Functional analysis of multiple nifB genes of Paenibacillus strains in synthesis of Mo-, Fe- and V-nitrogenases

Qin Li1,2, Haowei Zhang1, Liqun Zhang2 and Sanfeng Chen1*

Abstract

Background: Biological nitrogen fixation is catalyzed by Mo-, V- and Fe-nitrogenases that are encoded by nif, vnf and anf genes, respectively. NifB is the key protein in synthesis of the cofactors of all nitrogenases. Most diazotrophic Paenibacillus strains have only one nifB gene located in a compact nif gene cluster (nifBHDKENX(orf1)hesAnifV). But some Paenibacillus strains have multiple nifB genes and their functions are not known.

Results: A total of 138 nifB genes are found in the 116 diazotrophic Paenibacillus strains. Phylogeny analysis shows that these nifB genes fall into 4 classes: nifB1 class including the genes (named as nifB1 genes) that are the first gene within the compact nif gene cluster, nifBII class including the genes (named as nifB2 genes) that are adjacent to anf or vnf genes, nifBIII class whose members are designated as nifB3 genes and nifBIV class whose members are named as nifB4 genes are scattered on genomes. Functional analysis by complementation of the ∆nifB mutant of P. polymyxa which has only one nifB gene has shown that both nifB1 and nifB2 are active in synthesis of Mo-nitrogenase, while nifB3 and nifB4 genes are not. Deletion analysis also has revealed that nifB1 of Paenibacillus sabinense T27 is involved in synthesis of Mo-nitrogenase, while nifB3 and nifB4 genes are not. Complementation of the P. polymyxa ∆nifB-HDK mutant with the four reconstituted operons: nifB1anfHDGK, nifB2anfHDGK, nifB1vnfHDGK and nifB2vnfHDGK, has shown both that nifB1 and nifB2 were able to support synthesis of Fe- or V-nitrogenases. Transcriptional results obtained in the original Paenibacillus strains are consistent with the complementation results.

Conclusions: The multiple nifB genes of the diazotrophic Paenibacillus strains are divided into 4 classes. The nifB1 located in a compact nif gene cluster (nifBHDKENX(orf1)hesAnifV) and the nifB2 genes being adjacent to anf or vnf genes are active in synthesis of Mo-, Fe and V-nitrogenases, but nifB3 and nifB4 are not. The reconstituted anf system comprising 8 genes (nifB1anfHDGK and nifXhesAnifV) and vnf system comprising 10 genes (nifB1vnfHDGK and nifXhesAnifV) support synthesis of Fe-nitrogenase and V-nitrogenase in Paenibacillus background, respectively.

Keywords: Paenibacillus, nifB gene, Mo-nitrogenase, Alternative nitrogenases

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Mo-nitrogenase, some possess either of alternative Fe-nitrogenase and V-nitrogenase, or both. Each nitrogenase contains two components, a catalytic protein and a reductase [3–5]. For Mo-nitrogenase, MoFe protein is the catalytic protein and Fe protein is the reductase. The MoFe protein is an α₂β₂ heterotetramer (encoded by nifD and nifK) that contains two metal clusters: FeMo-co, a [Mo-7Fe-9S-C-homocitrate] cluster which serves as the active site of N₂ binding and reduction and the P-cluster, a [8Fe-7S] cluster which shuttles electrons to FeMo-co. The Fe protein (encoded by nifH) is a homodimer bridged by an intersubunit [4Fe-4S] cluster that serves as the obligate electron donor to the MoFe protein [6–8].

Like Mo-nitrogenase, alternative nitrogenases comprise an electron-delivery Fe protein (encoded by anfH in Fe-nitrogenase and encoded by vnfH in V-nitrogenase). The FeFe protein of Fe-nitrogenase encoded by anfDK and the VFe protein of V-nitrogenase encoded by vnfDK are homologous to the MoFe protein of Mo-nitrogenase. The alternative nitrogenases have either FeFe-co or FeV-co at the active site and also include an additional subunit (AnFG or VnFG) encoded by anfG or vnfG [9]. The FeFe-co is analogous to FeMo-co except for containing Fe in place of Mo [10], but FeV-co is a [V–7Fe–8S–C-homocitrate] cluster which replaces Mo with V and lacks one S compared to FeMo-co [11].

NifB has been demonstrated to be essential for the synthesis of all nitrogenases. NifB is a radical S-adenosyl methionine (SAM) enzyme that catalyzes the formation of NifB-co, a [8Fe-9S-C] cluster which is a common precursor for the syntheses of FeMo-co of Mo-nitrogenase, FeV-co of V-nitrogenase and FeFe-co of Fe-nitrogenase [12–14]. NifB-co is subsequently transferred to the scaffold protein NifEN, upon which mature cofactor is synthesized. The NifX protein is known to bind NifB-co and involved in NifB-co transfer [15].

The number, structure and properties of nifB genes show some variation among different diazotrophs. Azotobacter vinelandii and Rhodopseudomonas palustris possess only one nifB gene that is responsible for three types of nitrogenases and mutation of the nifB gene led to loss of all nitrogenases activities [16, 17]. Rhodobacter capsulatus with Mo-nitrogenase and Fe-nitrogenase carries two nifB genes that are located in two nif gene clusters [18] and either one of the two nifB genes was sufficient for nitrogen fixation via the Mo-dependent or Fe-dependent nitrogenase [19]. The cyanobacterium Anabaena variabilis ATCC 29,413 has two nifB genes for synthesis of two Mo-nitrogenases, but nifB1 is specifically expressed in heterocysts and nifB2 is specifically expressed in vegetative cells [20]. On the basis of NifB domain architecture, the NifB proteins are divided into three subfamilies [21, 22]. The first NifB subfamily has an N-terminal SAM-radical domain linked to a C-terminal NifX-like domain. A major of NifB proteins from Bacteria domain (e.g. A. vinelandii and Klebsiella oxytoca) belong to the first NifB subfamily. The second NifB subfamily contains a stand-alone SAM-radical domain and is found in Bacteria and Archaea domains. The third NifB subfamily has three domains including a NifN-like domain, a SAM-radical domain and a C-terminal NifX-like domain and is found in Clostridium species.

The Paenibacillus genus of the Firmicutes phylum is a large one that currently comprises 254 validly named species (https://www.bacterio.net/paenibacillus.html), more than 20 of which have the nitrogen fixation ability [23]. Comparative genome sequence analysis of 15 diazotrophic Paenibacillus strains have revealed that a compact nif gene cluster comprising 9–10 genes (nifH nifD nifK nifE nifN nifX (orf1) hesA nifV) encoding Mo-nitrogenase is conserved in the N₂-fixing Paenibacillus genus [24]. The 9 genes (nifBHDKENXhesAnifV) in Paenibacillus polymyxa WLY78 are organized as an operon under control of a σ⁷⁰ dependent promoter located in front of nifB gene [25]. In addition to the nif gene cluster, additional nif genes or anf or vnf genes are found in some diazotrophic Paenibacillus spp. For examples, Paenibacillus abciniae T27 has multiple nifB, nifH, nifE and nifN genes [26]. Paenibacillus forsythia T98 and Paenibacillus sophorae S27 have additional nif and anfDHGK genes, Paenibacillus zanthoxyli JH29 and Paenibacillus durus (previously called as Paenibacillus azotofixans) ATCC 35681 contain additional nif and vnfDHGKES [24]. Notably, in addition to the nifB gene in the compact nif gene cluster comprising 9–10 genes (nifB nifH nifD nifK nifE nifN nifX (orf1) hesA nifV) encoding Mo-nitrogenase, multiple nifB genes are found in some Paenibacillus species that carry additional nif genes or anf genes or vnf genes [24, 26]. However, functions of the multiple nifB genes are not known. In this study, we analyzed the distribution and phylogeny of the 138 putative NifB proteins from 116 diazotrophic Paenibacillus strains. All nifB genes in Paenibacillus fall into 4 classes: nifBI, nifBII, nifBIII and nifBIV. We demonstrate that only nifBI and nifBII are functional in synthesis of Mo-, Fe- and V-nitrogenase. The nifBIII and nifBIV may be not involved in nitrogen fixation. In addition, the reconstituted anf system comprising 8 genes (nifBanfHDKG and nifXnesAnifV) and vnf system comprising 10 genes (nifBvfnfHDKGEN and nifXnesAnifV) supported synthesis of Fe-nitrogenase and V-nitrogenase in Paenibacillus background, respectively. Our study will provide guidance for engineering nitrogenase into heterologous hosts.
Results

Classification of nifB genes of Paenibacillus genus

Here, the nitrogen fixation genes in the genomes of the 116 diazotrophic Paenibacillus strains taken from the RefSeq database are comparatively analyzed (Additional file 1: Table S1). A compact nif gene cluster composed of 9–10 genes (nifBHDKENXorf1hesAnifV) is conserved in all of the diazotrophic strains, in agreement with the previous studies [24]. In addition to the compact nif gene cluster encoding Mo-nitrogenase, 9 strains have additional anfHDGK encoding Fe-nitrogenase and 3 strains have additional vnfHDGKEN encoding V-nitrogenase.

A total of 138 NifB putative sequences are found in the 116 diazotrophic Paenibacillus strains. According to the nifB sequence similarity, the nifB genes were divided into 4 classes. The nifB1 class includes the nifB genes (named as nifB1 genes) that are the first gene in the compact nif gene cluster comprising 9–10 genes (nifB nifH nifD nifK nifE nifN nifX orf1 hesA nifV) and the genes linked to another nifH. The nifB1II class includes these genes (named as nifB2 genes) that are linked to additional copies of nifENXorf1) genes preceding anfHDGK or additional copies of nifENXorf1 orf1 preceding vnfHDGKEN. The genes (named as nifB3) of nifB1II class and the genes (named as nifB4) of nifBIV are scattered at different locations of genomes.

Of the 116 diazotrophic Paenibacillus strains, 105 strains have only one nifB and 11 strains have 2–4 nifB genes. Paenibacillus polymyxa WLY78 is a representative that has only a nifB1 located in the compact nif gene cluster consisting of 9 genes (nifBHDKENXhesAnifV) encoding Mo-nitrogenase (Fig. 1 and Additional file 1: Table S1). Paenibacillus subalaniae T27 is a representative strain with three nifB genes (nifB1, nifB3 and nifB4), but contained only Mo-nitrogenase. For the strains with both Mo- and V-nitrogenases, Paenibacillus zanthoxyli JH29 has nifB1, nifB2 and nifB3, but Paenibacillus durus DSM 1735 has nifB2, nifB3 and 2 copies of nifB1: one being located in the compact nif cluster and the other being linked to another nifH. For the strains with both Mo- and Fe-nitrogenases, Paenibacillus forsythiae T98 has three nifB genes (nifB1, nifB2 and nifB3), whereas Paenibacillus sophorae S27 has four nifB genes (nifB2, nifB3, and 2 copies of nifB1). The other 4 strains (Paenibacillus borealis FSL H70744, Paenibacillus sp. FSL H7-0357, Paenibacillus sp. HW567 and Paenibacillus camerounensis G4) with both Mo- and Fe-nitrogenases possess only one nifB gene. Organization of the nifB genes and other nitrogen fixation genes from 17 representatives of Paenibacillus strains is shown in Fig. 1.

Phylogeny and structure of Paenibacillus NifB proteins

Here, 138 putative NifB sequences from 116 diazotrophic Paenibacillus strains are used to construct a phylogenetic tree, with 11 NifB sequences from 10 diazotrophs (A. vinelandii, K. oxytoca, Bradyrhizobium japonicum, Clostridium kluyveri, Dehalobacter sp., Kyrpidia sp., Methanosarcina acetivorans, Methanococcus maripaludis, Frankia sp. EAN1pec, Nostoc sp. PCC 7120) as control (Fig. 2 and Additional file 1: Table S1). The phylogenetic tree has shown that all Paenibacillus putative NifB proteins form a large class which is separated from the NifB proteins from other diazotrophs. The data suggest that all Paenibacillus putative nifB genes have a common ancestor. The Paenibacillus putative NifB proteins are divided into 4 classes: NifB1, NifBII, NifBIII and NifBIV, which corresponded to the 4 nifB classes that are classified on basis of nifB sequence similarities. The NifB1, NifB2, NifB3 and NifB4 proteins corresponded to NifB1, NifBII, NifBIII and NifBIV classes, respectively. Phylogeny analyses have shown that the NifB1 proteins are emerged firstly in the diazotrophic Paenibacillus species, and NifB2, NifB3 and NifB4 may result from gene duplication.

Protein structure analysis showed that Paenibacillus NifB1, NifB2 and NifB4 have the same structure composed of an N-terminal SAM-radical domain and a C-terminal NifX-like domain. Most NifBIII members possesses the two domains, but the NifB3 proteins from the 2 strains (P. zanthoxyli JH29 and P. durnus DSM 1735) have only a SAM-radical domain. The Paenibacillus NifB1, NifB2, NifB3 and NifB4 proteins that possess both domains are composed of 427–505 amino acids (Additional file 1: Table S1) and have similarity (>57%) at amino acid levels. These proteins have a number of conserved motifs in the SAM-radical domain, including HPC motif, Cx3Cx2C motif, ExRP motif, AGPG motif, TTXN motif and Cx2CrxDAxG (Fig. 2). However, the NifB3 proteins of P. zanthoxyli JH29 and P. durnus DSM 1735 have only a SAM-radical domain that lacks the Cx2CrxDAxG motif. Sequence alignment of 13 NifB...
P. polymyxa WLY78
P. macerans 8244
P. borealis FSL H7-0744
Paenibacillus sp. FSL H7-0357
P. camerounensis G4
Paenibacillus sp. HW567
P. forsythia T98
P. stellifer DSM 14472
P. sophorae S27
P. riogramdensis SBR5
P. borealis DSM 13188
P. zantofixans JH29
P. dorus ATCC 35681
P. dorus DSM 1735
P. sabinae T27
P. rhizophilus 7197
Paenibacillus sp. IHB B 3415
proteins including NifB1, NifB2, NifB3 and NifB4 from 4 representatives of *Paenibacillus* strains (*P. polymyxa* WLY78, *P. sabinae* T27, *P. forsythia* T98 and *P. zanthoxyli* JH29) is shown in Additional file 1: Figure S1.

Transcription analysis of multiple *nifB* genes in medium containing only Mo or Fe or V

As described above, *P. sabinae* T27 with only Mo-nitrogenase has NifB1, NifB3 and NifB4, *P. zanthoxyli* JH29 with both Mo- and V-nitrogenases has NifB1, NifB2 and NifB3 and *P. forsythia* T98 with both Mo- and Fe-nitrogenases possesses NifB1, NifB2 and NifB3. The three species *P. sabinae* T27, *P. forsythia* T98 and *P. zanthoxyli* JH29 were used to investigate the transcriptions of the multiple *nifB* genes under different conditions by RT-qPCR. *Paenibacillus sabinae* T27 was cultivated in Mo-dependent N2-fixing condition, while *P. forsythia* T98 and *P. zanthoxyli* JH29 were cultivated...
in Mo-dependent and Fe-dependent or V-dependent N$_2$-fixing condition, respectively, with non-N$_2$-fixing condition of N-rich (LD medium) cultures as negative controls (Fig. 3). For _P. sabinae_ T27, the transcription level of nifB1 exhibited more than 2000-fold of increase under Mo-dependent N$_2$-fixing condition compared to under non-N$_2$-fixing condition, but the transcripts from nifB3 and nifB4 showed no differences under both conditions (Fig. 3a). For _P. forsythia_ T98 grown under both Mo-dependent and Fe-dependent condition, both nifB1 and nifB2 genes were highly transcribed, but nifB3 was not induced by N$_2$-fixing condition. The transcript level of nifB1 was much higher in Mo-dependent condition than in Fe-dependent condition, while the transcript level of nifB2 was higher in Fe-dependent condition than in Mo-dependent condition (Fig. 3b). For _P. zanthoxyli_ JH29 grown under both Mo-dependent and V-dependent conditions, the transcription of both nifB1 and nifB2 genes were activated, but nifB3 showed no differences in its expression under test conditions. The transcript level of nifB1 was higher in Mo-dependent condition than in V-dependent condition, while the transcript level of nifB2 was higher in V-dependent condition than in Mo-dependent condition (Fig. 3c). These results indicate that the nifB1 and nifB2 may be selectively expressed according to metal availability.

**Functional analysis of multiple nifB genes in synthesis of Mo-nitrogenase**

The nifB deletion mutant (ΔnifB) of _P. polymyxa_ WLY78 was here constructed by using recombination method as described in materials and methods. The _P. polymyxa_ ΔnifB mutant completely lost its nitrogenase activity and complementation by its nifB gene carried in a plasmid restored the nitrogenase activity (Fig. 4a). Thus, _P. polymyxa_ ΔnifB mutant was used as a host for complementation to investigate the functionality of the multiple nifB genes. Each nifB gene from _P. sabinae_ T27, _P. forsythia_ T98 and _P. zanthoxyli_ JH29 was cloned into a low-copy plasmid pRN5101[27, 28], in which the expression of these nifB genes were driven under the control of the nifB promoter of _P. polymyxa_ (details are provided in materials and methods). Among the 3 nifB genes of _P. sabinae_ T27, only the nifB1 can effectively restore the nitrogenase activity of the _P. polymyxa_ ΔnifB mutant, showing that the nifB1 gene was transcribed under nitrogen fixation condition and the translated NifB1 was functional. Both nifB1 and nifB2 from _P. forsythia_ T98 or _P. zanthoxyli_ JH29 can effectively restore nitrogenase activity of the _P. polymyxa_ ΔnifB mutant, but the nifB3 from _P. forsythia_ T98 or _P. zanthoxyli_ JH29 can not restore activity. The result suggests that both nifB1 and nifB2 are functional in synthesis of Mo-nitrogenase, but nifB3 product was not active.

To further examine the role of the multiple nifB genes, attempts to inactivate the nifB genes were made. Three single deletion mutants ΔnifB1, ΔnifB3 and ΔnifB4 of _P. sabinae_ T27 were successfully constructed. Deletion of nifB1 resulted to complete loss of nitrogenase activity. Whereas, the nitrogenase activities of ΔnifB3 or ΔnifB4 mutants were similar as that in wild-type _P. sabinae_ T27 (Fig. 4b). The data are consistent with the above described qRT-PCR and heterologous complementation results, confirming that both nifB3 and nifB4 are not involved in nitrogen fixation. However, attempts to inactivate the nifB genes of _P. forsythia_ T98 and _P. zanthoxyli_ JH29 were not successful, due to hardness of genetic transformation in these strains.
Functional analysis of \textit{nifB1} and \textit{nifB2} genes in synthesis of Fe- and V-nitrogenases

In order to investigate whether the \textit{nifB1} and \textit{nifB2} from \textit{P. forsythia} T98 and \textit{P. zanthoxyli} JH29 were active in synthesis of Fe-nitrogenase and V-nitrogenases, the \textit{ΔnifBHDK} and \textit{ΔnifBHDKEN} mutants of \textit{P. polymyxa} WLY78 which lost the ability to synthesize Mo-nitrogenase were constructed. As shown in Fig. 5, the \textit{nifB-HDK} and \textit{nifBHDKEN} of \textit{P. polymyxa} WLY78 carried in plasmid could restore the nitrogenase activity to 90% wild-type level in the complementary strain (\textit{ΔnifBHDK/nifBHDK}) and (\textit{ΔnifBHDKEN/nifBHDKEN}), suggesting that the mutants can be used as a host for complementation study of alternative nitrogenases.

Two new operons \textit{nifB1anfHDGK} and \textit{nifB2anfHDGK} of \textit{P. forsythia} T98 under the control of the \textit{P. polymyxa} WLY78 \textit{nifB} promoter were constructed (Fig. 5). Each of the reconstituted \textit{nifB1anfHDGK} and \textit{nifB2anfHDGK} operons of \textit{P. forsythia} T98 carried in the recombinant plasmids can enable \textit{P. polymyxa} \textit{ΔnifBHDK} mutant to have nitrogenase activity in medium containing Fe and lacking Mo. The data suggest that either \textit{nifB1} or \textit{nifB2} together with \textit{anfHDGK} of \textit{P. forsythia} can support synthesis of Fe-nitrogenase in the heterologous host \textit{P. polymyxa} which originally has only Mo-nitrogenase system. Furthermore, in order to investigate whether \textit{nifE} and \textit{nifN} genes (designed \textit{nifE2} and \textit{nifN2} genes) preceding \textit{anfHDGK} of \textit{P. forsythia} T98 were functional, another new operon \textit{nifB2E2N2anfHDGK} of \textit{P. forsythia} T98 was constructed (Fig. 5). Then, \textit{nifB2E2N2anfHDGK} and \textit{nifB2anfHDGK} carried in the recombinant plasmids are individually used to complement \textit{ΔnifBHDKEN} mutant of \textit{P. polymyxa} WLY78. As shown in Fig. 5, either \textit{nifB2E2N2anfHDGK} or \textit{nifB2anfHDGK} can support \textit{ΔnifBHDKEN} mutant of \textit{P. polymyxa} WLY78 to have nitrogenase activity in medium containing Fe and lacking Mo. Like the \textit{P. forsythia} T98 that was capable of diazotrophic growth, the reconstituted \textit{nifB/anf}-complemented strains can grow in liquid media with dinitrogen as the sole nitrogen source (Fig. S2). The results indicated that that \textit{nifEN} is not necessary for the biosynthesis and the reconstituted \textit{anf} system composed of 8 genes (\textit{nifBanfHDGK} of \textit{P. forsythia})

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure5}
\caption{Schematic map and nitrogenase activity of the \textit{ΔnifBHDK} and \textit{ΔnifBHDKEN} mutants of \textit{P. polymyxa} and the complementary strains carrying \textit{nifB1anfHDGK}, \textit{nifB2anfBHDGK}, \textit{nifB2E2N2anfBHDGK} of \textit{P. forsythia} T98, respectively and the complementary strains carrying \textit{nifB1vmfHDGK}, \textit{nifB2vmfHDGK}, \textit{nifB2vmfHDKGEN} of \textit{P. zanthoxyli} JH29, respectively. a Schematic map of the \textit{P. polymyxa} \textit{ΔnifBHDK} and \textit{P. polymyxa} \textit{ΔnifBHDKEN} mutants and the complementary strains. b The nitrogenase activity of the \textit{P. polymyxa} \textit{ΔnifBHDK} and \textit{P. polymyxa} \textit{ΔnifBHDKEN} mutants and the complementary strains. Activity was measured by acetylene reduction assay. The complementary strains carrying \textit{nifB1anfHDGK}, \textit{nifB2anfBHDGK} and \textit{nifB2E2N2anfBHDGK} were cultivated in Fe-dependent conditions. The complementary strains carrying \textit{nifB1vmfHDGK}, \textit{nifB2vmfHDGK} and \textit{nifB2vmfHDKGEN} were cultivated in V-dependent conditions. Error bars indicate the SD observed from at least three independent experiments.}
\end{figure}
Fig. 5 (See legend on previous page.)
T98 and nifXhesAnifV of P. polymyxa WLY78) can support synthesis of Fe-nitrogenase to fix nitrogen.

Similarly, two new operons nifB1vnfHDGK and nifB2vnfHDGK of P. zanthoxyli JH29 under the control of the nifB promoter of P. polymyxa WLY78 were constructed (Fig. 5a). Each of the nifB1vnfHDGK and nifB2vnfHDGK operons of P. zanthoxyli JH29 carried in the recombinant plasmids can enable P. polymyxa ΔnifBHDK mutant to have nitrogenase activity in medium containing V and lacking Mo (Fig. 5b). The data suggest that either of nifB1 or nifB2 together with vnfHDGK of P. zanthoxyli JH29 can support synthesis of V-nitrogenase. Furthermore, a new operon comprising nifB2 and vnfHDGKEN under the control of the nifB promoter of P. polymyxa WLY78 was constructed. The reconstituted operons nifB2vnfHDGKEN and nifB2vnfHDGK of P. zanthoxyli JH29 are individually used to complement ΔnifBHDKEN mutant of P. polymyxa WLY78. The operon nifB2vnfHDGKEN can effectively enable ΔnifBHDKEN mutant of P. polymyxa WLY78 to synthesize V-nitrogenase (Fig. 5). Our data demonstrate that the reconstituted vnf system with vnfEN exhibited higher nitrogenase activity compared to the reconstituted vnf system with nifEN. However, the nifB2vnfHDGK operon of P. zanthoxyli JH29 can not complement the ΔnifBHDKEN mutant of P. polymyxa WLY78, suggesting that the vnfEN or nifEN was required for the biosynthesis of VFe-co. The diazotrophic growth tests showed that all the reconstituted nifB/vnf-complemented strains lacking ΔnifBHDK/ΔnifB2vnfHDGK strain grew as well as the P. zanthoxyli JH29 (Additional file 1: Figure S2). The results indicated that the reconstituted vnf system composed of 10 genes (nifBvnhfHDGK of P. zanthoxyli JH29 and nifENXhesAnifV of P. polymyxa WLY78 or nifBvnhfHDGK of P. zanthoxyli JH29 and nifXhesAnifV of P. polymyxa WLY78) can support synthesis of V-nitrogenase to fix nitrogen.

Discussion

Most of the diazotrophs carried a single copy of nifB. However, our results demonstrated that 2–4 nifB genes were distributed in Paenibacillus strains having additional nif genes or anf genes or vnf genes. The occurrence of multiple nifB copies appears to be specific to diazotrophic Paenibacillus. In addition, the presence of nifB1 immediately upstream of the structural genes nifHDK and presence of nifB2 close to the structural genes anfHDGK or vnfHDGK also seem to characterize the genus. Our analyses have revealed that all nifB genes in Paenibacillus fall into 4 classes and their encoded products have a N-terminal SAM-radical domain linked to a C-terminal NifX-like domain. However, the NifB3 protein of P. zanthoxyli JH29 or P. durus DSM 1735 is a stand-alone SAM-radical protein which is adjacent to a NifX-like protein.

To confirm the accuracy of the nifB3 at DNA sequence level, a DNA fragment including both of the coding regions of a SAM-radical protein and a NifX-like protein was PCR amplified from P. zanthoxyli JH29 (Additional file 1: Figure S3). Sequence analysis have shown that the NifB3 protein of P. zanthoxyli JH29 is really a stand-alone SAM-radical protein that linked to a NifX-like protein. We deduce that the nifB3 gene of P. zanthoxyli JH29 or P. durus DSM 1735 is divided to two genes: one encoding a SAM-radical protein and the other encoding a NifX-like protein during evolution. The NifB proteins with only a SAM-radical domain are distributed in some bacteria and in most archaea [21]. However, a stand-alone SAM-radical domain in the NifB3 proteins of P. zanthoxyli JH29 and P. durus DSM 1735 lacks the C-terminal Cxx2CRxDaXG motif that binds an Fe-S cluster necessary for NifB-co synthesis [29]. The NifB proteins with three domain architectures comprising a NifN-like domain, SAM-radical domain and a NifX domain are widely distributed in Clostridium genus [21]. However, the NifB proteins with three domain architectures are not found in Paenibacillus, although both Paenibacillus and Clostridium are genera of the Firmicutes phylum.

The canonical NifB protein contains a SAM-radical domain and a NifX-like domain. We have found that some N2-fixing Paenibacillus strains possess NifX-like protein that shows higher sequence similarity value with the C-terminal domain of NifB compared with that of NifX protein family. These proteins with only a NifX-like domain are also found in other diazotrophs, but they were eliminated from their studies [21]. Here, the transcription and function of the nifX-like genes from P. sabinae T27, P. forsythia T98 and P. zanthoxyli JH29 are investigated. Generally, the nifX-like gene in Paenibacillus strains is linked together with nifH or other gene. In P. sabinae T27, the nifX-like gene is located within the nifH nifX-like nifN nifE cluster and is significantly transcribed under N2-fixing condition compared to non-N2-fixing condition (Additional file 1: Figure S4a). One possible reason is that the nifX-like and nifH were cotranscribed from a common promoter, consistent with previous studies that transcript of the nifH and nifX-like (previously called as nifB) increased under nitrogen fixation condition [26, 30]. However, the transcription of nifX-like gene linked together with gldA gene in P. forsythia T98 or P. zanthoxyli JH29 was not upregulated under N2-fixing condition than non-N2-fixing condition (Additional file 1: Figure S4b, c). Complementation experiments demonstrate that NifX-like proteins of P. sabinae T27, P. forsythia T98 and P. zanthoxyli JH29 could not resume the nitrogenase activity of P. polymyxa ΔnifB mutant (Additional file 1: Figure S4d), indicating that these NifX-like proteins can not substitute NifB. It was reported that...
NifX-like domain of NifB is not required for nitrogen fixation but may perform complementary functions that are beneficial for FeMo-co biosynthesis [21].

Complementation studies revealed that either NifB1 or NifB2 protein can support any type of nitrogenase activity. However, expression analysis showed that nifB1 exhibited the greatest increase in expression under Mo-dependent N2-fixing condition compared to alternative N2-fixing condition and nifB2 is even more induced under alternative N2-fixing condition compared to Mo-dependent N2-fixing condition. This implies that the NifB1 or NifB2 are specifically expressed under different conditions compared to P. forsythia T98 exhibited high nitrogenase activities.

P. sabinae, P. zanthoxyli T27, JH29 and WLY78 mutants for the biosynthesis of VFe-co, but the VnfEN A. vinelandii, NifEN can substitute for VnfEN in V-nitrogenase is dependent on either nifEN or vnfEN. In contrast, synthesis of nitrogenase in P. polymerxla is dependent on either nifEN or vnfEN. Our current result also confirms that VnfEN is the preferred scaffold for FeV-co maturation [34, 35]. Our result also confirms that VnfEN is more effective in FeV-co biosynthesis than NifEN.

Many efforts have been directed at engineering diazotrophic eukaryotes, one of the main hurdles is achieving NifB activity. Recent studies have found that the expressed NifB from the methanogen Methanocaldo- coccus infernus in the yeast cell was in a soluble form, while the expressed NifB from A. vinelandii in the yeast cells formed aggregates [36, 37]. In addition, the minimal number of genes required for nitrogen fixation is also the crucial step toward this goal. The Paenibacillus strains has some interesting features for engineering of eukaryotic N2 fixation, such as minimal nif gene cluster and additional nif and anf or vnf genes. Our study may provide guidance for screening nif genes to sort the best candidates to generate efficient nitrogenase. Given widespread findings of terrestrial Mo limitation [38], the minimal Fe- nitrogenase and V- nitrogenase systems described here have practical potentials in engineering nitrogen fixation.

Materials and methods
Phylogenetic analysis
The 138 putative nifB gene sequences of the 116 N2-fixing Paenibacillus strains and 11 putative nifB gene sequences of 10 other diazotrophs (Frankia, EAN1pec, Nostoc sp. PCC7120, Bradyrhizobium japonicum USDA 6, Krypidia spormannii CVV65, Clostridium kluveri DSM 555, Dehalobacter sp. CF, A. vinelandii DJ, K. oxytoca KONIH1, Methanococcus maripaludis S2 and Methano- sarcina aceticivorans C2A) from the NCBI RefSeq database (last accessed July 2019) are shown in Table S1. Multiple alignment of amino acid sequences was performed by ClustalW (version 2.1) [39]. A maximum-likelihood phylogenetic tree of Paenibacillus species was constructed using PhyML (version 3.0) software [40].

Plasmids, strains and growth conditions
Strains and plasmids used in this work are listed in (Additional file 1: Table S2). Paenibacillus strains were routinely grown in LD medium (2.5 g/L NaCl, 5 g/L yeast and 10 g/L tryptone) at 30°C with shaking under aerobic condition. For nitrogen fixation, Paenibacillus strains were grown in nitrogen-limited medium (0.3 g/L glutamate) under anaerobic condition. Nitrogen-limited medium used in this study contains 10.4 g/L of NaN3HP04, 3.4 g/L of KH2PO4, 26 mg/L of CaC12·2H2O, 30 mg/L of MgSO4·7H2O, 0.3 mg/L of MnSO4·H2O, 0.3 g/L of ferric citrate, 7.6 mg/L of Na2MoO4·2H2O, 10 µg/L of p-aminobenzoic acid, 5 µg/L of biotin, and 4 g/L glucose, with 0.3 g/L glutamate as the nitrogen source. Escherichia coli JM109 was used as routine cloning host. Thermo-sensitive vector pRN5101 [27,
nitrogen-limited medium that was depleted of molybdenum cells were collected, washed, and resuspended in nitrogen-free medium (nitrogen-limited medium without glutamate) under N$_2$ atmosphere, with initial OD$_{600}$ of 0.3. After 48 h, OD$_{600}$ was detected.

For diazotrophic growth, *Paenibacillus* strains and complementary strains were initially grown overnight in LD medium at 30°C. Cells were collected by centrifugation, and the pellet was washed three times with sterilized water and then resuspended in nitrogen-free medium (nitrogen-limited medium without glutamate) under N$_2$ atmosphere, with initial OD$_{600}$ of 0.3. After 48 h, OD$_{600}$ was detected.

Acetylene reduction assays for nitrogenase activity
Nitrogenase activity was measured by acetylene reduction assays as described previously [25]. For Mo-nitrogenase activity, *P. polymyxa* WLY78 and its derivatives were individually grown overnight in 50 mL of liquid LD media for 16 h at 30°C with shaking at 200 rpm. The culture was collected by centrifugation, and the pellet was washed three times with sterilized water and then resuspended in a 26 mL sealed tube containing 4 mL of nitrogen-limited medium to a final OD$_{600}$ of 0.3 to 0.5. The headspace in the tube was then evacuated and replaced with argon gas. After C$_2$H$_2$ (10% of the headspace volume) was injected into the test tubes, the cultures were incubated at 30 °C for 2–4 h and with shaking at 200 rpm. Then, 100 μL of gas was withdrawn through the rubber stopper with a gas tight syringe and manually injected into the gas chromatograph HP6890 to quantify ethylene production. The nitrogenase activity was expressed into the gas chromatograph HP6890 to quantify ethylene production. The nitrogenase activity was expressed in nmol C$_2$H$_4$/mg protein/hr. To assess Fe-nitrogenase activity, Mo-starved *Paenibacillus* cells were grown in nitrogen-limited medium that was depleted of molybdenum by Schneider et al. [41]. For V-nitrogenase activity, 30 μM Na$_2$VO$_4$ was added to the nitrogen-limited medium to take place of Na$_2$MoO$_4$. All treatments were in three replicates and all the experiments were repeated three or more than three times.

Transcription analysis
Transcription analyses of *nifB* genes were investigated by real-time quantitative PCR (RT-qPCR). *Paenibacillus sabinae* T27 was grown in nitrogen-limited medium containing Mo (Na$_2$MoO$_4$), while *P. zanthoxyli* JH29 and *P. forsythia* T98 were grown in Mo-free nitrogen-limited media containing Fe and V, respectively. For negative controls, these bacteria were individually grown in LD medium which has excess nitrogen medium to inhibit nitrogen fixation. These *Paenibacillus* strains were grown at 30°C with shaking under anaerobic condition. The bacterial cells were harvested after cultivation for 4 h cultivation. Total RNA was extracted with Trizol (Takara Bio, Tokyo, Japan) according to the manufacturer’s instructions. The integrity and size distribution of the RNA was verified by agarose gel electrophoresis, and the concentration was determined spectrophotometrically. Remove of genome DNA and synthesis of cDNA were performed using RT Prime Mix according to the manufacturer’s specifications (Takara Bio, Tokyo, Japan). Primers for *nif* genes and 16S rDNA used for RT-qPCR are listed in Additional file 1: Table S3. RT-qPCR was performed on Applied Biosystems 7500 Real-Time System and detected by the SYBR Green detection system with the following program: 95°C for 15 min, 1 cycle; 95°C for 10 s and 65°C for 30 s, 40 cycles. The relative expression level was calculated using the 2$^{-\Delta\DeltaCT}$ method [42]. Each experiment was performed in triplicate.

Construction of Δ*nifB*, Δ*nifBHDK* and Δ*nifBHDKEN* mutants of *P. polymyxa*
The *nifB*, *nifBHDK* and *nifBHDKEN* deletion mutants of *P. polymyxa* WLY78 were constructed by a homologous recombination method. The upstream (ca. 1 kb) and downstream (ca. 1.0 kb) fragments flanking the coding region of *nifB* or *nifBHDK* or *nifBHDKEN* were amplified by PCR from the genomic DNA of *P. polymyxa* WLY78 using Super-Fidelity DNA Polymerase (Vazyme Biotech Co., Ltd., Nanjing, China), respectively. The two fragments flanking coding region of *nifB* or *nifBHDK* or *nifBHDKEN* were then fused with BamH I digested pRNs101 vector using Gibson assembly master mix (New England Biolabs, Ipswich, USA), generating the recombinant plasmids pRDnifB, pRDnifBHDK and pRDnifBHDKEN, respectively. Then, each of these recombinant plasmids was transformed into *P. polymyxa* WLY78 as described by Wang et al., [43]. Subsequently, marker-free deletion mutants (the double-crossover transformants) Δ*nifB*, Δ*nifBHDK* and Δ*nifBHDKEN* were selected from the initial Em$^\text{R}$ transformants after several rounds of non-selective growth at 39 °C and then confirmed by PCR amplification and sequencing analysis. The primers used for the PCR amplifications were listed in Additional file 1: Table S3.

Construction of plasmids for complementation of the *P. polymyxa* Δ*nifB* mutant
Here, 9 *nifB* genes from *P. sabinae* T27, *P. forsythia* T98 and *P. zanthoxyli* JH29 were used to complement the *P. polymyxa* Δ*nifB* mutant. These *nifB* genes include *nifB1*, *nifB3* and *nifB4* of *P. sabinae* T27, *nifB1*, *nifB2* and *nifB3* of *P. forsythia* T98 and *nifB1*, *nifB2* and *nifB3* of *P. zanthoxyli* JH29. The coding region of each *nifB* gene from *P. sabinae* T27, *P. forsythia* T98 and *P. zanthoxyli* JH29 and a 310 bp promoter region of *nifB* in the *nifBHDKEN*XhesAnifV operon of *P. polymyxa* WLY78 were PCR amplified. Then, The PCR products of the *nifB* coding region
and the promoter region were fused together with vector pRN5101 using Gibson assembly master mix, yielding the recombinant plasmid. The recombinant plasmid was transformed to *P. polymyxa* WLY78 *nifB* mutant for complementation. The primers used in fusion were listed in Additional file 1: Table S3.

**Construction of Δ*nifB1*, Δ*nifB3* and Δ*nifB4* mutants of *P. sabinae* T27 and complementation strain**

Three *nifB* deletion mutants in *P. sabinae* T27 including Δ*nifB1*, Δ*nifB3* and Δ*nifB4* were constructed via homologous recombination using the suicide plasmid pRN5101 as described above. The upstream and downstream fragments flanking the coding region of *nifB1*, *nifB3* and *nifB4* were PCR amplified from the genomic DNA of *P. sabinae* T27, respectively. The primers used for deletion mutagenesis are listed in Additional file 1: Table S3. The upstream and downstream fragments of three *nifB* genes were then fused with BamH I-digested vector pRN5101 in Gibson assembly master mix, generating the three recombinant plasmids pRD*nifB1*, pRD*nifB3* and pRD*nifB4*. Then, each of these recombinant plasmids was electroporated into *P. sabinae* T27, and the deletion mutants were screened and confirmed by PCR and sequencing.

For complementation of Δ*nifB1*, a DNA fragment carrying the *nifB1* ORF (1377 bp) and its own promoter (549 bp) was PCR amplified and then ligated to pRN5101 and then transformed to *P. sabinae* T27 Δ*nifB1*, generating the *nifB1* complemented strain *nifB1/nifB1*. The primers used here are listed in Additional file 1: Table S3.

**Construction of the recombinant plasmids for complementation of the *P. polymyxa* Δ*nifBHDK* or Δ*nifBHDKEN* mutant**

For construction recombinant plasmids of alternative nitrogenases in *P. polymyxa*, the coding regions of the *nifB1*, *nifB2*, the *anfHDGK* and *nifE2N2anfHDGK* operon were amplified from the genome of *P. forsythia* T98, respectively. Also, a 310 bp promoter region of *nifB* in the *nifBHDK* operon *P. polymyxa* WLY78 was PCR amplified. Then, the PCR amplified promoter, *nifB1* or *nifB2* and the *anfHDGK* or *nifE2N2anfHDGK* operon were in order linked to vector pRN5101 using Gibson assembly master mix, yielding the recombinant plasmid carrying the reconstituted *nifB1anfHDGK* operon or *nifB2anfHDGK* operon or *nifB2E2N2anfHDGK* operon. The expression of *nifB1anfHDGK* or *nifB2anfHDGK* or *nifE2N2anfHDGK* was under control of the *P. polymyxa* *nifB* promoter. Finally, these plasmids were individually transformed into Δ*nifBHDK* or Δ*nifBHDKEN* mutant of *P. polymyxa* WLY78.

Similarly, the *nifB1*, *nifB2*, *vnfHDGK* and *vnfHDGKEN* operon were amplified from the genome of *P. zanthoxyli* JH29, respectively. A 310 bp promoter region of *nifB* in the *nifBHDK* operon *P. polymyxa* WLY78 was PCR amplified. Then, the three fragments including the promoter, *nifB1* or *nifB2* and *vnfHDGK* or *vnfHDGKEN* operon were in order fused together with vector pRN5101 using Gibson assembly master mix, yielding the recombinant plasmid carrying the reconstituted operon *nifB1vnfHDGK* or *nifB2vnfHDGK* or *nifB2vnfHDGKEN*. The expression of *nifB1vnfHDGK* or *nifB2vnfHDGK* or *nifB2vnfHDGKEN* was under control of the *P. polymyxa* *nifB* promoter. Finally, these plasmids were individually transformed into Δ*nifBHDK* mutant or Δ*nifBHDKEN* of *P. polymyxa* WLY78.

**Supplementary Information**

The online version contains supplementary material available at https://doi.org/10.1186/s12934-021-01629-9.

**Acknowledgements**

We thank Dr. Sihshou Wang for his guidance in phylogenetic analysis and helpful discussion.

**Authors’ contributions**

QL performed all experiments, and drafted the manuscript. HWZ participated in strain construction. LQZ assisted in the writing. SFC conceived the study, guided its coordination and wrote the manuscript. All authors read and approved the final manuscript.

**Funding**

This work was supported by the National Natural Science Foundation of China (No. 32000048) and the National Key Research and Development Program of China (No. 2019YFA0904700).

**Availability of data and materials**

All data generated or analysed during this study are included in this published article and are available from the corresponding author on reasonable request.

**Declarations**

**Ethics approval and consent to participate**

Not applicable.
Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

Author details
1 State Key Laboratory for Agrobiotechnology, College of Biological Sciences, China Agricultural University, Beijing 100193, People’s Republic of China. 2 Key Laboratory of Pest Monitoring and Green Management, Ministry of Agriculture and Rural Affairs, and College of Plant Protection, China Agricultural University, Beijing 100193, People’s Republic of China.

Received: 19 April 2021  Accepted: 10 July 2021  Published online: 19 July 2021

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