Biotechnol Lett
https://doi.org/10.1007/s10529-018-2576-6

ORIGINAL RESEARCH PAPER

Plant growth-promoting bacterium *Pseudomonas fluorescens* FR1 secrets a novel type of extracellular polyhydroxybutyrate polymerase involved in abiotic stress response in plants

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Received: 26 April 2018 / Accepted: 22 May 2018

Abstract
Objectives Identification of novel microbial factors contributing to plant protection against abiotic stress.
Results The genome of plant growth-promoting bacterium *Pseudomonas fluorescens* FR1 contains a short mobile element encoding a novel type of extracellular polyhydroxybutyrate (PHB) polymerase (PhbC) associated with a type I secretion system. Genetic analysis using a *phbC* mutant strain and plants showed that this novel extracellular enzyme is related to the PHB production *in planta* and suggests that PHB could be a beneficial microbial compound synthesized during plant adaptation to cold stress.
Conclusion Extracellular PhbC can be used as a new tool for improve crop production under abiotic stress.

Keywords Abiotic stress · Extracellular · Polyhydroxybutyrate (PHB) · PHB polymerase (PhbC) · Plants

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Introduction

The polyhydroxybutyrate (PHB) polymer is a highly reduced microbial storage compound, whose production was originally related with nutrient storage for survival under starvation conditions (Lopez et al. 1995). The key enzyme for the production of this storage compound is the intracellular PHB polymerase (PhbC) that recognizes specifically 3-hydroxybutyryl-CoA as substrate for polymerization (Steinbuchel and Hein 2001). While PHB is synthetized by this specific intracellular enzyme and PHB-producing strains normally have only one copy of *phbC* gene, this polymer can be degraded by both specific and unspecific varieties of PHB depolymerases (PhaZ) which are ubiquitous components of both prokaryotic and eukaryotic cells (Smithen et al. 2013). Moreover, an individual microorganism commonly codifies for several PhaZ enzymes (Eggers and Steinbuchel 2014). Previously, we showed that natural PHB-producing *Pseudomonas* strains have an uncommon strong tolerance to abiotic stress and that their PHB genes were incorporated recently by horizontal transfer events via mobile elements. Importantly, we demonstrated that the increased abiotic stress tolerance of these natural PHB-producing *Pseudomonas* strains is due to the ability of PHB to act as radical-scavenging antioxidant, osmolyte and molecular chaperone in the prevention of heat-, cold-, and saline-stress damage in free-living microbes (Pascuan

Published online: 24 May 2018

et al. 2015; Ayub et al. 2006, 2007, 2009, 2004; Soto et al. 2012; Fox et al. 2014). While these studies provide robust evidences for the function of PHB in abiotic stress adaptation in free-living microbes, the role of PHB as a plant growth-promoting factor remains unclear. Here we identify and characterize the role of a novel type of extracellular PhbC from bacterium FR1, a PHB-producing *Pseudomonas fluorescens* strain (Fig. 1a) isolated from the leaves of abiotic stress-tolerant Pangola grass.

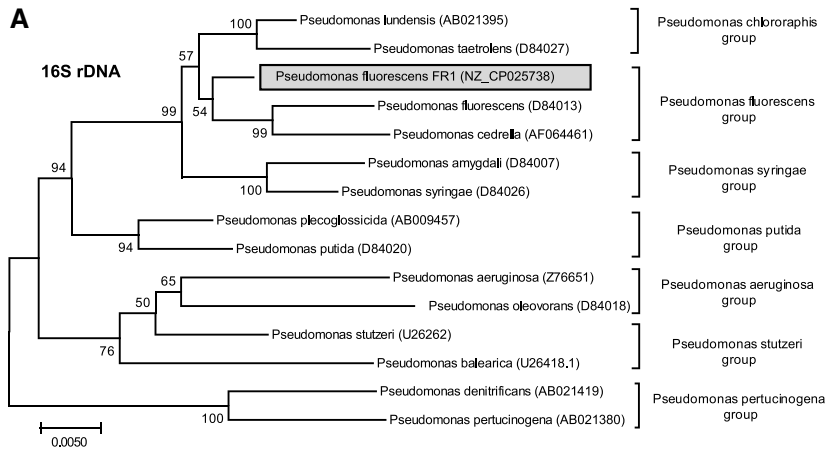
Materials and methods

The genomic DNA of strain FR1 was isolated from overnight cultures by using Wizard Genomic DNA Purification Kit (#A1120-Promega). As a result, a total of 18.09 mega reads (1.73 Gp) were obtained from Ilumina Hi-seq 1500 technology and assembled de novo as previously (Jozefkowicz et al. 2017). Annotations were carried out using NCBI Prokaryotic Genome Annotation Pipeline (Tatusova et al. 2016). The genome of strain FR1 was deposited in GenBank (accession number CP025738). Evolutionary analysis was conducted using MEGA7 software (http://www.megasoftware.net/). Protein sequences were aligned using the ClustalW program. Phylogenetic tree was constructed using the neighbor-joining (NJ) method, p-distance model, bootstrap analysis of 500 resamples and root on midpoint. Genomic synteny comparisons and detections of IR and GGXGXD repeats were performed by using LASTZ and Repeat Finder plugins into Geneious v10.1 software (https://www.geneious.com/). Operon were predicted by the FGENESB software (Mavromatis et al. 2007). Transmembrane helices in PhbC enzymes were predicted by PHOBIUS software (http://phobius.sbc.su.se). CoA release activities of the PhbC from FR1 in the extracellular and intracellular extracts were determined by spectrophotometric assays (Gerngross et al. 1994). The *phbC* mutant strain (FR1c) derived from strain FR1 was constructed as previously described (Ayub et al. 2009). Wheat plants were grown under greenhouse conditions and inoculated with *Pseudomonas* strains as previously described (Fox et al. 2016). Leaf chlorophyll and PHB levels in 3-month-old wheat plants grown under 21 °C (optimal conditions) or 0 °C (cold stress) for 24 h were measured with a portable chlorophyll meter and gas chromatography as previously described (Moriconi et al. 2013; Soto et al. 2012). GFP chromosomal tagging of *Pseudomonas* strains, plant colonization and bacterial survival assays were carried out by vector pBK-miniTn7-gf2 and by selective minimal medium NL as described previously (Fox et al. 2016).

Fig. 1 Identification and characterization of a novel type of extracellular PhbC from plant growth-promoting bacterium *Pseudomonas fluorescens* FR1. **a** Strain FR1 clustered within the *Pseudomonas fluorescens* group. Phylogenetic relationships among representative species of the genus *Pseudomonas* based on Neighbor-Joining analysis of the 16S rDNA gene. Bootstrap percentages of 50% or more are indicated at the branch points. Strain FR1 studied in this work is boxed. **b** Genome features of strain FR1. **c** The *phbC* gene of strain FR1 is located within a mobile genetic element, named Tn-PHB, which is absent in other related *Pseudomonas fluorescens* species. In this synthetic analysis, orthologous genes are shown in the same color, the orthologous block of genes is represented with a gray shadow, and arrows represent gene orientation. Tn-PHB is flanked by two inverted repeats (IR), and contains two operons (Tn and *phbC-hlyD-hlyB-tolC*), which are probably related to the transposition of this mobile element and the biosynthesis of PHB outside microbial cells, respectively. In addition with the typical conserved catalytic domain (light blue), extracellular enzyme PhbC from strain FR1 contains three transmembrane helices (green) and three GGXGXD export signals in the *C*-t region (yellow), which are absent in the intracellular enzyme PhbC from model strains *Ralstonia eutropha* H16 (H16) and *Azotobacter vinelandii* DJ (DJ). **d** Leaf chlorophyll level, **e** PHB levels and **f** colony forming units (CFU) in 3-month-old FR1-treated, FR1C-treated or uninoculated wheat plants at either optimal growth conditions or under cold stress (0 °C) for 24 h. PHB (wt%) = total PHB content with respect to the cell dry weight. Values are means ± SE of six replicates. The significance is indicated as $^{**}P < 0.01$, $^{***}P < 0.001$, ANOVA

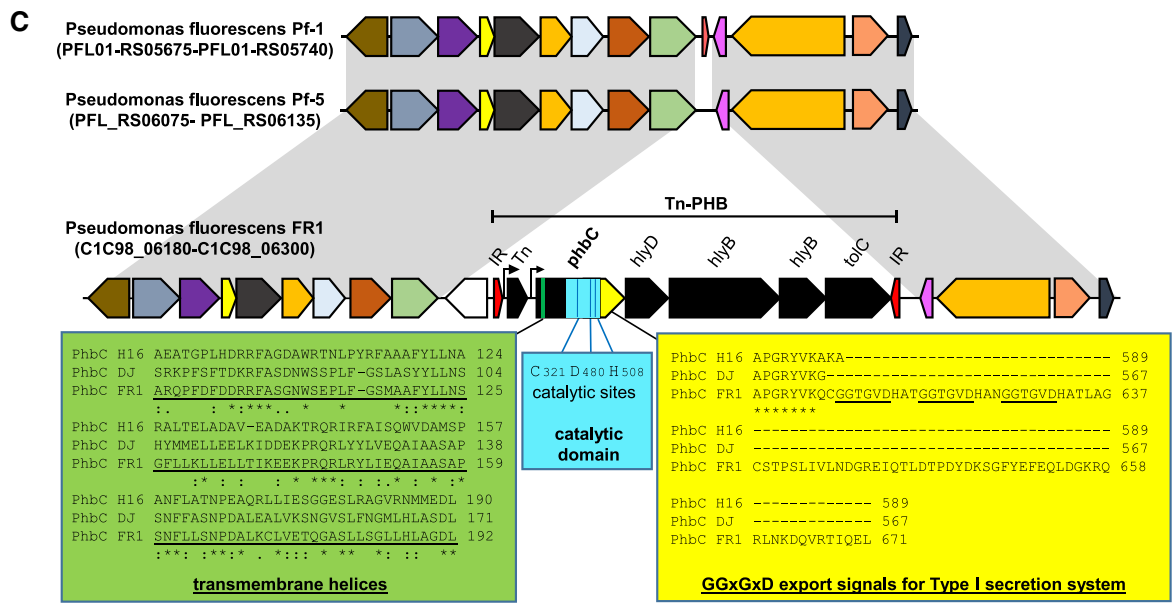
Results and discussion

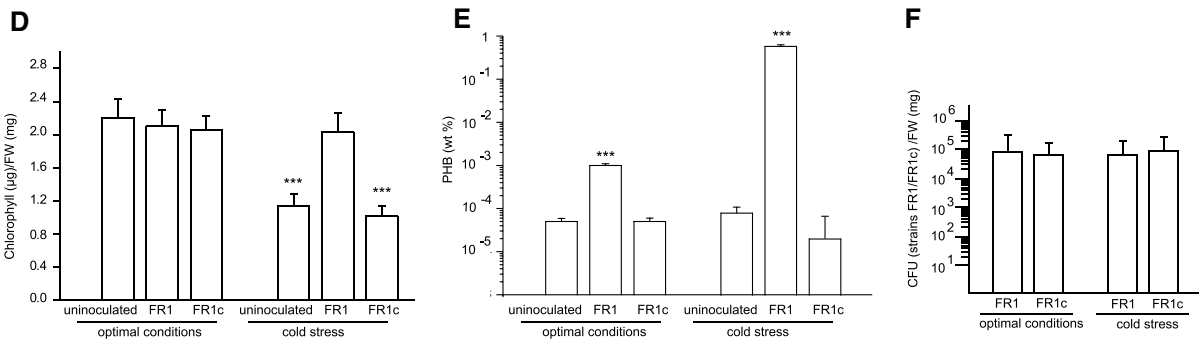
Genome analysis showed that the complete genome sequence of the bacterium FR1 consists of a circular chromosome (6,859,291 bp), and that the G+C content (60.8%) is similar to that of other *P. fluorescens* strains (Fig. 1b). Interestingly, we identified one copy of the *phbC* gene, which codified for the polyhydroxybutyrate (PHB) polymerase (PhbC) that recognizes specifically 3-hydroxybutyryl-CoA as substrate for polymerization (Steinbuchel and Hein 2001). In concordance with other reports showing that PHB synthesis is not an ancestral feature of the genus *Pseudomonas* (Ayub et al. 2007; Yan et al. 2008), a



B

Genome features of Pseudomonas fluorescens FR1	
Features	Values
Genome size	6,869,291 bp
G+C content	60.8 %
Total genes	6,321
Coding genes	6,235
Pseudo genes	229
rRNA	16
tRNA	66





10-kb block containing the *phbC* gene was found to be within a mobile genetic element (called Tn-PHB) unique to strain FR1 (Fig. 1c), suggesting that the *phbC* gene was acquired by horizontal transfer. The Tn-PHB is flanked by a 19 bp (5'-GAAATAGGGGT-CAGACCAC-3') inverted repeat (IR), which might be

part of its site-specific recombination site (Fig. 1c). Contrary to the typical *phbC* gene clustering with the *phbA* (β-thiolase) and *phbB* (3-ketoacyl-CoA reductase) genes for the intracellular production of PHB from acetyl-CoA, known as the ABC pathway (Fox et al. 2014), the *phbC* gene from FR1 is part of the same operon that also contains a complete Type I secretion system which can secrete proteins containing GGXGXD motifs in the *C*-t region (Fig. 1c). In contrast to the classical intracellular enzyme PhbC from model strains such as *Ralstonia eutropha* H16 and *Azotobacter vinelandii* DJ (Wittenborn et al. 2016), the PhbC from FR1 contains three transmembrane helices and three tandem GGXGXD repeats within its *N*-t and *C*-t regions, respectively, suggesting a extracellular localization of this polymerase. In agreement with this analysis, high levels of extracellular (0.035 U/mg total protein), but not intracellular (< 0.005 U/mg total protein) PhbC activity was observed in pure cultures of strain FR1. We also studied the putative relevance of PHB production on the beneficial effects promoted by strain FR1 in wheat plants by using a *phbC* mutant strain (FR1c). The non-inoculated wheat plants and the wheat plants inoculated with the mutant strain FR1c showed a significantly lower level of chlorophyll (Fig. 1d) and PHB production (Fig. 1e) under cold stress than in optimal conditions. In contrast, FR1-treated wheat plants were able to maintain the chlorophyll levels (Fig. 1d) and to produce high levels of PHB (Fig. 1e) under cold stress. In addition, the *phbC* mutation seems not to have affected wheat colonization under optimal conditions and bacterial survival under cold stress (Fig. 1f), which is consistent with a mutation in a plant growth promotion gene that has no pleiotropic effects on bacterial fitness *in planta* conditions. These results suggest that strain FR1 can be used as an inoculant to improve the production of wheat under low temperature environments, and that PHB could be a beneficial compound synthesized by endophytic bacteria under abiotic stress conditions.

Conclusions

During the last decades, the biotechnological relevance of PHB has been mainly focused on the production of this biodegradable polyester as a natural candidate for supplanting the ordinary plastic derived from non-renewable fossil fuels. In the last years, it has been described the capability of PHB to increase the stress tolerance of free-living *Pseudomonas* strains, expanding the ecological role of PHB in microbial life. Our findings of the use of PhbC-secreting endophytic *Pseudomonas* strains as a chassis to engineer the transfer of the ability to PHB biosynthesis in crops, open the way to the production of not only stress tolerant crops but also PHB biodegradable plastic using light energy. Finally, *in planta* production of PHB by natural strains can avoid the genetic manipulations of plants, and then, constitutes a promising alternative of the traditional production of transgenic stress-tolerant crops.

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