Characterization and analysis of \textit{nifH} genes from \textit{Paenibacillus sabinae} T27

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\textbf{A B S T R A C T}

\textit{Paenibacillus sabinae} T27 (CCBAU 10202=DSM 17841) is a gram-positive, spore-forming diazotroph with high nitrogenase activities. Three \textit{nifH} clusters were cloned from \textit{P. sabinae} T27. Phylogenetic analysis revealed that \textit{NifH}1, \textit{NifH}2 and \textit{NifH}3 cluster with \textit{Cyano}bacteria. Each of the coding regions of \textit{nifH1}, \textit{nifH2} and \textit{nifH3} from \textit{P. sabinae} T27 under the control of the \textit{nifH} promoter of \textit{Klebsiella pneumoniae} could partially restore nitrogenase activity of \textit{K. pneumoniae} \textit{nifH}\textsuperscript{−} mutant strain 1795, which has no nitrogenase activity. This suggests that the three \textit{nifH} genes from \textit{P. sabinae} T27 have some function in nitrogen fixation. RT-PCR showed that all three \textit{nifH} genes were expressed under nitrogen-fixing growth conditions. Using promoter vectors which have promoterless \textit{lacZ} gene, three putative promoter regions of \textit{nifH} genes were identified.

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1. Introduction

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

The nucleotide sequences for coding regions of \textit{nifHDK} genes among all nitrogen-fixing organisms are highly conserved. However, the copy numbers and arrangement of \textit{nifH}, \textit{nifD}, and \textit{nifK} are different among the different diazotrophic bacteria. In many microorganisms, including \textit{Klebsiella pneumoniae}, \textit{Azospirillum brasilense}, \textit{Glucanacetobacter diazotrophicus} and \textit{Herbaspirillum seropedicae}, there is only one \textit{nifH} gene and the \textit{nifHDK} genes are transcribed as a single unit. However, some diazotrophs have multiple \textit{nifH} genes. It is reported that \textit{Clostridium pasteurianum} has six \textit{nifH}(\textit{nifH1}) and \textit{nifH}-like (\textit{nifH2}, \textit{nifH3}, \textit{nifH4}, \textit{nifH5} and \textit{nifH6}) genes whose sequence identities range between 68 and 99.3\% within the \textit{nifH} coding regions (Chen 2004; Wang et al. 1988).

\textit{Bacillus} and \textit{Paenibacillus} are Gram-positive, spore-forming bacteria and can survive even in strict environments. The members of nitrogen-fixing \textit{Bacillus} and \textit{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, \textit{Paenibacillus azotofixans} contains three copies of \textit{nifH} (Choo et al. 2003) and \textit{Paenibacillus massiliensis} T7 contains a \textit{nifBHDKEN} cluster (Zhao et al. 2006).

\textit{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 \textit{nifH} genes are cloned, and their activities and putative promoter regions are characterized.

2. Materials and methods

2.1. Media and growth conditions

\textit{P. sabinae} T27 was cultured in soypeptone medium, which contains (11): 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 \textit{K. pneumoniae} and \textit{P. sabinae} T27 contains (11): Na\textsubscript{2}HPO\textsubscript{4}.12H\textsubscript{2}O 26.3 g; KH\textsubscript{2}PO\textsubscript{4} 3.4 g; biotin 10 μg; CaCl\textsubscript{2}.2H\textsubscript{2}O 26 mg; MgSO\textsubscript{4} 30 mg; MnSO\textsubscript{4}.H\textsubscript{2}O 0.33 mg; ferric citrate 36 mg; Na\textsubscript{2}MoO\textsubscript{4}.2H\textsubscript{2}O 7.6 mg; p-aminobenzoic acid 10 μg; glucose 4 g; glutamic acid 1 g.

2.2. Southern blot analysis

The genomic DNA of \textit{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, Nor I, Hind III, Cla I and Apa I. The 324-bp \textit{nifH} fragment was PCR amplified from \textit{P. sabinae} T27 genomic DNA by using two degenerate primers \textit{nifH}P1 and \textit{nifH}P2 (Ding et al. 2005), labeled using DIG-DNA Labeling and Detection Kit purchased from Roche
2.3. Cloning of nif genes

The genomic DNA of P. sabinae T27 was digested by HindIII 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 (Norlander 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 EcoRI 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 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 Pst I and Xba I; then inserted into the corresponding sites 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 CO2) to an approximate OD600 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™ 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 MgCl2), 0.5 μl (10 mM dNTPs), 0.3 μl Taq DNA polymerase and 19 μl H2O. 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...
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).

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**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.)

**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, Cyanobacter sp. strain ATCC 51142; Fal-Ari3, Frankia alni strain Ari3; Fsp-EUIK1, Frankia sp. strain EU1K1; Fsp-FaC1, Frankia sp. strain FaC1; Fsp, Fischerella sp. strain UTEx1931; Gdi, Gluconacetobacter diazotrophicus; Hse, Herbaspirillum seropedicae; Kpn, Klebsielra pneumoniae; Mba, Methanocarcina barkeri; Mma, Methanococcus maripaludis; Mth (H), Methanothermobacter thermautotrophicus str. Delta H; Mmnr, Methanothermobacter marburgensis strain Marburg; Mth, Methanothermococcus thermolithotrophicus; Paz, Paenibacillus azotofixans; Pba, Plectonema boryanum; Ret, Rhizobium etli; Pma, Paenibacillus massiliensis strain T7; Rle, Rhizobium leguminosarum; Rsp, Rhizobium sp. strain NGR234; Sme, Sinorhizobium meliloti; Tle, Acidithiobacillus ferrooxidans; Tsp, Trichodesmium sp. strain IMS101.
3. Results

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

Three nifH clusters were isolated from P. sabiniae T27. Firstly, a 324-bp fragment of the nifH1 gene was amplified from genomic DNA using two degenerate primers nifH1P and nifH2P (Ding et al. 2005) and thus labeled with digoxigenin. The Southern blot hybridization revealed P. sabiniae T27 contains more than one nifH1 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 nifH1. 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: HMs83798, HMs83799, and HMs583800. 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. azotofoxans which is a species closely related to P. sabiniae T27 (Choo et al. 2003). P. azotofoxans NifH1 and NifH2 clustered with Cyanobacterium, P. azotofoxans NifH3 clustered with NifH proteins of Archaea. P. sabiniae T27 NifH1, NifH2 and NifH3 clustered with Cyanobacterium, P. azotofoxans NifH3 clustered with NifH proteins of Archaea. P. sabiniae T27 NifH1, NifH2 and NifH3 have 52–95% DNA identities with nifH1, nifH2 and nifH3 of P. azotofoxans, respectively. The DNA identities of nifH1, nifH2 and nifH3 of P. sabiniae T27 have 52–95% DNA identities with nifH1, nifH2 and nifH3 of P. azotofoxans, respectively. The DNA identities of nifH1, nifH2 and nifH3 of P. sabiniae 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. sabiniae T27 can complement the K. pneumoniae nifH- mutant

To further study the functions of the nifH1, nifH2 and nifH3 of P. sabiniae T27, K. pneumoniae nifH mutant strain 1795, which has no nitrogenase activity, was complemented with the three nifH genes of P. sabiniae 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. sabiniae 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. sabiniae T27 nifH genes

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

In order to identify P. sabiniae T27 nifH promoter regions, promoter probe vector pPR97T 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 pPR97T 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. sabiniae T27 are repressed by oxygen and ammonium chloride; and there are at least three functional nifH genes in P. sabiniae T27.
Table 1

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<th>Strains</th>
<th>β-GA (U)</th>
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<th>β-GA (U)</th>
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<tr>
<td>E. coli JM109 (pPR9TT)</td>
<td>3 ± 3</td>
<td>B. cereus 3005 (ppLacZ)</td>
<td>0 ± 0</td>
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<tr>
<td>E. coli JM109 (pPR9TT-P1)</td>
<td>989 ± 43</td>
<td>B. cereus 3005 (pLacZ-P1)</td>
<td>66 ± 3</td>
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<tr>
<td>E. coli JM109 (pPR9TT-P3)</td>
<td>302 ± 25</td>
<td>B. cereus 3005 (pLacZ-P3)</td>
<td>359 ± 21</td>
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<tr>
<td>E. coli JM109 (pPR9TT-P5)</td>
<td>54 ± 7</td>
<td>B. cereus 3005 (pLacZ-P5)</td>
<td>10 ± 1</td>
</tr>
<tr>
<td>E. coli JM109 (pPR9TT-Psp7H)</td>
<td>2 ± 1</td>
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4. Discussion

P. sabinea T27 was used as model organism in our study because it has high nitrogenase activities (Ma et al. 2007). But P. sabinea T27 is difficult to transform with foreign genes carried in a plasmid; despite attempts of electroporation 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; Heliotcicetia chlorum nifB is separated with nifHDK; there is no nifB in Methanococcus maripaludis. P. sabinea T27 nifB clustered with nifHDK which is similar with P. azotofixans. P. sabinea 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. sabinea T27 with lacZ fusions are successfully expressed in E. coli JM109. The data suggests that transcriptions of P. sabinea 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 N2-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.

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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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