501 (Fig. 2). Therefore, our results suggest that horizontally transferred PHB production capability can regulate the activity of heterologous nitrogenases.

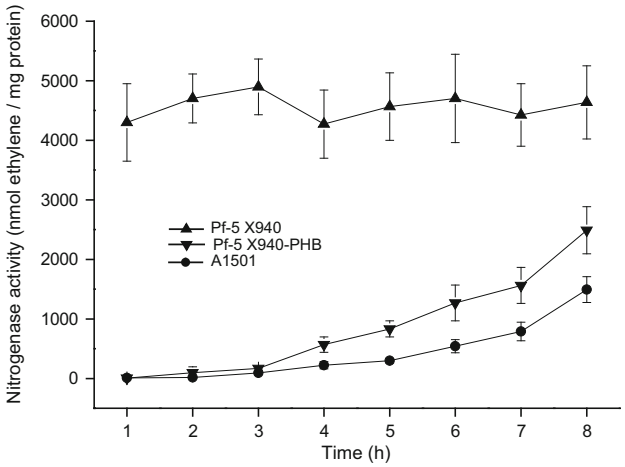

Fig. 2 Nitrogenase activity of the natural strain A1501 and recombinant strains Pf-5 X940 and Pf-5 X940-PHB under nitrogen-limiting conditions. Values are the mean ± SD of three independent measurements

Discussion

In previous studies, we showed that horizontal transfer of PHB genes is a mechanism of adaptability to high stress environments such as cold (Ayub et al. 2004, 2006, 2007, 2009) and salinity (Fox et al. 2014; Soto et al. 2011, 2012). However, the specific functions of heterologous PHB production can be different in each environmental context. For example, in the Antarctic strain *Pseudomonas* sp. 14-3, PHB modules the availability of reducing equivalents, contributing to alleviating the oxidative stress produced by low temperatures (Ayub et al. 2009), whereas in the halotolerant bacterium *Pseudomonas* sp. CT13, the PHB monomer acts both as an osmolyte and a chemical chaperone regulating the osmotic pressure of cells and preventing protein aggregation under salt conditions, respectively (Soto et al. 2012). It has also been described that the acquisition of production of PHB genes can displace the production of native polymers, probably due to competition for available energy and reducing power (Catone et al. 2014). In addition, recent experiments in *Pseudomonas* backgrounds further support the interactions between acquired genetic elements, exhibiting the relevance to study these types of phenomena to understand bacterial evolution (San Millan et al. 2015).

Nitrogen fixation is an endergonic process that normally requires a highly reducing microenvironment, whose regulation must be exquisitely controlled to avoid excessive waste of energy (Soto et al. 2013). Biotechnology research proposes the production of recombinant strains expressing constitutive nitrogenase activity for biological nitrogen fixation in non-legume crops (Geddes et al. 2015). In natural conditions, it is expected that nitrogen fixation genes incorporated by horizontal transfer are rapidly eliminated by natural selection because the transferred genes consume excess energy and reducing power. In fact, transformed strains constitutively expressing the nitrogenase complex also release ammonium into the extracellular medium (Bali et al. 1992; Brewin et al. 1999; Setten et al. 2013; Van Dommelen et al. 2003; Zhang et al. 2012). Consequently, in addition to its impact on the intrinsic growth rates, transfer of nitrogen fixation genes probably favors the growth of microbial competitors. In this context, and given the high efficiency in horizontal gene transfer through mobile elements in prokaryotes, the acquisition of PHB production may have been the most-parsimonious adaptation of novel diazotrophs in response to selection pressure on growth under energy-limiting microbial-competing conditions. Similar to our results, it has been recently described the importance of the host evolution for the maintenance of horizontally acquired genes related to dichloromethane consumption in *Methylobacterium* strains, supporting the relevance of genome evolution in the fixation of new genes (Michener et al. 2014a, b).

Conclusion

The presence of gene transfer of *nif* genes among bacteria in natural conditions has been discussed almost since the identification of nitrogen fixation genes, but the constraints for the adaptation of nitrogenase to their novel hosts have not yet been analyzed in depth. In this article, we showed that heterologous PHB production can regulate heterologous nitrogenase activity, suggesting that the transferred PHB production capability is a simple mechanism to mitigate the fitness cost derived from the acquisition of nitrogen fixation genes.

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