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Recombineering *Pseudomonas protegens* CHA0: An innovative approach that improves nitrogen fixation with impressive bactericidal potency

Fangnan Yu[a,b], Xiaoshu Jing[a], Xiaochen Li[a], Hailong Wang[a], Hanna Chen[b], Lin Zhong[a], Jia Yin[a,b], Deng Pan[c], Yulong Yin[b], Jun Fu[a], Liqiu Xia[b], Xiaoying Bian[a], Qiang Tu[a,*], Youming Zhang[a,b,*]

[a] *Shandong University–Helmholtz Institute of Biotechnology, State Key Laboratory of Microbial Technology, School of Life Science, Suzhou Institute of Shandong University, Shandong University, Qingdao, 266237, People's Republic of China*

[b] *Hunan Provincial Key Laboratory of Microbial Molecular Biology, State Key Laboratory of Developmental Biology of Freshwater Fish, College of Life Science, Hunan Normal University, Changsha 410081, People's Republic of China*

[c] *Jinan Yian Biology Institute, Shandong Yian Biological Engineering Co. Ltd., Jinan, 250100, People's Republic of China*

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ABSTRACT

Pseudomonas protegens CHA0 is a well-characterized, root-colonizing bacterium with broad-spectrum biocontrol ability. Therefore, it has a great potential to curb plant diseases and to partly replace synthetic chemical pesticides that are harmful to humans. Here, we obtained the multifunctional mutant CHA0-Δ*retS-Nif* via Red/ET recombineering technology. After deletion of the *retS* gene and integration of the nitrogen-fixing gene island (*Nif*) into the CHA0 genome, the resulting mutant, CHA0-Δ*retS-Nif*, manifested improved both bactericidal activity and biological nitrogen-fixation function. A pot experiment of *Arabidopsis thaliana* indicated that the strain CHA0-Δ*retS-Nif* promoted plant growth via expressing several secondary factors, such as the antibiotic 2,4-diacetylphloroglucinol (2,4-DAPG) and nitrogenase. In order to grow this biocontrol strain at an industrial level, the growth conditions in a 1 L continuous-flow fermenter were optimized to 28 °C, pH of 7.0, and 600 rpm. Moreover, growth experiments in a 5 L fermenter with these optimal growth conditions yielded the maximum cell density, providing vital insights for the industrialization and large-scale fermentation of *P. protegens* CHA0 for further applications. CHA0-Δ*retS-Nif* possesses both bactericidal and nitrogen-fixation activities and thus could be used as a biological agent to enhance crop production.

1. Introduction

Microorganisms are often applied in fields and orchards to improve soil fertility, plant growth, and crop health (Haas and Defago, 2005), and they are generally utilized in large quantities to achieve maximum efficacy. The usage of large-scale bacterial inoculants in agricultural began in the early 20th century, with much of their purpose related to the N-cycle (Vogel, 1922), nitrogen being an indispensable factor for crop production (Boddey and Dőbereiner, 1988). For better yield, huge amounts of chemical fertilizers were used in agriculture, which caused various environmental problems such as decrements in soil organic matter and fertility together with deterioration of the physical and chemical properties of the soil (Sun et al., 2012). The ideal solution would be to increase the amount of *Rhizobium* ssp. released to enhance the N-fixation potential of leguminous plants (Haas and Defago, 2005; Ma et al., 2007). However, previous studies emphasized that newly developed, distinctive strains failed to compete with ones introduced earlier and that had become indigenous to the soil (Paul and Clark, 2014). Such established strains may also impede the introduction of new strains. Furthermore, many other organisms (e.g., *Mycorrhiza*, *Bacillus* ssp., *Pseudomonas* ssp., *Trichoderma* ssp.) have already been released into agricultural crops and soils for nutrient ingestion improvement and plant growth promotion (Girlanda et al., 2001). Promisingly, so far none of these beneficial bacteria have been reported to produce adverse effects on the environment.

Pseudomonas protegens, a Gram-negative rod-shaped bacterium isolated from soils and plant roots, has also been studied extensively in recent decades for its biocontrol capabilities and higher application value in agriculture (Ma et al., 2007). The most obvious benefit of the strain, which is categorized as a plant growth-promoting rhizobacterium (PGPR) (Yang, 2012), is the control of soil-borne plant pathogens to enhance productivity and plant health. *P. protegens* has

* Corresponding authors at: Shandong University–Helmholtz Institute of Biotechnology, State Key Laboratory of Microbial Technology, School of Life Science, Suzhou Institute of Shandong University, Shandong University, Qingdao, 266237, People's Republic of China.

E-mail addresses: tuqiang1986@163.com (Q. Tu), zhangyouming@sdu.edu.cn (Y. Zhang).

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proven to be an efficient biocontrol strain against target pathogens and fungus because of its diverse biocontrol mechanisms, including competition for nutritional iron through production of ferritin (Wang et al., 2010); effective rhizosphere colonization (Barahona et al., 2011); induction of pathogen resistance in plants (Diyansah et al., 2014); and the enormous production of secondary metabolites (Nagarajkumar et al., 2005), such as 2,4-DAPG, pyoluteorin, phenazine, and pyrrolnitrin (Almario et al., 2013). *P. protegens* strain has the potential to impact the growth of bacteria, fungi, and even nematodes in soil, and hence, the widely used model strain *P. protegens* CHA0 was used in this study.

P. protegens CHA0 was isolated from tobacco roots and plays a strong role in the prevention and treatment of tobacco black root and wheat take-all diseases caused by *Thielaviopsis basicola* (Shaukat and Siddiqui, 2003). Additionally, previous research reported that the *retS*-encoded sensing kinase RetS could negatively regulate the expression of the antibiotic 2,4-DAPG, resulting in lower bactericidal activity of *P. aeruginosa* and *P. protegens* (Goodman et al., 2009; Brencic et al., 2009).

Genetic engineering has been applied to bacteria with various biological activities to improve the synthesis of diverse natural products (Ongley et al., 2013). For example, the crucial factor nitrogenase (encoded by *nif* gene clusters from the *P. stutzeri* A1501 strain and *Klebsiella pneumonia*) has been transferred into different heterologous hosts, such as *Escherichia coli* (Dixon and Postgate, 1972; Han et al., 2015), *P. fluorescens* Pf-5 (Setten et al., 2013; Fox et al., 2016), and *P. putida* MT20-3 (Postgate and Kent, 1987), conferring on them the ability to fix nitrogen. These results indicate that *nif* genes can be successfully expressed in obligate aerobic heterologous microbes. However, the methods used for genetic engineering of the gene clusters are often inefficient and time-consuming. Red/ET recombineering is a powerful and effective DNA genetic engineering tool that can be used for direct cloning of large genome sequences (Zhang et al., 1998, 2000; Bian et al., 2012; Fu et al., 2012). In this study, we used recombineering to improve *P. protegens* CHA0 for agricultural applications. Firstly, the *retS* gene was deleted from the chromosome of CHA0 by recombineering to enhance its biocontrol activity. Afterwards, a 49-kb *Nif* nitrogen-fixing gene island (Table S3) from the *P. stutzeri* DSM4166 genome, was transferred into CHA0-Δ*retS* mutant strains using Red/ET recombination, generating the final mutant strain, CHA0-Δ*retS-Nif*, which could successfully express nitrogenase. Moreover, the optimal fermentation conditions were established for CHA0-Δ*retS-Nif,* providing a framework for large-scale fermentation of the mutant in industrial settings.

2. Materials and methods

Strains and plasmids used in this research are shown in Table S1. Sequences of all the primers used in this work are listed in Table S2. All restriction enzymes, Taq polymerase, and DNA markers are purchased from New England Biolabs (UK).

2.1. Bacterial strains and growth conditions

All recombineering experiments were performed in *E. coli* strain GB2005; this strain and its derivatives were cultured in low-salt LB medium (LSLB, tryptone 10 g/L, yeast extract 5 g/L, NaCl 1 g/L, pH adjusted to 7.0 using 1 mol/L NaOH) containing antibiotics as needed (kanamycin [kan], 15 μg/mL; and gentamycin [genta], 15 μg/mL) with shaking at 200 rpm at 37 °C. The following strains were used: GB2005, derived from DH10B by deletion of *fhuA*, *ybcC,* and *recET* (Fu et al., 2008, 2010); GB05-red, derived from GB2005 by insertion of the P_{BAD}-*gbaA* cassette at the *ybcC* locus (Fu et al., 2010; Fu et al., 2012); and GB05-dir, derived by integrating the P_{BAD}-*ETgA* operon into the *ybcC* locus in GB2005 (Fu et al., 2012). The integration in GB05-dir ablates expression of *ybcC*, which encoded a putative exonuclease similar to that encoded by Redα. *E. coli* ET12567, the donor strain for intergeneric conjugation with *P. protegens* CHA0, was cultured in LSLB medium at 37 °C overnight (Buntin et al., 2010). *P. protegens* CHA0 and its mutant derivatives were grown at 30 °C in KB medium (K_2HPO_4 1.5 g/L, $MgSO_4{\cdot}7\ H_2O$ 1.5 g/L, peptone 20 g/L, glycerin 10 mL/L, pH 7.0) as described previously (Iavicoli et al., 2003).

2.2. Red/ET recombineering

All transgenic approaches were performed as previously described (Fu et al., 2012; Wang et al., 2016). For Red/ET recombineering, 0.3 μg of a linear DNA fragment (either a PCR product or a fragment obtained from restriction enzyme digestion) was electroporated into 50 μL Red/ET-competent *E. coli* cells (such as GB-red cells or GB-dir cells). After electroporation, colonies were grown on LSLB agar plates under selection for the target antibiotic resistance gene and were then examined for the intended Red/ET recombination product by restriction analysis with suitable enzymes.

All PCR reactions were carried out using Taq polymerase (Invitrogen GmbH, Karlsruhe, Germany) according to the manufacturer's protocol. For the amplification of the ~1000 bp cassette with high GC content, DMSO was added to a final concentration of 3%. PCR was performed using an Eppendorf master cycler with the following conditions: 10 min at 95 °C, denaturation at 95 °C (30 s), annealing at 58 °C (30 s), and extension at 72 °C (35 s); 35 cycles. The PCR products were used directly without any purification.

2.3. retS gene deletion from the chromosome of P. protegens CHA0

Plasmid pBBR1-Rha-TEGpsy-kan could express a special recombinase for *Pseudomonas* strains (manuscript under preparation) (Fig. S4), and 0.3 μg of this plasmid was electrotransferred into the wild-type CHA0 strain to obtain the mutant CHA0-P for subsequent homologous recombination steps. Next, the PCR product containing the *loxM-genta* cassette (0.3 μg in 2 μL) and corresponding homologous arms was electroporated into recombineering proficient competent cells of CHA0-P. Recombinants CHA0::Δ*retS-genta-loxM* were selected on KB plates containing 15 μg/mL of genta. Then 0.3 μg of plasmid pCM157, which expressed IPTG-inducible Cre enzyme (Marx and Lidstrom, 2002) (Fig. S4), was electrotransferred into CHA0::Δ*retS–genta-loxM* to remove the gentamycin resistance gene (Fig. 1). Nine final recombinant colonies of CHA0-Δ*retS* were randomly selected for PCR verification using primers check-5 and check-3 (Table S2), and ones showing the correct amplicon were verified by sequencing (Fig. S1).

The oligonucleotides RetS-Genta-loxM-5' and RetS-Genta-loxM-3' were used for insertion of the gentamycin resistance gene.

2.4. Engineering of the nitrogen-fixing gene island Nif

The genomic DNA of *P. stutzeri* DSM4166 (GenBank accession no. NC_017532.1) was isolated and digested with *Afl* II and *Ssp* I to release the 49-kb DNA fragment containing the entire *Nif* gene island. Next, the *Nif* gene island was directly cloned into the pBeloBAC11 vector via linear-linear homologous recombination mediated with RecET (Wang et al., 2016), using primers nif 1–4 (Table S2). Then, the MycoMar transposition cassette was integrated into the BAC vector containing the *Nif* gene island using Redαβ recombineering to form expression plasmid pBeloBAC11-oriT-TnpA-*genta-Nif* (Fig. 2). The resulting plasmid was checked by gentamicin selection and restriction digestion analysis using *Kpn* I (Fig. S2) and then transformed into *E. coli* ET12567 for further study.

2.5. Conjugation of P. protegens CHA0

pBeloBAC11-oriT-TnpA-*genta-Nif,* the expression construct containing the *Nif* gene island, was introduced into the chromosomes of *P. protegens* CHA0 WT and CHA0-Δ*retS* by amphipathic conjugation. The selection of integrant was carried out on KB medium agar plates containing genta (15 μg/mL), and transformants were randomly screened