Microbiological Research 218 (2019) 58–65

Contents lists available at ScienceDirect

Microbiological Research

journal homepage: www.elsevier.com/locate/micres

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*

ARTICLE INFO

Key words:
Pseudomonas protegens CHA0
retS gene
Nitrogen-fixation island (*Nif*)
Antibacterial activity
Fermentation conditions

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).

https://doi.org/10.1016/j.micres.2018.09.009
Received 24 November 2017; Received in revised form 6 August 2018; Accepted 28 September 2018
Available online 06 October 2018
0944-5013/

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

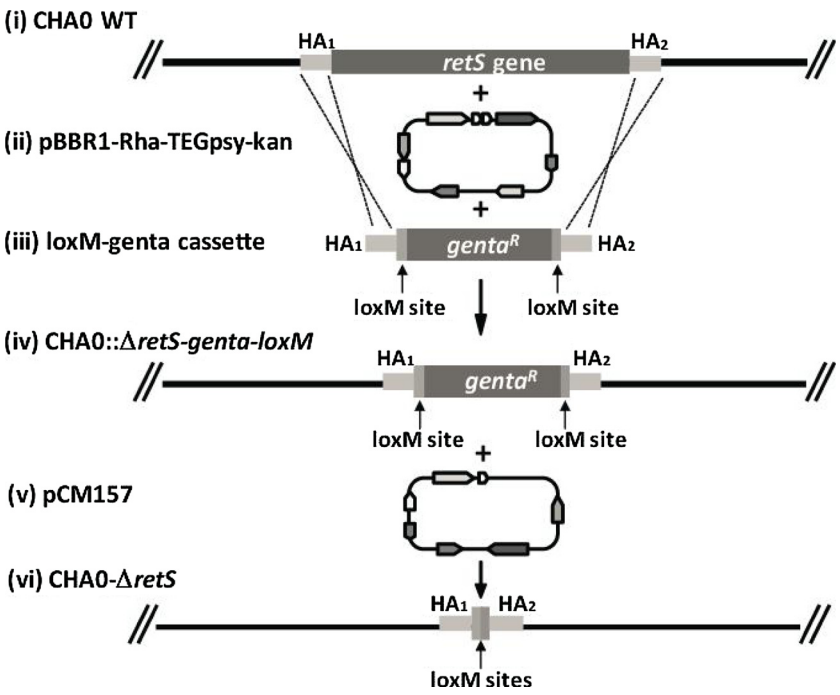

Fig. 1. Deletion of *retS* gene from the chromosome of *P. protegens* CHA0. In the first step, the plasmid pBBR1-Rha-TEGpsy-kan (ii) was transferred into the wild-type CHA0 strain (i) in preparation for subsequent homologous recombination. In the second step, the DNA cassette *loxM-genta* (iii) with corresponding homologous arms (including the loxM site) was generated by PCR and electrotransferred into the CHA0 strain containing pBBR1-Rha-TEGpsy-kan to delete the *retS* gene, generating the mutant strain CHA0::Δ*retS–genta-loxM* (iv). In the third step, plasmid pCM157 (v), which expresses IPTG-inducible Cre enzyme, was transferred into CHA0::Δ*retS-genta-loxM* (iv). After introduction of IPTG, the gentamycin-resistance gene was deleted from the chromosome resulting in the unmarked recombinant strain CHA0-Δ*retS*.

by colony PCR for successful *Nif* gene insertion, using four pairs of apra-check primers (primers apra-check-1 to apra-check-8, Table S2). Another pair of primers (check-5 and check-3) was used to verify that the introduced *Nif* nitrogen-fixing gene island was derived from *P. protegens* CHA0 rather than from *E. coli* ET12567 (Fig. S3). The verified transformants were named CHA0-*Nif* and CHA0-Δ*retS-Nif* (Fig. 2).

2.6. Expression and analysis of nitrogenase production

Nitrogenase activity in CHA0 strains was determined using the standard acetylene reduction assay (Cannon et al., 1976). First, activated strains that were grown on solid medium were inoculated into KB medium and cultured at 30 °C, 200 rpm for 8 h. Bacteria were then collected by centrifugation at 5000 rpm for 10 min at 4 °C, washed three times with 0.85% saline solution, and resuspended to achieve an OD_{600} of 1.0 in nitrogen-free medium. Next, 18 mL of nitrogen-free medium plus 2 mL of the bacterial suspension were added to a 100 mL anaerobic flask. At the same time, the headspace air was replaced with high-purity argon. Oxygen (1%) was then added, and the mixture was incubated at 30 °C for 6 h with shaking at 250 rpm. Afterwards, 10% of the mixed gas was extracted, and 10% acetylene gas was injected while maintaining the culture conditions. Samples were taken after 4 h of incubation. To assess activity, 100 µL of mixed gas from the bottle was gathered with a sterile syringe and injected into a Shimadzu GC2010 gas chromatograph to measure ethylene yields. The protein content was determined by the Bradford method (Bradford, 1976). All measurements were repeated at least three times, and the data were analyzed by Graph-Pad Prism 6 (GraphPad Software, Inc.) (Fig. 3C and 3D).

2.7. Assay of antibacterial activity

The antibacterial activity of CHA0 strains was determined with *Bacillus subtilis* (from our laboratory) as the reporter in an inhibition zone assay (Lehrer et al., 1991). Four 6-mm diameter, sterile filter pieces were placed on nearly dry LSLB plates. Cultures of *P. protegens* adjusted to an OD_{600} of 1.5 were spotted onto the filters, and 1 mL *Bacillus* samples were spotted onto the LSLB medium. After overnight incubation at 30 °C, all the cells were killed by UV irradiation on a transilluminator for 5 min. Antibiotic production by *P. protegens* was revealed by measuring growth inhibition zones around the filters (Fig. 3A).

2.8. Quantitative analysis of 2,4-DAPG by high-performance liquid chromatography (HPLC) and mass spectrometry (MS)

CHA0-Δ*retS-Nif* was cultured in 30 mL KB medium at 150 rpm and 30 °C for 24 h. Next, 1 mL of Amberlite XAD16 was added to the fermentation liquid, followed by shaking for 24 h, and then centrifugation at 8000 rpm for 10 min to collect the precipitate. Then, 30 mL ethyl acetate was mixed with the precipitate followed by shaking overnight. After vacuum concentration of the ethyl acetate mixture, 1 mL of methanol was added in preparation for HPLC detection. For HPLC, we used a reversed-phase column (2.1 x 100 mm, 2.2 µm, Thermo Scientific Acclaim TM C18) at 30 °C with the following detection conditions: mobile phase, composed of 0.1% aqueous acetic acid (solvent A) and acetonitrile (solvent B); elution procedure, 0–5 min, 5% solvent B; 5–20 min, 5%–95% solvent B; 20–25 min, 95% solvent B; flow rate 0.5 mL/min. An ultraviolet (UV) light detector monitored at 250 nm, 270 nm, 290 nm, and 310 nm (2,4-DAPG, λ = 270 nm) (Xie et al., 2016). MS measurements were performed on an amaZon velocity mass spectrometer (Bruker Daltonics, Bremen, Germany) and ultra-high resolution Qq-Time-of-Flight mass spectrometer (Bruker Daltonics) using a standard ESI (electrospray ionization) source. Mass spectra were acquired in a positive ion mode of 200–2000 m/z with automatic MS^2 fragmentation. Determination of 2,4-DAPG substances was based on the secondary MS (Xie Y et al. 2016). The experiments were independently performed three times.

2.9. Real-time PCR analysis

CHA0-Δ*retS-Nif* and control strains were cultured in KB medium for 24 h and then transferred to nitrogen-free medium for 6 h. Total RNA was extracted from strains CHA0-Δ*retS-Nif*, DSM4166, and CHA0 WT using Bioteke RNAPure. cDNA was obtained using the PrimeScript RT Reagent Kit with gDNA Eraser (Perfect Real Time, Takara). The expression of genes *nifD*, *nifK*, *nifN*, *nifM*, *nifQ*, *nifS*, and *nifT* from the nitrogen-fixing gene island was normalized to the expression of the housekeeping gene *GAPDH* by real-time PCR using SYBR Premix Ex Taq

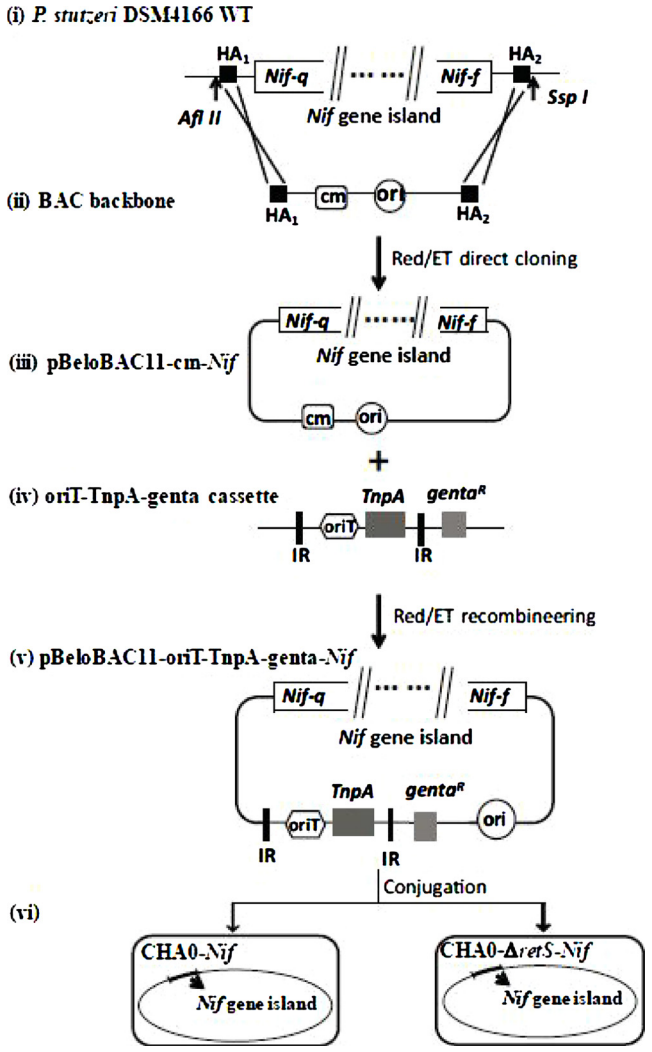

Fig. 2. Insertion of the *Nif* nitrogen-fixing gene island into *P. protegens* CHA0 by Red/ET recombineering. Genomic DNA of *P. stutzeri* DSM4166 (i) was digested with restriction endonucleases *Afl* II and *Ssp* I (restriction sites indicated by upward-pointing vertical arrows, ↑) to release the linear DNA fragment containing the 49-kb *Nif* gene island. Another linear DNA cassette containing the BAC backbone (ii) was generated by PCR amplification, and then these two linear DNA fragments were co-transformed into *E. coli* strain GB05-dir to form plasmid pBeloBAC11-cm-*Nif* (iii) via Red/ET direct cloning. After insertion of the oriT-TnpA-*genta* cassette (iv), the final expression plasmid pBeloBAC11-oriT-TnpA-*genta-Nif* (v) harboring a full-sized *Nif* gene island was obtained by Red/ET recombineering. This plasmid (v) was then transferred into *P. protegens* CHA0 strains by conjugation. Two types of transformants, CHA0-*Nif* and CHA0-Δ*retS-Nif*, were selected for further research (vi).

II (Tli RNaseH Plus), and the relative gene expression levels ($-\Delta CT$) were measured automatically (Setten et al., 2013). The experiments were independently performed three times.

2.10. Inoculation tests

For the inoculation assay, seeds of *Arabidopsis thaliana* Columbia-0 were surface-sterilized and vernalized for 3 days at 4 °C in darkness. Later, plants were disinfected in 2% hypochlorite and then incubated in a growth chamber at 22 °C under light/dark cycles of 16 h/8 h, with light intensities of 80 μmol m^{-2} s^{-1}, in 100% vermiculite cultures. *Arabidopsis* was grown in 1/2 MS medium (Cho and Seo, 2005) with or without a nitrogen source (NH_4NO_3 or KNO_3) for 4 weeks. After transplantation and further culturing for 7 days, *Arabidopsis* plants were inoculated either with wild-type samples (CHA0, DSM4166) or recombinant bacteria (CHA0-Δ*retS-Nif*) prepared as follows: 1 mL of overnight culture was grown at 30 °C in KB medium, centrifuged, and then resuspended in 1 mL of saline solution. Then, the 1 mL doses of the bacterial solution, containing 8×10^8–1×10^9 colony forming units, were applied to the roots of *A. thaliana* Columbia-0 once a day for three consecutive days. Four treatments were used in this test: (1) a negative control without nitrogen fertilizer; (2) application of wild-type *P. protegens* strain CHA0 as another negative control; (3) application of *P. stutzeri* DSM4166, the origin of the *Nif* nitrogen-fixing gene island, as a positive control; and (4) application of CHA0-Δ*retS-Nif* as the experimental group. For evaluation of plant growth, rosette diameter was measured between the two largest leaves at the recommended stage of development (Schwachtje et al., 2012) by Image J (Fig. 3).

2.11. Fermentation tests

To determine optimal growth conditions for industrial production, the CHA0-Δ*retS-Nif* mutant strains were cultured in a 1 L fermenter with KB medium for 96 h using single factor experiments testing temperature, pH, and speed (rpm). In order to enlarge the production scale of the mutant biocontrol bacterium, subsequent fermentations were also performed in a 5 L fermenter with the optimal growth conditions. All fermentation experiments were performed in duplicate.

3. Results

3.1. Construction of the CHA0-ΔretS mutant by deletion of the retS gene

To obtain a mutant strain with better bactericidal activities, the 2.8-kb *retS* gene was replaced with a genta-resistance gene in the chromosome of CHA0 using the Red/ET homologous recombination technique (Fig. 1, Fig. S5). The deletion was verified by PCR in ten random recombinant strains, and then in the second recombineering step, the genta-resistance gene was deleted by site-specific recombination between the two *loxM* site sequences. The sequencing results for nine random transformants manifested that all of them were the correct CHA0-Δ*retS* mutants.

3.2. Direct cloning of the Nif gene island and transfer of nif genes into CHA0

To express nitrogenase activity, the entire *Nif* gene island was transferred into a CHA0-Δ*retS* mutant and into the CHA0 WT strain using plasmid pBeloBAC11-oriT-TnpA-*genta-Nif* (Fig. 2 and S2). After the conjugation experiment, successful integration of the *nif* genes into the genomes of the final transformants, CHA0-*Nif* and CHA0-Δ*retS-Nif*, was confirmed by PCR verification (Fig. 2 and S3).

3.3. Antibacterial activity and 2,4-DAPG yield in CHA0 mutants

The antibacterial activity of the recombinant strains was compared with that of the parental CHA0 strain using *Bacillus subtilis* as the indicator strain. As shown in Fig. 3A, the inhibition zones generated by CHA0-Δ*retS* and CHA0-Δ*retS-Nif* were 2.7 cm in diameter, whereas those of CHA0 and CHA0-*Nif* were only 2.3 cm. These results indicated that strains without the *retS* gene generated significantly larger inhibition zones. Moreover, we analyzed the metabolite profile using HPLC, and the results revealed that the yield of the antibiotic 2,4-DAPG was significantly higher, by approximately 100-fold, in CHA0-Δ*retS* mutants than in CHA0 (Fig. S5). These findings suggest that deletion of *retS* strengthened the antibacterial ability of the CHA0 strain by improving production of 2,4-DAPG.

3.4. Nitrogenase activity and pot experiments

In order to characterize the heterologous nitrogenase complex,

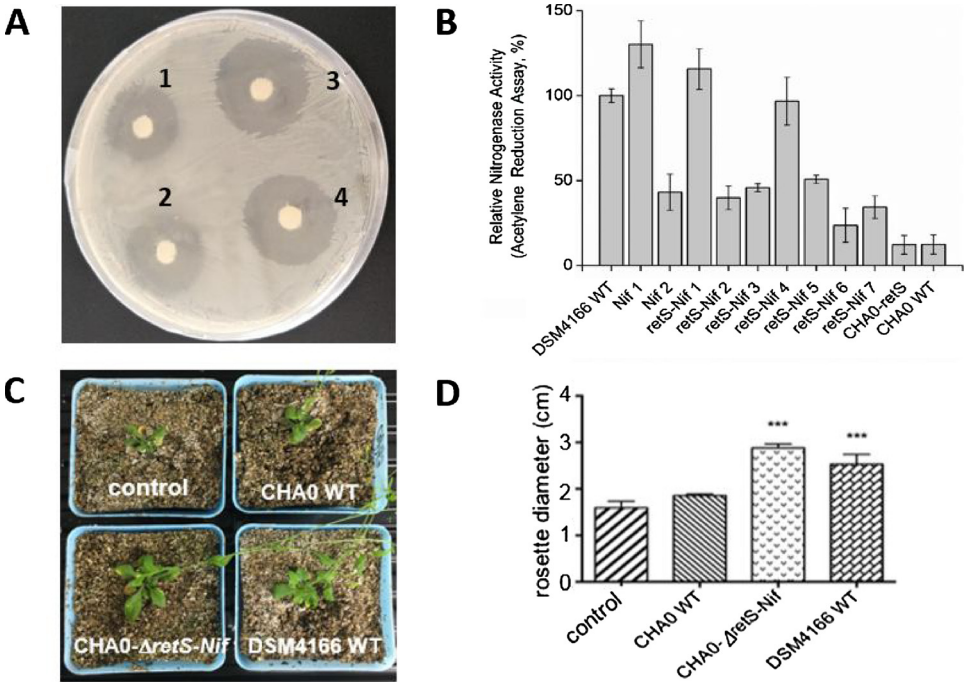

Fig. 3. Biological activity analysis of CHA0 mutants. (A) Inhibition zone assay for CHA0 and mutants with *Bacillus subtilis* as the indicator strain. 1, CHA0 WT; 2, CHA0-*Nif;* 3, CHA0-Δ*retS*; 4, CHA0-Δ*retS-Nif*. (B) Influence of *retS* deletion or *Nif* integration on nitrogenase activity of CHA0 transformants. The activity of native nitrogenases expressed by DSM4166 WT was given a value of 100% and was compared with the activity of 11 other strains: Nif 1 and Nif 2, two transformants of CHA0 containing an integrated *Nif* island; retS-Nif 1 through retS-Nif 7, seven individual transformants of CHA0-Δ*retS* containing an integrated *Nif* island; CHA0-Δ*retS,* the CHA0 strain lacking the *retS* gene; and CHA0 WT. (C) *Arabidopsis thaliana* pot experiment. Plants were grown without application of any nitrogen fertilizer and were left uninoculated (control) or were inoculated with CHA0 WT, the recombinant strain CHA0-Δ*retS-Nif*, or DSM4166 WT. (D) Diameter of *A. thaliana* rosette leaves. The same experimental groups were used as shown in (C). Results are the mean of at least three independent measurements. Standard error of the mean is indicated. ***P < 0.001, statistically significant difference between the sample and control, using Student's t-test.

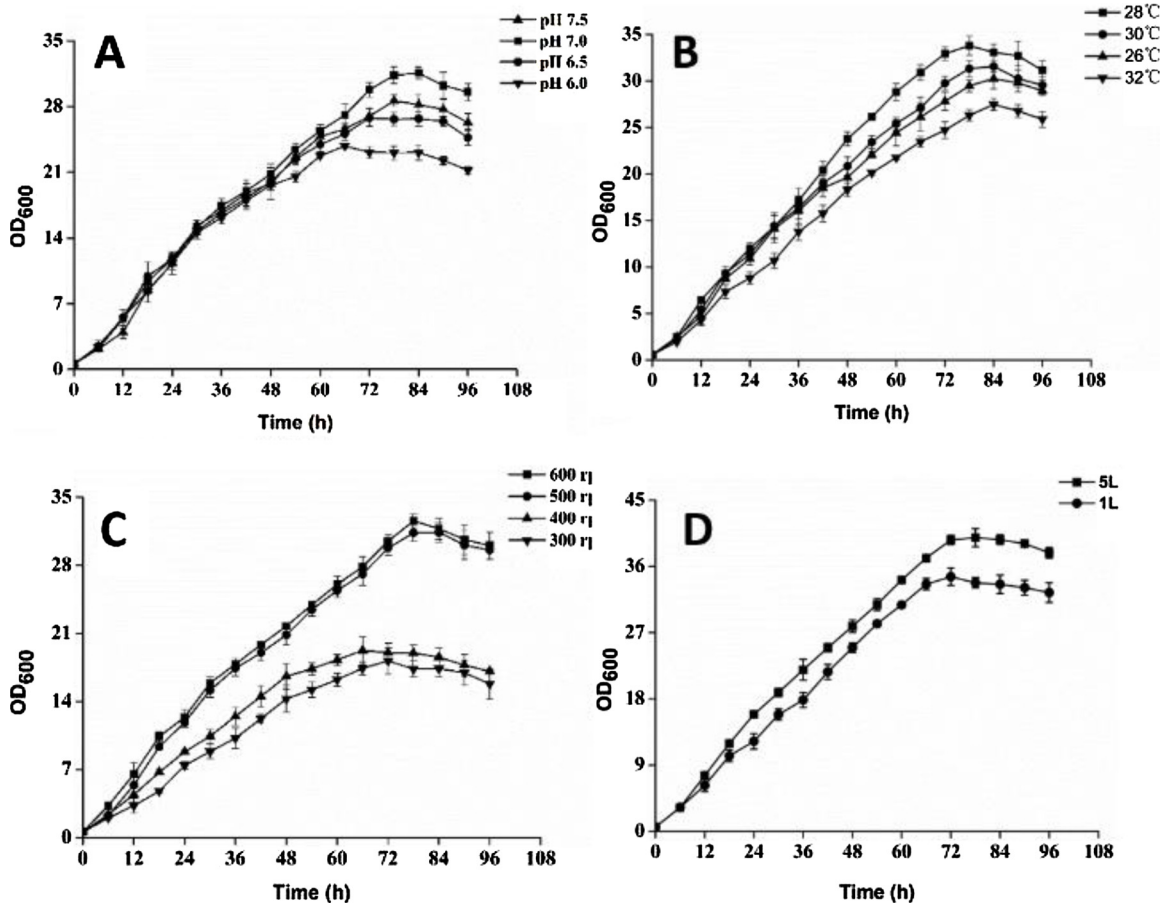

Fig. 4. Fermentation of CHA0-Δ*retS-Nif* under different growth conditions. Each experiment was repeated three times, and growth of the cultures was measured by optical density. Bars represent standard error of the mean. (A–C) Single factor experiments to evaluate the effects of changes in (A) pH, (B) temperature, and (C) rotational speed (rpm) on growth. Cultures were grown in a 1 L fermenter. (D) Comparison of growth of CHA0-Δ*retS-Nif* in 1 L and 5 L fermenters using optimal growth conditions (28 °C, pH 7.0, and rotational speed of 600 rpm) determined from panels A–C.

nitrogenase activity in CHA0-*Nif* and CHA0-Δ*retS-Nif* in KB medium was evaluated using the acetylene reduction assay (Cannon et al., 1976). The activity of nitrogenases in wild-type DSM4166 represented 100% activity in each case. All the CHA0-based transformants containing integrated *nif* genes exhibited nitrogenase activity (Fig. 3B), whereas only minimal nitrogenase activity was detected in wild-type CHA0 and CHA0-Δ*retS*. The mutants with higher nitrogenase activity (such as transformants retS-Nif1 and retS-Nif2) were selected for pot experiments with *Arabidopsis thaliana,* with the results revealing that mutant retS-Nif1 was the optimal candidate as it had the highest nitrogenase activity among the transformants.

We assessed the effect of inoculation with CHA0-Δ*retS-Nif* on the growth of *Arabidopsis* (Fig. 3C). The plant was grown in 1/2 MS medium with or without nitrogen (NH_4NO_3 or KNO_3), and inoculated with CHA0 WT, DSM4166 WT, or CHA0-Δ*retS-Nif,* or not inoculated (non-inoculated control). After six weeks, the uninoculated plants and plants inoculated with CHA0 WT showed less growth than plants in this same medium inoculated with wild-type DSM4166 and CHA0-Δ*retS-Nif* (Fig. 3C). This growth enhancement in medium without nitrogen appears to be due to inoculation with the recombinant bacterium CHA0-Δ*retS-Nif* (Fig. 3D), consistent with the high nitrogenase activity levels in this strain (Fig. 3B). Additionally, according to our results, the mutant strain CHA0-Δ*retS-Nif* has a higher capacity than DSM4166 WT to fix nitrogen.

For further evaluation of the expression level of the *nif* genes in CHA0-Δ*retS-Nif*, we selected the best transformant, retS-Nif1, for real-time PCR analysis. The results showed that the expression levels of *nifD*, *nifK*, *nifN*, *nifM*, *nifQ*, *nifS,* and *nifT* in CHA0-Δ*retS-Nif* were higher than in DSM4166 (Fig. S6). As expected, no amplification was detected for CHA0 WT.

3.5. Fermentation optimization for mutant strain CHA0-ΔretS-Nif

To investigate the physiological characteristics of strain CHA0-Δ*retS-Nif*, several growth conditions were evaluated in a 1 L fermenter using single factor experiments. Comparing the effects under different experimental conditions, there was a slight inflection in the specific growth rate and bacterial production, perhaps resulting from the metabolic burden of expressing a substantial number of secondary metabolites (Fig. 4). As shown in Fig. 4A, during the first 48 h of incubation, different pH conditions had no significant effect on bacterial densities nor on growth rates. However, after 48 h, final cell densities differed markedly among pH conditions, with a pH of 7.0 optimal for growth of CHA0-Δ*retS-Nif*. Using a pH of 7.0, we next varied the temperature (Fig. 4B), with results indicating maximum growth at 28 °C. Finally, different rotational speeds were tested at pH 7.0 and 28 °C (Fig. 4C). Overall, the optimal fermentation conditions for CHA0-Δ*retS-Nif* in a 1 L fermenter include a pH of 7.0, 28 °C, and a rotational speed of 600 rpm.

For the preparation of pilot plant experiments, the target strain CHA0-Δ*retS-Nif* was cultivated under the same optimal fermentation conditions in a 5 L fermenter. As shown in Fig. 4D, the bacterial growth rate in the logarithmic growth phase was markedly higher in the 5 L fermenter than in the 1 L fermenter. Furthermore, the final bacterial density was higher in the 5 L fermenter, with an OD_{600} value of 40 compared to an OD_{600} value of 33.0 for the 1 L fermenter. However, the overall growth patterns were similar, with higher growth rates in the early logarithmic phase compared to the middle and late logarithmic phases.

4. Discussion

Pseudomonas protegens is a particularly useful biocontrol agent against bacterial species, and strains CHA0 and Pf-5 can produce a variety of antibiotics and secondary metabolites. Pf-5 has been especially useful as a model strain and widely used to understand the secondary metabolites that are produced by *P. protegens,* in addition to its native protease biosynthetic pathways (Kidarsa et al., 2013). However, comparison of the whole genome sequences of CHA0 and Pf-5 revealed that CHA0 has a larger genome and contains additional genes that contribute to its industrial value. To name only a few, CHA0 has Cluster 2 genes for a nitrite/nitrate assimilation operon, indicating that CHA0 is more effective at reducing nitrate content in soil. CHA0 also has the following notable genes: Cluster S3 genes, which code for a penicillin-binding protein; Cluster S10 genes that encode a formate dehydrogenase; Cluster S13 genes that encode McrBC restriction enzymes; and Cluster S11 and Cluster S12 genes, a number of silencing gene clusters that are capable of expressing secondary metabolites (Takeuchi et al., 2014). The presence of the above-mentioned gene clusters in the CHA0 genome suggests that CHA0 is a new and attractive alternative for the application of *P. protegens* in biocontrol, and potentially in the construction and industrialization of genetically engineered strains.

As previously mentioned, antibiotic production increased in *P. protegens* CHA0 mutants upon *retS* gene deletion from the chromosome (Humair et al., 2009). The results of the inhibition zone test against *Bacillus subtilis* and metabolic analysis of 2,4-DAPG with HPLC suggest that the mutant strain increased 2,4-DAPG production after *retS* gene deletion, thereby enhancing its ability to inhibit *Bacillus subtilis* growth. However, the size of the inhibition zone was nearly unaltered in the CHA0-*Nif* mutant, which contains the *Nif* gene island, when compared to the wild-type CHA0 strain. Interestingly, the results for CHA0-Δ*retS* were similar to those of CHA0-Δ*retS-Nif,* which is also a *retS* knockout strain but has an integrated nitrogen-fixation operon. Thus, the introduction of the nitrogen-fixing gene island had no direct effect on the antibacterial activity of CHA0 strains.

In our work, the *Nif* gene island, consisting of many various genes from the non-anaerobic bacteria *P. stutzeri* DSM4166, was heterologously expressed in the affinitive strain *P. protegens* CHA0. The constitutive nitrogenase activity of the various CHA0-Δ*retS-Nif* transformants indicates that the Nif proteins were successfully expressed in all of the transformants, although the extent of nitrogenase activity differed among them. This variation probably occurred because the *Nif* gene island was randomly inserted into the genome of mutant CHA0 strains, and the positioning of *Nif* gene island at different genomic locales would affect the expression of Nif proteins. Importantly, the growth of *A. thaliana* after application of CHA0-Δ*retS-Nif* bacteria was superior to that of the control groups, and even superior to plants exposed to the original strain DSM4166 with integration of the *Nif* gene island. These results might be explained by the biocontrol activity of *P. protegens* CHA0, which has its own biocontrol mechanisms, autotrophic lifestyle, and has been demonstrated to promote plant growth. Therefore, the new biocontrol strain CHA0-Δ*retS-Nif* has diverse effects on plant growth after gaining nitrogen-fixing ability.

Engineering large clusters of genes encoding complex functional enzymes for natural product biosynthesis has been formidable via conventional technology. To genetically engineer the large *Nif* gene island, Red/ET recombineering and direct cloning, a potent technique based on *in vivo* homologous recombination in *E. coli*, was used in this work (Zhang et al., 1998, 2000; Fu et al., 2008, 2012). This method allows the cloning of large gene clusters directly from genomic DNA, without the construction and screening of genomic libraries (Fu et al., 2012). An additional benefit of this technology is that it operates irrespective of restriction enzyme sites as well as the size of the DNA fragments to be shuffled. Recently, this technique had been applied in studies of various natural product biosynthetic gene clusters, including syringolins from *Pseudomonas syringae* (Bian et al., 2012), glidobactins and luminmycins from *Burkholderia* DSM7029 (Bian et al., 2015), edeines from *Brevibacillus brevis* X23, and bacillomycins from *Bacillus amyloliquefaciens* FZB42 (Liu et al., 2016). Compared with previous methods of *Nif* gene island construction (Dixon and Postgate, 1972; Postgate and Kent, 1987; Setten et al., 2013; Han et al., 2015; Fox et al., 2016), our approach is convenient and cost-effective with high accuracy. Hence, Red/ET recombineering is a preferred choice for research

and reconstruction of such complex DNA regions of interest.

In order to optimize the growth conditions for the mutant strain CHA0-Δ*retS-Nif*, we investigated the effects of pH, temperature, and rotational speed using single factor experiments. Afterwards, our analyses suggest that the optimal growth conditions include a temperature of 28 °C, pH of 7.0, and 600 rpm. However, despite the great potential of this genetically modified strain, it is essential not to underestimate the potential complications in its use. The recombinant strain may work well in some regions or be replaced by indigenous microorganisms, possibly depending on the physical and chemical properties of the local environment. Increasing the inoculum size of the introduced strain may be one way to overcome potential problems. We anticipate that our engineered strains will be industrialized by large-scale fermentation in the future, using the growth conditions we test with the 5 L fermenter.

In conclusion, here we represent a new approach to developing nitrogen-fixing recombinant bacteria for application in advancing plant growth under nitrogen-deficient conditions. Our analyses have also laid the groundwork for small-scale production and pilot-plant scale production of these strains for use in agro-biotechnology.

Author contributions

FY, XJ, QT and YZ planned the projects. FY, XL, HW, HC, JY, LZ and DP performed the molecular biology experiments. FY and XJ performed the activity verification experiments. YY, JF, LX, XB, QT, and YZ performed the data analyses and wrote the manuscript. All authors discussed the results and commented on the manuscript.

Conflict of interest

Author Deng Pan was employed by Shandong Yian Biological Engineering Co. Ltd. All other authors declare no competing interests.

Acknowledgements

This work was supported by funding from the Recruitment Program of Global Experts (1000 plan), the Project of Taishan Industry Leading Talents in Shandong Province (LJNY201603), China Postdoctoral Science Foundation Grant (No. 2018M632669), the Major Basic Program of Natural Science Foundation of Shandong Province (ZR2017ZB0212), Science and Technology Development Program of Suzhou (SYG201507), the Program of Introducing Talents of Discipline to Universities (B16030) and the National Natural Science Foundation of China (31700004) and Huxiang Youth Excellence project (2017RS3029). The authors acknowledged Dr. Vinothkannan Ravichandran's help in proofreading this manuscript.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.micres.2018.09.009.

References

Almario, J., Prigent-Combaret, C., Muller, D., et al., 2013. Effect of clay mineralogy on iron bioavailability and rhizosphere transcription of 2,4-diacetylphloroglucinol biosynthetic genes in biocontrol *Pseudomonas protegens*. Mol. Plant Microbe Interact. 26, 566–574.

Barahona, E., Navazo, A., Martínez-Granero, F., et al., 2011. *Pseudomonas fluorescens* F113 mutant with enhanced competitive colonization ability and improved biocontrol activity against fungal root pathogens. Appl. Environ. Microbiol. 77, 5412–5419.

Bian, X., Huang, F., Stewart, F.A., et al., 2012. Direct cloning, genetic engineering, and heterologous expression of the syringolin biosynthetic gene cluster in *E. coli* through Red/ET recombineering. ChemBioChem 13, 1946–1952.

Bian, X., Huang, F., Wang, H., Klefisch, T., Müller, R., Zhang, Y., 2015. Heterologous production of glidobactins/luminmycins in *Escherichia coli* Nissle containing the glidobactin biosynthetic gene cluster from *Burkholderia* DSM7029. ChemBioChem 15, 2221–2224.

Boddey, R.M., Dőbereiner, J., 1988. Nitrogen fixation associated with grasses and cereals: recent results and perspectives for future research. Plant Soil 108, 53–65.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Brencic, A., McFarland, K.A., Mcmanus, H.R., et al., 2009. The GacS/GacA signal transduction system of *Pseudomonas aeruginosa* acts exclusively through its control over the transcription of the RsmY and RsmZ regulary small RNAs. Mol. Microbiol. 73, 434–445.

Buntin, K., Irschik, H., Weissman, K.J., Luxenburger, E., Blöcker, H., Müller, R., 2010. Biosynthesis of thuggacins in myxobacteria: comparative cluster analysis reveals basis for natural product structural diversity. Chem. Biol. 17, 342–356.

Cannon, F.C., Dixon, R.A., Postgat, J.R., 1976. Derivation and properties of F-prime-factors in *Escherichia-coli* carrying nitrogen-fixation genes from *Klebsiella-pneumoniae*. J. Gen. Microbiol. 93, 111–125.

Cho, U.H., Seo, N.H., 2005. Oxidative stress in *Arabidopsis thaliana* exposed to cadmium is due to hydrogen peroxide accumulation. Plant Sci. 168, 113–120.

Dixon, R.A., Postgate, J.R., 1972. Genetic transfer of nitrogen fixation from *Klebsiella pneumoniae* to *Escherichia coli*. Nature 237, 102–103.

Diyansah, B., Aini, L.Q., Hadiastono, T., 2014. The effect of PGPR (Plant Growth Promoting Rhizobacteria) *Pseudomonas fluorescens* and *Bacillus subtilis* on the health of leaf mustard plant (*Brassica juncea* L.) infected by TuMV (Turnip Mosaic Virus). J. Tropic. Plant Protect 1, 30–38.

Fox, A.R., Soto, G., Valverde, C., et al., 2016. Major cereal crops benefit from biological nitrogen fixation when inoculated with the nitrogen-fixing bacterium *Pseudomonas protegens* Pf-5 X940. Environ. Microbiol. 18, 3522–3534.

Fu, J., Wenzel, S.C., Perlova, O., et al., 2008. Efficient transfer of two large secondary metabolite pathway gene clusters into heterologous hosts by transposition. Nucleic Acids Res. 36, e113.

Fu, J., Teucher, M., Anastassiadis, K., et al., 2010. A recombineering pipeline to make conditional targeting constructs. Meth. Enzymol. 477, 125–144.

Fu, J., Bian, X., Hu, S., et al., 2012. Full-length RecE enhances linear-linear homologous recombination and facilitates direct cloning for bioprospecting. Nat. Biotech. 30, 440–446.

Girlanda, M., Perotto, S., Moenneloccoz, Y., et al., 2001. Impact of biocontrol *Pseudomonas fluorescens* CHA0 and a genetically modified derivative on the diversity of culturable fungi in the cucumber Rhizosphere. Appl. Environ. Microbiol. 67, 1851–1864.

Goodman, A.L., Merighi, M., Hyodo, M., et al., 2009. Direct interaction between sensor kinase proteins mediates acute and chronic disease phenotypes in a bacterial pathogen. Genes Dev. 23, 249–259.

Haas, D., Defago, G., 2005. Biological control of soil-borne pathogens by fluorescent pseudomonads. Nature Rev. Microbiol. 3, 307–319.

Han, Y., Lu, N., Chen, Q., et al., 2015. Interspecies transfer and regulation of the *Pseudomonas stutzeri* A1501 nitrogen fixation island in *Escherichia coli*. J. Microbiol. Biotechnol. 25, 1339–1348.

Humair, B., González, N., Mossialos, D., et al., 2009. Temperature-responsive sensing regulates biocontrol factor expression in *Pseudomonas fluorescens* CHA0. ISME J. 3, 955–965.

Iavicoli, A., Boutet, E., Buchala, A., et al., 2003. Induced systemic resistance in *Arabidopsis thaliana* in response to root inoculation with *Pseudomonas fluorescens* CHA0. Mol. Plant Microbe Interact. 16, 851–858.

Kidarsa, T.A., Shaffer, B.T., Goebel, N.C., et al., 2013. Genes expressed by the biological control bacterium *Pseudomonas protegens* Pf-5 on seed surfaces under the control of the global regulators GacA and RpoS. Environ. Microbiol. 15, 716–735.

Lehrer, R.I., Rosenman, M., Harwig, S.S., et al., 1991. Ultrasensitive assays for endogenous antimicrobial polypeptides. J. Immunol. Methods 137, 167–173.

Liu, Q.S., Shen, Q.Y., Bian, X., et al., 2016. Simple and rapid direct cloning and heterologous expression of natural product biosynthetic gene cluster in *Bacillus subtilis* via Red/ET recombineering. Sci. Rep. 6, 34623.

Ma, C.T., Hu, Q., Yang, D.K., 2007. Progress of the prevention and treatment of the profitable soil microorganism for plants diseases. Shandong Sci. 20, 61–67.

Marx, C.J., Lidstrom, M.E., 2002. Broad-host-range cre-lox system for antibiotic marker recycling in gram-negative bacteria. Biotechniques 33, 1062–1067.

Nagarajkumar, M., Jayaraj, J., Muthukrishnan, S., et al., 2005. Detoxification of oxalic acid by *Pseudomonas fluorescens* strain pfMDU2: implications for the biological control of rice sheath blight caused by *Rhizoctonia solani*. Microbiol. Res. 160, 291–298.

Ongley, S.E., Bian, X., Neilan, B.A., et al., 2013. Recent advances in the heterologous expression of microbial natural product biosynthetic pathways. Nat. Prod. Rep. 30, 1121–1138.

Paul, E.A., Clark, F.E., 2014. Soil microbiology and biochemistry. J. Range Manage. 51, 254.

Postgate, J.R., Kent, H.M., 1987. Qualitative evidence for expression of *Klebsiella pneumoniae nif* in *Pseudomonas putida*. J. Gen. Microbiol. 133, 2563–2566.

Schwachtje, J., Karojet, S., Kunz, S., et al., 2012. Plant-growth promoting effect of newly isolated rhizobacteria varies between two *Arabidopsis ecotypes*. Plant Signal Behav. 7, 623–627.

Setten, L., Soto, G., Mozzicafreddo, M., et al., 2013. Correction: Engineering *Pseudomonas protegens* Pf-5 for nitrogen fixation and its application to improve plant growth under nitrogen-deficient conditions. PLoS One 8, e63666.

Shaukat, S.S., Siddiqui, I.A., 2003. Impact of biocontrol agents *Pseudomonas fluorescens* CHA0 and its genetically modified derivatives on the diversity of culturable fungi in the rhizosphere of mungbean. J. Appl. Microbiol. 95, 1039–1048.

Sun, Z., Duan, X., Xie, Y., et al., 2012. Nutrient supplying capacity of typical black soil and fertilizer use efficiency of soybean in Heilongjiang province. Chin. Agric. Sci. Bull. 28, 46–51.

Takeuchi, K., Noda, N., Someya, N., 2014. Complete genome sequence of the biocontrol

strain *Pseudomonas protegens* Cab57 discovered in Japan reveals srain-specific diversity of this species. PLoS One 9, e93683.
Vogel, 1922. Impfung von Hülsenfrüchten und Nichtleguminosen. Zeitschrift Für Pflanzenernährung Und Düngung 1, 531–535.
Wang, P., Hui, L.I., Qiu, Y.X., et al., 2010. Siderophores produced by *Pseudomonas fluorescens* P13 against *Sclerotinia sclerotiorum*. J. Shanghai Normal Univ. 39, 200–203.
Wang, H., Li, Z., Jia, R., et al., 2016. RecET direct cloning and Redαβ recombineering of biosynthetic gene clusters, large operons or single genes for heterologous expression. Nat. Protoc. 11, 1175–1190.
Xie, Y., Liu, Z., Zhang, G., et al., 2016. A rifampicin-resistant (rpoB) mutation in *Pseudomonas protegens* Pf-5 strain leads to improved antifungal activity and elevated production of secondary metabolites. Res. Microbiol. 167, 625–629.
Yang, Y., 2012. The antibiotic metabolites genes of *Pseudomonas fluorescens*. China Biotechnol. 32, 100–106.
Zhang, Y., Buchholz, F., Muyrers, J.P., et al., 1998. A new logic for DNA engineering using recombination in *Escherichia coli*. Nat. Genet. 20, 123–128.
Zhang, Y., Muyrers, J.P., Testa, G., et al., 2000. DNA cloning by homologous recombination in *Escherichia coli*. Nat. Biotechnol. 18, 1314–1317.