Microbiological Research 167 (2012) 596–601

Contents lists available at SciVerse ScienceDirect

Microbiological Research

journal homepage: www.elsevier.de/micres

Characterization and analysis of *nifH* genes from *Paenibacillus sabinae* T27

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ARTICLE INFO

Article history:
Received 6 February 2012
Received in revised form 16 May 2012
Accepted 17 May 2012

Keywords:
Paenibacillus sabinae
nifH
Nitrogenase
Promoter

ABSTRACT

Paenibacillus sabinae T27 (CCBAU 10202=DSM 17841) is a gram-positive, spore-forming diazotroph with high nitrogenase activities. Three *nifH* clusters were cloned from *P. sabinae* T27. Phylogenetic analysis revealed that NifH1, NifH2 and NifH3 cluster with Cyanobacterium. Each of the coding regions of *nifH1*, *nifH2* and *nifH3* from *P. sabinae* T27 under the control of the *nifH* promoter of *Klebsiella pneumoniae* could partially restore nitrogenase activity of *K. pneumoniae nifH*$^-$ mutant strain 1795, which has no nitrogenase activity. This suggests that the three *nifH* genes from *P. sabinae* T27 have some function in nitrogen fixation. RT-PCR showed that all three *nifH* genes were expressed under nitrogen-fixing growth conditions. Using promoter vectors which have promoterless *lacZ* gene, three putative promoter regions of *nifH* genes were identified.

1. Introduction

Nitrogen fixation is mainly governed by nitrogen fixation (*nif*) genes whose expressions are strictly regulated by environmental oxygen and ammonium. The *nifD* and *nifK* genes specify α and β subunits, respectively, of the molybdenum iron protein (dinitrogenase) and the iron protein (dinitrogenase reductase) is encoded by *nifH* (Rubio and Ludden 2005).

The nucleotide sequences for coding regions of *nifHDK* genes among all nitrogen-fixing organisms are highly conserved. However, the copy numbers and arrangement of *nifH*, *nifD*, and *nifK* are different among the different diazotrophic bacteria. In many microorganisms, including *Klebsiella pneumoniae*, *Azospirillum brasilense*, *Gluconacetobacter diazotrophicus* and *Herbasipirillum seropedicae*, there is only one *nifH* gene and the *nifHDK* genes are transcribed as a single unit. However, some diazotrophs have multiple *nifH* genes. It is reported that *Clostridium pasteurianum* has six *nifH* (*nifH1*) and *nifH*-like (*nifH2*, *nifH3*, *nifH4*, *nifH5* and *nifH6*) genes whose sequence identities range between 68 and 99.9% within the *nifH* coding regions (Chen 2004; Wang et al. 1988).

Bacillus and *Paenibacillus* are Gram-positive, spore-forming bacteria and can survive even in strict environments. The members of nitrogen-fixing *Bacillus* and *Paenibacillus* have great potential for use as a bacterial fertilizer in agriculture. Presently, there are only a few reports about nitrogen fixation in these bacteria. For example, *Paenibacillus azotofixans* contains three copies of *nifH* (Choo et al. 2003) and *Paenibacillus massiliensis* T7 contains a *nifBHDKENX cluster* (Zhao et al. 2006).

Paenibacillus sabinae T27 is a Gram-positive, spore-forming diazotroph which is a novel species isolated and named by our lab and it has high nitrogenase activities (Ma et al. 2007). Currently, nothing is known about the nitrogen fixation of this bacterium. In this study, three *nifH* genes are cloned, and their activities and putative promoter regions are characterized.

2. Materials and methods

2.1. Media and growth conditions

P. sabinae T27 was cultured in soypeptone medium, which contains (1 l): 5 g soypeptone, 3 g beef extract, 5 g NaCl, pH 7.0. Optimal growth occurs at 30 °C in aerobic conditions.

The nitrogen limited medium for nitrogenase measurement of *K. pneumoniae* and *P. sabinae* T27 contains (1 l): $Na_2HPO_4{\cdot}12H_2O$ 26.3 g; KH_2PO_4 3.4 g; biotin 10 μg; $CaCl_2{\cdot}2H_2O$ 26 mg; $MgSO_4$ 30 mg; $MnSO_4{\cdot}H_2O$ 0.33 mg; ferric citrate 36 mg; $Na_2MoO_4{\cdot}2H_2O$ 7.6 mg; p-aminobenzoic acid 10 μg; glucose 4 g; glutamic acid 1 g.

2.2. Southern blot analysis

The genomic DNA of *P. sabinae* T27 was extracted using the procedure described by Marmur and Yoon (Marmur 1961; Yoon et al. 1996), and digested thoroughly by restriction enzymes *Pst* I, *EcoR* I, *Sal* I, *Not* I, *Hind* III, *Cla* I and *Apa* I. The 324-bp *nifH* fragment was PCR amplified from *P. sabinae* T27 genomic DNA by using two degenerate primers *nifHP1* and *nifHP2* (Ding et al. 2005), labeled using DIG-DNA Labeling and Detection Kit purchased from Roche

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http://dx.doi.org/10.1016/j.micres.2012.05.003

(Germany). Southern blot hybridization was carried out using the labeled *nifH* fragment as a probe following the instructions from this kit.

2.3. Cloning of nif genes

The genomic DNA of *P. sabinae* T27 was digested by *Hind*III and separated on an agarose gel. After southern blot analysis, the DNA regions containing the *nifH* gene were purified and the products were ligated to pUC18 (Norrander et al. 1983). Then, ligated products were used to transform *Escherichia coli* JM109 competent cells and positive clones possessing *nifH* fragments were selected by colony-PCR with the primers *nifHP1* and *nifHP2*, and then sequenced. Three fragments containing *nifH* genes were cloned. The first fragment contains *nifH1* clustered with partial *nifB1* and partial *nifD*. The second fragment contains *nifH2* alone. The third fragment contains *nifH3* and an ABC transporter gene *t2*. With the self-ligated products as a template, after genomic DNA of *P. sabinae* T27 was digested by *Sac* II, inverse PCR was performed with the primers inverse pcr 1 and inverse pcr 2 to clone *nifB1* and its regulation region. The genomic DNA of *P. sabinae* T27 was digested by *EcoR* I and *Pst* I, then separated on an agarose gel. After southern blot analysis, the DNA regions containing the *nifH* gene were purified and the products were ligated to pUC18 which was digested by the same enzymes. Next, the ligated products were transformed to *E. coli* competent cells and the positive clones possessing *nifH* fragments were selected by colony-PCR with the primers *nifHP1* and *nifHP2*, and then sequenced. Using this method, *nifX-like gene* and partial *nifN* were cloned. In addition, TAIL-PCR was performed with the four shorter arbitrary degenerate primers (AD1, AD2, AD3 and AD4) described by Liu and Whittier (1995) and three nested sequence-specific primers Sp1, Sp2 and Sp3, designed based on the partial *nifD* sequence to obtain the full-length *nifD* and its downstream nucleotides, and specific primers st1, st2 and st3 were designed based on *t2* to obtain the *t1* and its upstream nucleotides. The sequence assembly and the ORF analysis was performed with the software DNAMAN (Version 5.2.2, Lynnon Biosoft, Canada) and the BLAST (Basic Local Alignment Search Tool) from NCBI website.

2.4. Construction of plasmids

In order to determine the function of *nifH* genes, overlap PCR (Ho et al. 1989) was performed to fuse *nifH1*, *nifH2* and *nifH3* ORF of *P. sabinae* T27 with the promoter of *nifH* gene from *K. pneumoniae*. The primers used in fusion were A1, A2, B1 and B4 for *nifH1*, A1, A2, B1 and B2 for *nifH2*, A1, A4, B5 and B6 for *nifH3*, respectively. Then, the fusion PCR products were individually ligated to pVK100 (Knauf and Nester 1982) and transformed to the mutant strain 1795 (Chang et al. 1988).

To determine the *nifH* promoter regions of *P. sabinae* T27 in *E. coli*, the three DNA fragments directly upstream of *nifH* genes P1, P3 and P5 were amplified by PCR, digested with *Kpn* I and *Xho* I, and then ligated to pPR9TT carrying *lacZ* coding region (Santos et al. 2001), following digestion by the same two enzymes. The primers used in the process were q1 up and q1 down for promoter region P1, q3 up and q3 down for promoter region P3, q5 up and q5 down for promoter region P5, respectively.

To determine the *nifH* promoter activities of *P. sabinae* T27 in *Bacillus*, the *lacZ* coding region was separated from pPR9TT and digested with *Kpn* I and *Xba* I; then inserted into the corresponding site of the plasmid pBE2 (Guo et al. 1991) which could replicate in *Bacillus*, yielding plasmid ppLacZ. Subsequently, each of the three promoter regions for *nifH* genes of *P. sabinae* T27 were cloned by PCR and ligated to ppLacZ, yielding recombinant plasmids.

2.5. RT-PCR

In order to study expression of the three *nifH* genes, RT-PCR was performed. The primers used were RT-1 and RT-2 for *nifH1*, RT-3 and RT-4 for *nifH2*; and RT-31 and RT-32 for *nifH3*, respectively.

Fresh cultures of *P. sabinae* T27 grown under 2 mM glutamic acid anaerobically (an anaerobic condition was produced by the addition of CO_2) to an approximate OD_{600} 0.2 were used to extract total RNAs by Aurum Total RNA Fatty and Fibrous Tissue Kit (BioRad). The first strand of cDNA was synthesized with random primers using the RevertAid^{TM} First Strand cDNA Synthesis Kit from Fermentas.

The conditions of the RT-PCR reactions: 25 μl reactions containing 1 μl cDNA, 0.5 μl each of forward and reverse primer, 2.5 μl PCR buffer, 1.7 μl (25 mM $MgCl_2$), 0.5 μl (10 mM dNTPs), 0.3 μl Taq DNA polymerase and 19 μl H_2O. Reactions were cycled at 95 °C for 3 min; 30 cycles of 95 °C for 20 s, 58 °C for 20 s, and 72 °C for 30 s; and finally, 72 °C for 10 min.

2.6. Enzyme assays of nitrogenase and β-galactosidase

The activity of β-galactosidase was measured using o-nitrophenyl-β-D-galactopyranoside as the substrate via the method described by Miller and expressed in Miller units (Miller 1972). For measurement, of nitrogenase activity, the *K. pneumoniae* cells were grown overnight, collected and washed twice with

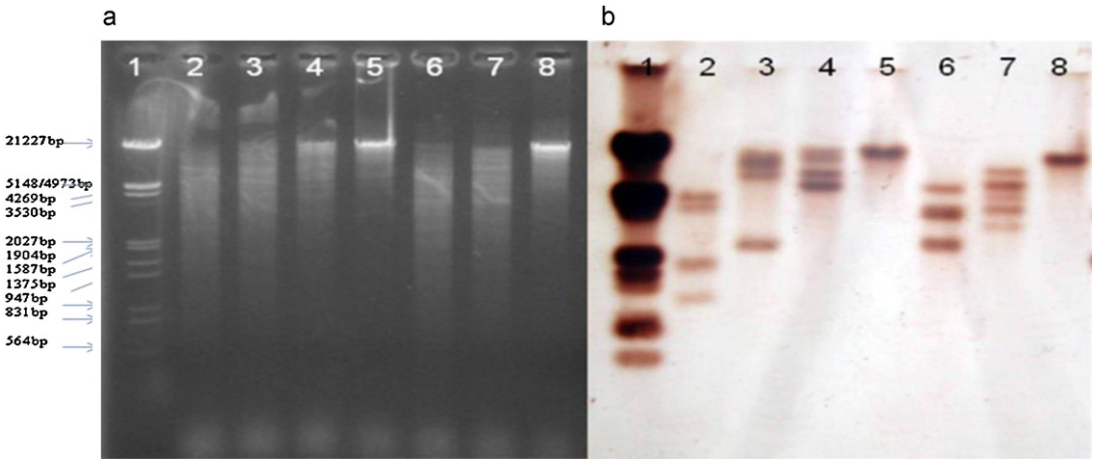

Fig. 1. Genomic DNA restriction profile of *P. sabinae* T27 by different enzymes (a) and southern blot profile with the 324-bp *nifH* label (b). Lane 1: λDNA/*Hin*d III+ *Eco*RI; Lane 2: DNA/*Pst*I; Lane 3: DNA/*Eco*RI; Lane 4: DNA/*Sal*I; Lane 5: DNA/*Not*I; Lane 6: DNA/*Hin*d III; Lane 7: DNA/*Cal*I; Lane 8: DNA/*Apa*I.

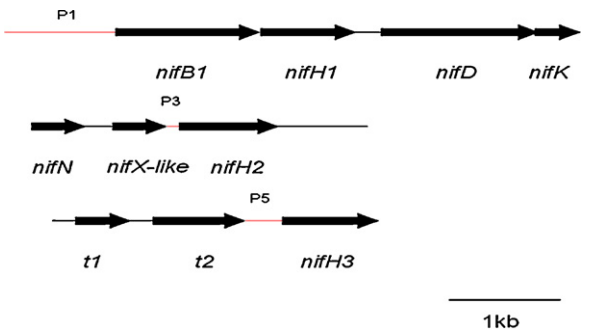

Fig. 2. Organization of *nif* genes from *P. sabinae* T27. The *nifH1* gene is together with *nifB1*, *nifD* and *nifK*; *nifH2* is together with *nifX-like* and *nifN*; and *nifH3* is clustered with two ABC transporter genes *t1* and *t2*. Three putative promoter regions of *nifH* genes are shown in red color. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

nitrogen limited medium; then resuspended in culture to OD_{600} 0.1 with the nitrogen limited medium. Using a vacuum pump, an anaerobic condition was produced by the addition of argon, and, simultaneously acetylene was added. Strains were incubated at 30 °C, under 10% (v/v) acetylene in the atmosphere for 20 h then analyzed for ethylene production by Gas Chromatography (HP6890, the software is HP CHEMSTATION Ver. 5.01). Nitrogenase activity was expressed as nmol ethylene h^{-1} mg^{-1} protein. The concentration of protein was measured by the method of Bradford (1976).

2.7. Phylogenetic analysis

The deduced amino acid sequences of the *nifH* gene products from the five strains were aligned, using CLUSTAL_X software (Thompson et al. 1997) with NifH sequences held in GenBank. A phylogenetic tree was generated using the neighbor-joining method with the software package TREECONW (Van de Peer and De Wachter 1994).

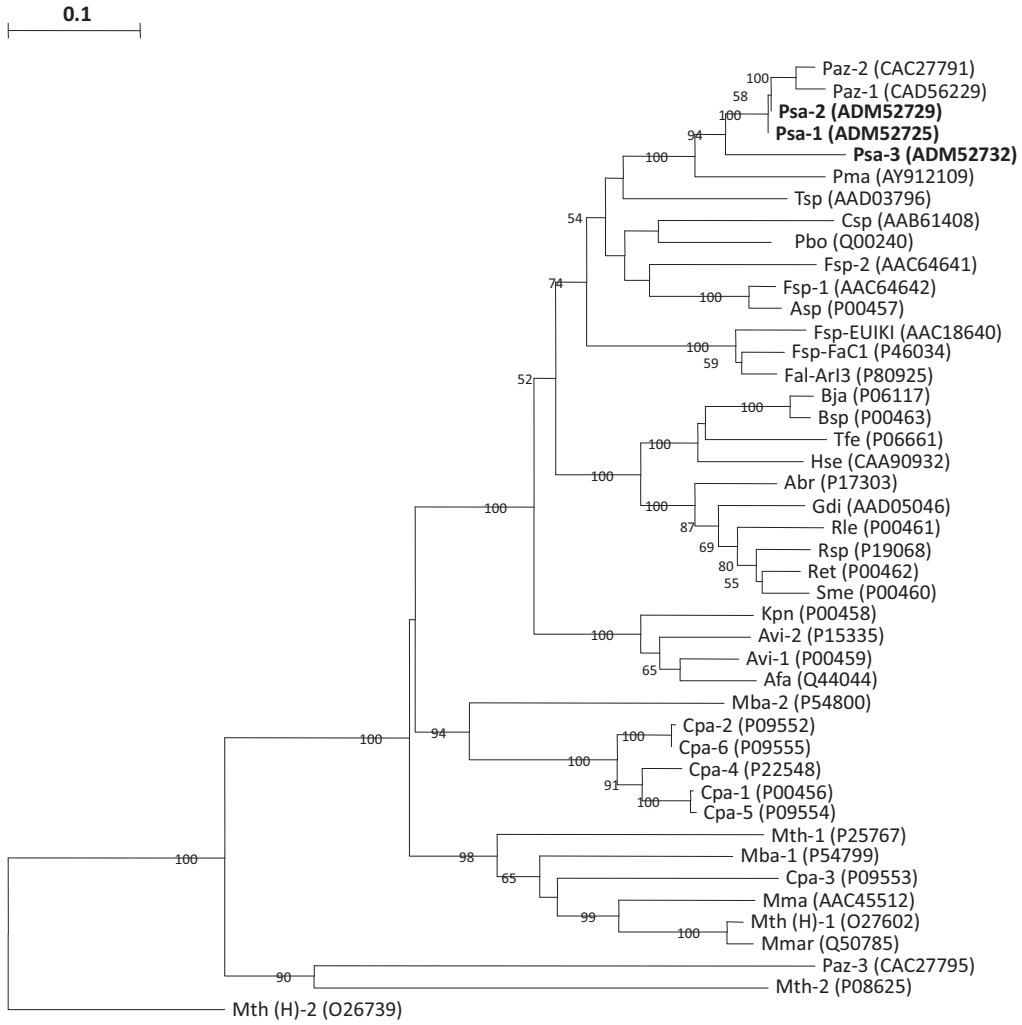

Fig. 3. Tree showing phylogeny of NifH polypeptide sequences, constructed by the neighbor-joining method. Graphic representation of the tree was made using TREECONW software. The database accession numbers are indicated after the abbreviations. The data was analyzed with 100 bootstrap values. The values presented above the nodes are the bootstrap values generated. Bootstrap values below 50% are not shown. The scale bar represents 0.1 substitution per site. Abbreviations: Abr, *Azospirillum brasilense*; Afa, *Alcaligenes faecalis*; Asp, *Nostoc* sp. strain PCC7120; Avi, *Azotobacter vinelandii*; Bsp, *Bradyrhizobium* sp. strain ANU289; Bja, *Bradyrhizobium japonicum*; Cpa, *Clostridium pasteurianum*; Csp, *Cyanothece* sp. strain ATCC 51142; Fal-ArI3, *Frankia alni* strain ArI3; Fsp-EUIK1, *Frankia* sp. strain EUIK1; Fsp-FaC1, *Frankia* sp. strain FaC1; Fsp, *Fischerella* sp. strain UTEX1931; Gdi, *Gluconacetobacter diazotrophicus*; Hse, *Herbaspirillum seropedicae*; Kpn, *Klebsiella pneumoniae*; Mba, *Methanosarcina barkeri*; Mma, *Methanococcus maripaludis*; Mth (H), *Methanothermobacter thermoautotrophicus* str. Delta H; Mmar, *Methanothermobacter marburgensis* strain Marburg; Mth, *Methanothermococcus thermolithotrophicus*; Paz, *Paenibacillus azotofixans*; Pbo, *Plectonema boryanum*; Ret, *Rhizobium etli*; Pma, *Paenibacillus massilensis* strain T7; Rle, *Rhizobium leguminosarum*; Rsp, *Rhizobium* sp. strain NGR234; Sme, *Sinorhizobium meliloti*; Tfe, *Acidithiobacillus ferrooxidans*; Tsp, *Trichodesmium* sp. strain IMS101.

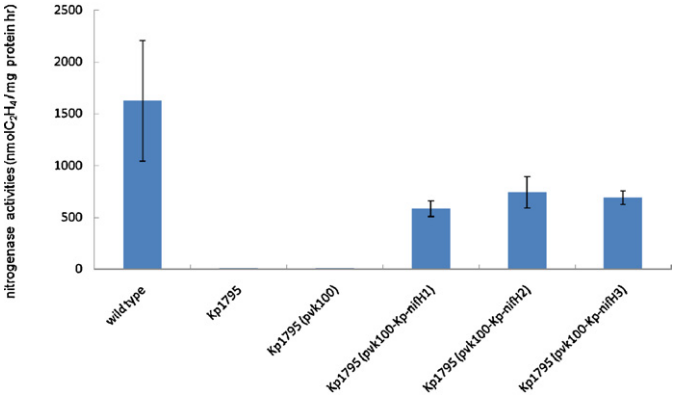

Fig. 4. The nitrogenase activities of complementary strains and control strains. Wild-type: *K. pneumoniae* (Kp) strain UN; Kp1795: *K. pneumoniae nifH* mutant strain 1795; Kp1795 (pvk100): Kp1795 carrying vector pvk100; Kp1795 (pvk100-Kp-nifH): Kp1795 carrying Kp *nifH* promoter and coding region of *P. sabinae nifH1* or *nifH2* or *nifH3* in vector pvk100.

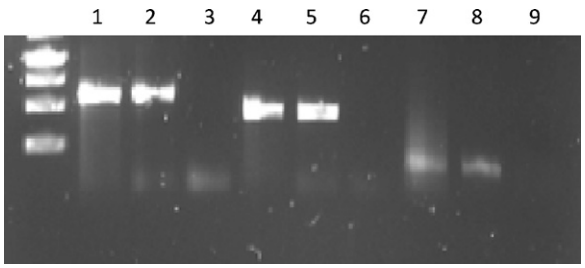

Fig. 5. Transcriptional analysis of three *nifH* genes from *P. sabinae* T27, using RT-PCR in limited fixed nitrogen (2 mM glutamic acid) anaerobically. Lane 1: genomic DNA was used as template, primers were designed for *nifH1*; Lane 2: cDNA was used as template, primers were designed for *nifH1*; Lane 3: RNA was used as template, primers were designed for *nifH1*; Lane 4: genomic DNA was used as template, primers were designed for *nifH2*; Lane 5: cDNA was used as template, primers were designed for *nifH2*; Lane 6: RNA was used as template, primers were designed for *nifH2*; Lane 7: genomic DNA was used as template, primers were designed for *nifH3*; Lane 8: cDNA was used as template, primers were designed for *nifH3*; Lane 9: RNA was used as template, primers were designed for *nifH3*.

3. Results

3.1. Cloning of three nifH clusters from P. sabinae T27

Three *nifH* clusters were isolated from *P. sabinae* T27. Firstly, a 324-bp fragment of the *nifH* gene was amplified from genomic DNA using two degenerate primers *nifHP1* and *nifHP2* (Ding et al. 2005) and thus labeled with digoxigenin. The southern blot hybridization revealed *P. sabinae* T27 contains more than one *nifH* genes (Fig. 1). Furthermore, three *nifH* clusters were cloned through screening a plasmid library combined with inverse PCR and TAIL-PCR (Fig. 2). Moreover, sequence comparison and BLAST analysis revealed the first 5272-bp fragment contains a 1377-bp *nifB1* followed by 879-bp *nifH1*, 1458-bp *nifD* and 319-bp partial *nifK*. The 2949-bp second fragment contains 473-bp partial *nifN*, followed by 495-bp *nifX-like* and 891-bp *nifH2*. The 3014-bp third fragment contains a 495-bp ABC transporter gene (*t1*), followed by an 822-bp ABC transporter gene (*t2*) and 873-bp *nifH3*. The sequencing data obtained in this study have been deposited in the GenBank database under the following accession numbers: HM583798, HM583799, and HM583800. The sequence identities of *nifH1*/*nifH2*, *nifH1*/*nifH3*, *and nifH2*/*nifH3* are 96.57%, 78% and 78.81%, respectively. These results are consistent with Choo's study that found there are three copies of *nifH* in *P. azotofixans* which is a species closely related to *P. sabinae* T27 (Choo et al. 2003). *P. azotofixans* NifH1 and NifH2 clustered with Cyanobacterium, *P. azotofixans* NifH3 clustered with NifH proteins of Archaea. *P. sabinae* T27 NifH1, NifH2 and NifH3 clustered with Cyanobacterium (Fig. 3). The *nifH1*, *nifH2* and *nifH3* of *P. sabinae* T27 have 52–95% DNA identities with *nifH1*, *nifH2* and *nifH3 of P. azotofixans*, respectively. The DNA identities of *nifH1*, *nifH2* and *nifH3* of *P. sabinae* T27 with *nifH1*, *nifH2*, *nifH3*, *nifH4*, *nifH5* and *nifH6* of *C. pasteurianum* range between 56% and 60%, respectively.

3.2. Each of the three nifH genes from P. sabinae T27 can complement the K. pneumoniae nifH⁻ mutant

To further study the functions of the *nifH1*, *nifH2* and *nifH3* of *P. sabinae* T27, *K. pneumoniae nifH* mutant strain 1795, which has no nitrogenase activity, was complemented with the three *nifH* genes of *P. sabinae* T27 under the control of *K. pneumoniae nifH* promoter. As shown in Fig. 4, complementary strains carrying *nifH1* or *nifH2* or *nifH3* partially restored the nitrogenase activity of *K. pneumoniae nifH* mutant strain 1795 to 40%, 46% and 33% activity of the wild-type strain UN, respectively, while the control strain Kp1795 and Kp1795 (pvk100) carrying vector pvk100 did not have any nitrogenase activities. The data suggests that all three *P. sabinae* T27 *nifH* genes are functional in *K. pneumoniae*.

3.3. RT-PCR analysis of nifH gene expression

RT-PCR analysis was performed to assess whether all three *nifH* genes were transcribed in nitrogen-fixing growth conditions (2 mM glutamic acid and no oxygen). As shown in Fig. 5, all three *nifH* genes were expressed in mRNA levels.

3.4. Three putative promoter regions of P. sabinae T27 nifH genes

Because plasmids cannot be transformed to *P. sabinae* T27 successfully, a preliminary research about the putative promoter regions of *P. sabinae* T27 *nifH* genes were done by using heterologous expression analysis systems (*E. coli* and *Bacillus cereus*).

In order to identify *P. sabinae* T27 *nifH* promoter regions, promoter probe vector pPR9TT was used. Following other researchers (Chang et al. 2007; Charania et al. 2009; Santos and Correia 2007), the DNA fragments directly upstream of *nifH1*, *nifH2* and *nifH3* ORF (P1, P3 and P5) (Fig. 2) were in-frame translationally fused to promoterless *lacZ* in vector pPR9TT or ppLacZ and the plasmids were transformed to *E. coli* JM109 and *B. cereus* B905, respectively. As shown in Table 1, the three putative promoter-*lacZ* fusions are expressed in *E. coli* JM109. The β-galactosidase activities of *E. coli* transformants carrying promoter regions P1, P3 and P5 with *lacZ* fusions are 989, 302 and 54 miller units, respectively, suggesting that all three promoter regions have activities in *E. coli*. Comparative analysis showed that there was no β-galactosidase activity in *E. coli* JM109 transformants carrying *A. brasilense nifH* promoter-*lacZ* translational fusion, most likely due to lack of activator NifA in *E. coli* (Table 1).

As shown in Table 1, the three promoter regions for *nifH* genes are also expressed in *B. cereus* B905. The β-galactosidase activities of *B. cereus* transformants carrying promoter regions P1, P3 and P5 with *lacZ* fusions are 66, 359 and 10 miller units, respectively. Compared to those of *E. coli* transformants, the β-galactosidase activities of *B. cereus* transformants were much lower.

All things considered, we demonstrate that the nitrogenase activities of *P. sabinae* T27 are repressed by oxygen and ammonium chloride; and there are at least three functional *nifH* genes in *P. sabinae* T27.

Table 1
The β-galactosidase activities (β-GA) of the three putative promoter regions of *nifH* with *lacZ* fusions in *E. coli* wild-type JM109 and *B. cereus* B905. *E. coli* JM109 (pPR9TT-Psp7H): *E. coli* JM109 transformants carrying *A. brasilense nifH* promoter region with *lacZ* fusions in vector pPR9TT.

Strains	β-GA (U)	Strains	β-GA (U)
E. coli JM109 (pPR9TT)	3 ± 3	*B. cereus* B905 (ppLacZ)	0 ± 0
E. coli JM109 (pPR9TT-P1)	989 ± 43	*B. cereus* B905 (ppLacZ-P1)	66 ± 3
E. coli JM109 (pPR9TT-P3)	302 ± 25	*B. cereus* B905 (ppLacZ-P3)	359 ± 21
E. coli JM109 (pPR9TT-P5)	54 ± 7	*B. cereus* B905 (ppLacZ-P5)	10 ± 1
E. coli JM109 (pPR9TT-Psp7H)	2 ± 1		

4. Discussion

P. sabinae T27 was used as model organism in our study because it has high nitrogenase activities (Ma et al. 2007). But *P. sabinae* T27 is difficult to transform with foreign genes carried in a plasmid; despite attempts of electro-transformation and protoplast transformation in our study, we were unsuccessful. It is commonly considered that there are difficulties in transformation of some gram-positive, spore-forming bacteria.

Although the promoter experiments were done in *E. coli* and *Bacillus*, the results can still reveal important information. This is because *Bacillus* is closely related to *Paenibacillus* and *E. coli* is a model system for research. For example, heterologous expression analysis in *E. coli* were also used by other researchers (Morales et al. 1986; Valderrama et al. 1996; Bermejo et al. 1998; Visnapuu et al. 2008).

K. pneumoniae nifB is separated with *nifHDK*; *Heliobacteria chlorum nifB* is separated with *nifHDK*; there is no *nifB* in *Methanococcus maripaludis*. *P. sabinae* T27 *nifB* clustered with *nifHDK* which is similar with *P. azotofixans*. *P. sabinae* T27 has three *nifH* genes, In many microorganisms, including *K. pneumoniae*, *A. brasilense*, there is only one nifH gene.

In most diazotrophs, activator NifA are required for transcription of *nif* genes including *nifH*. It is well known that there is no activator NifA in *E. coli*. However, the three putative promoter regions P1, P3 and P5 for *nifH* genes of *P. sabinae* T27 with *lacZ* fusions are successfully expressed in *E. coli* JM109. The data suggests that transcriptions of *P. sabinae nifH* genes are not dependent on activator NifA. There are similar reports that *C. pasteurianum* lacks a *nifA*-like gene and that more than one *nifH* gene under N_2-fixing growth conditions were functional (Asami and Kiwamu 2006; Chen 2004; Wang et al. 1988).

There are only few reports about nitrogen fixation in nitrogen-fixing *Paenibacillus*. Our study will enrich knowledge of nitrogen fixation in these bacteria.

Acknowledgements

We thank Professor Yaoping Zhang, Professor Qi Wang for providing strain *K. pneumoniae* 1795 and *B. cereus* 905, respectively. This work is supported by National Nature Science Foundation of China (Grant No. 30870076) and the National "973" Project (Grant No. 2010CB126504).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.micres.2012.05.003.

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