Functional Analysis of Multiple nifB Genes of Paenibacillus Strains in Synthesis of Mo-, Fe- and V-Nitrogenase

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Research

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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:** We have analyzed the genomes of the 116 diazotrophic *Paenibacillus* strains and found that some *Paenibacillus* strains have 2-4 *nifB* genes. Phylogeny analysis shows that all *nifB* genes in *Paenibacillus* fall into 4 subclasses: the *nifB1* being the first gene within the compact *nif* gene cluster, the *nifB2* being adjacent to *anf* or *vnf* genes, the other *nifB3* and *nifB4* being scattered on genomes. Transcriptional results demonstrate that *nifB1* exhibits the greatest increase in expression under Mo-dependent conditions and *nifB2* is even more induced under alternative fixation conditions. Functional analyses by complementation of the Δ*nifB* and Δ*nifBHDK* mutant of *P. polymyxa* WLY78 which has only one *nifB* gene and only Mo-nitrogenase showed that both *nifB1* and *nifB2* are active in synthesis of Mo-, Fe and V-nitrogenase. The *nifB3* and *nifB4* genes were not significantly expressed under N₂-fixing conditions and could not restore the nitrogenase activity of *P. polymyxa* Δ*nifB* mutant, suggesting that *nifB3* and *nifB4* genes were not involved in nitrogen fixation. In addition, reconstruction of *anf* system comprising 8 genes (*nifBanfHDGK* and *nifXhesAnifV*) and *vnf* system comprising 10 genes (*nifBvnfHDGKEN* and *nifXhesAnifV*) supported synthesis of Fe-nitrogenase and V-nitrogenase in *P. polymyxa*, respectively.

**Conclusions:** Our data and analysis reveal the contents and distribution of *nifB* genes in *Paenibacillus*. We demonstrated that the transcriptions of *nifB* being adjacent to *nif* or *anf* or *vnf* genes significantly expressed under N₂-fixation conditions and are active in synthesis of Mo-, Fe and V-nitrogenase. Our study also provides guidance for engineering nitrogen fixation genes into heterologous hosts for nitrogen fixation.

**Background**

Biological nitrogen fixation, a process unique to some bacteria and archaea (called diazotrophs), is catalyzed by nitrogenase and plays an important role in world agriculture [1]. There are three known nitrogenase designated as the Mo-nitrogenase, V-nitrogenase and Fe-nitrogenase that are encoded by *nif*, *vnf*, and *anf*, respectively [2]. Nitrogen fixation is mainly catalyzed by Mo-nitrogenase, which is found in all diazotrophs. In addition to 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 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 29413 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 (nifBHDKENXhesAnifV) 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, P. sabinae T27 has addition nif genes, including nifB, nifH, nifE and nifN. P. forsythia T98 and P. Sophorae S27 have additional nif and anfDHGK genes, P. zanthoxyli JH29 and P. durus (previously called as P.
azotoxans) ATCC 35681 contain additional nif and vnfDHGKEN genes. Notably, more than one copy of nifB genes were 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 subclasses: nifB1, nifB2, nifB3 and nifB4. We demonstrate that only nifB1 and nifB2 are functional in synthesis of Mo-, Fe- and V-nitrogenase. The nifB3 and nifB4 genes are not involved in nitrogen fixation. Our results define a minimal requirement of 8 and 10 genes for synthesis of the Fe-nitrogenase and V-nitrogenase in *P. polymyxa*, respectively, thus providing guidance for engineering nitrogenase into heterologous hosts in the absence of Mo.

**Results**

The nifB genes of *Paenibacillus* genus

Here, the nitrogen fixation genes in the genomes of the 116 diazotrophic *Paenibacillus* strains taken from the RefSeq database were comparatively analyzed (Additional file 1: Table S1). A compact nif gene cluster composed of 9–10 genes (nifBHDKENX(orf1)hesAnifV) was 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 had additional anfHDGK encoding Fe-nitrogenase and 3 strains had additional vnfHDGKEN encoding V-nitrogenase.

A total of 138 NifB putative sequences were found in the 116 diazotrophic *Paenibacillus* strains. According to the nifB position and sequence similarity, the nifB genes were divided into 4 classes. The nifB1 was designated as the one that is the first gene in the compact nif gene cluster comprising 9–10 genes (nifB nifH nifD nifK nifE nifN nifX (orf1) hesA nifV). The nifB2 was linked to additional copies of nifENXorf(fer) genes preceding anfHDGK or additional copies of nifENXorf orf preceding vnfHDGKEN or orf preceding vnfHDGKEN. The nifB3 and nifB4 were scattered at different locations with sequence divergence.

Of the 116 diazotrophic *Paenibacillus* strains, 105 strains had only one nifB and 11 strains had 2–4 nifB genes. *P. polymyxa* WLY78 was a representative that has only a nifB1 located in the compact nif gene cluster consisting of 9 genes (nifBHDKEXhesAnifV) encoding Mo-nitrogenase (Fig. 1 and Additional file 1: Table S1). *P. sabinae* T27 was 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, *P. zanthoxyli* JH29 had nifB1, nifB2 and nifB3, but *P. durus* ATCC 35681 had 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, *P. forsythiae* T98 had three nifB genes (nifB1, nifB2 and nifB3), whereas *P. sophorae* S27 had four nifB genes (nifB2, nifB3, and 2 copies of nifB1. The other 4 strains (*P. borealis* FSL H70744, *Paenibacillus* sp. FSL H7-0357, *Paenibacillus* sp. HW567 and *P. camerounensis* G4) with both Mo- and Fe-nitrogenases possessed only one nifB gene. Organization of the nifB genes and other nitrogen fixation genes from 17 representatives of *Paenibacillus* strains was shown in Fig. 1.
Phylogeny and Structure of *Paenibacillus* NifB proteins

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

Protein structure analysis showed that *Paenibacillus* NifB1, NifB2 and NifB4 proteins had the same structure composed of an N-terminal SAM-radical domain and a C-terminal NifX-like domain. Most NifB3 proteins possessed the two domains. But the NifB3 proteins from the 2 strains (*P. zanthoxyli* JH29 and *P. durus* DSM 1735) had only a SAM-radical domain. The *Paenibacillus* NifB1, NifB2, NifB3 and NifB4 proteins that possessed both domains were composed of 427–505 amino acids (Additional file 1: Table S1) and had similarity (> 57%) at amino acid levels. These proteins had a number of conserved motifs in the SAM-radical domain, including HPC motif, Cx3Cx2C motif, ExRP motif, AGPG motif, TxTxN motif and Cx2CRxDAxG (Fig. 2). However, NifB3 proteins of *P. zanthoxyli* JH29 and *P. durus* DSM 1735 had only a SAM-radical domain that lacks the Cx2CRxDAxG motif. Sequence alignment of 13 NifB 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) was shown in Additional file 2: 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 had NifB1, NifB3 and NifB4, *P. zanthoxyli* JH29 with both Mo- and V-nitrogenases had NifB1, NifB2 and NifB3 and *P. forsythia* T98 with both Mo- and Fe-nitrogenases possessed NifB1, NifB2 and NifB3. Here, 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. *P. sabinae* T27 was cultivated in Mo-dependent nitrogen fixation conditions, while *P. forsythia* T98 and *P. zanthoxyli* JH29 were cultivated in Mo-dependent and Fe-dependent or V-dependent nitrogen fixation conditions, respectively, with non-nitrogen fixing conditions of N-rich (LD medium) cultures as negative controls (Fig. 3). For *P. sabinae* T27 under Mo-dependent condition, nifB1 was significantly transcribed, but the other two genes nifB3 and nifB4 were nearly not expressed (Fig. 3a). For *P. forsythia* T98 under both Mo-dependent and Fe-dependent conditions, both nifB1 and nifB2 genes were transcribed, but nifB3 was nearly not expressed. 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 under both Mo-dependent and V-dependent conditions, both nifB1 and nifB2 genes were transcribed, but nifB3 was nearly not detected. 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 indicated 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 nearly completely lost the nitrogenase activity and its nifB gene carried in plasmid can restore the nitrogenase activity (Fig. 4). 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 the same result with transcription data that only nifB1 gene was upregulated under nitrogen fixation condition. 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, in agreement with the transcription data and suggesting that both nifB1 and nifB2 were functional in synthesis of Mo-nitrogenase.

Functional analysis of nifB1 and nifB2 genes in synthesis of Fe- and V-nitrogenases

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

Two new operons nifB1anfHDGK and nifB2anfHDGK of P. forsythia T98 under the control of the P. polymyxa WLY78 nifB promoter were constructed (Fig. 5). Each of the reconstituted nifB1anfHDGK and nifB2anfHDGK operons of P. forsythia T98 carried in the recombinant plasmids can enable P. polymyxa ΔnifBHDK mutant to have nitrogenase activity in medium containing Fe and lacking Mo. The data suggest that either nifB1 or nifB2 together with anfHDGK of P. forsythia can support synthesis of Fe-nitrogenase in the heterologous host P. polymyxa which originally has only Mo-nitrogenase system. Furthermore, in order to investigate whether nifE and nifN genes (designed nifE2 and nifN2 genes) preceding anfHDGK of P. forsythia T98 were functional, another new operon nifB2E2N2anfHDGK of P. forsythia T98 was constructed (Fig. 5). Then, nifB2E2N2anfHDGK and nifB2anfHDGK carried in the
recombinant plasmids are individually used to complement ΔnifBHDKEN mutant of _P. polymyxa_ WLY78. As shown in Fig. 5, either nifB2E2N2anfHDGK or nifB2anfHDGK can support ΔnifBHDKEN mutant of _P. polymyxa_ WLY78 to have nitrogenase activity in medium containing Fe and lacking Mo. Like the _P. forsythia_ T98 that was capable of diazotrophic growth, the reconstituted nifB/anf-complemented strains can grow in liquid media with dinitrogen as the sole nitrogen source (Fig. S2). The results indicated that _nifEN_ is not necessary for the biosynthesis and the reconstituted anf system composed of 8 genes (nifBanfHDGK of _P. forsythia_ 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 excluding ΔnifBHDKEN/nifB2vnfHDGK strain grew as well as the _P. zanthoxyli_ JH29 (Additional file 3: Figure S2). The results indicated that the reconstituted vnf system composed of 10 genes (nifBvnfHDGK of _P. zanthoxyli_ JH29 and nifENXhesAnifV of _P. polymyxa_ WLY78 or nifBvnfHDGKEN 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 subclasses and their encoded products have a N-terminal SAM-radical domain linked to a C-terminal NifX-like domain. However, the NifB3 proteins of _P. zanthoxyli_ JH29 and _P. durus_ DSM 1735 with V-nitrogenases are a SAM-radical protein linked 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 4: Figure S3). Sequence analysis have shown that the NifB3 protein of \textit{P. zanthoxyli} JH29 is really a stand-alone SAM-radical protein that linked to a NifX-like protein. We deduce that the \textit{nifB3} gene of \textit{P. zanthoxyli} JH29 or \textit{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 \textit{P. zanthoxyli} JH29 and \textit{P. durus} DSM 1735 lacks the C-terminal Cx2CRxDAxG 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 \textit{Clostridium} genus [21]. However, the NifB proteins with three domain architectures are not found in \textit{Paenibacillus}, although both \textit{Paenibacillus} and \textit{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 N$_2$-fixing \textit{Paenibacillus} strains possess NifX-like protein that shows high sequence similarity with the C-terminal domain of NifB but not with 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 \textit{P. sabinae} T27, \textit{P. forsythia} T98 and \textit{P. zanthoxyli} JH29 are investigated. Generally, the \textit{nifX}-like gene in \textit{Paenibacillus} strains is linked together with \textit{nifH} or other gene. In \textit{P. sabinae} T27, the \textit{nifX}-like gene is located within the operon \textit{nifHEN} in the order of \textit{nifH nifX}-like \textit{nifEN} and is significantly transcribed under nitrogen-fixing conditions (Additional file 5: Figure S4a). This could be \textit{nifX}-like and \textit{nifH} are organized as one operon and the previous reports that the transcription of \textit{nifH} genes was up-regulated under nitrogen fixation condition [26, 30]. However, the \textit{nifX}-like gene is linked together with \textit{gldA} gene in \textit{P. forsythia} T98 and \textit{P. zanthoxyli} JH29 and both \textit{nifX}-like genes were nearly not expressed (Additional file 5: Figure S4b, c). Complementation experiments demonstrate that NifX-like proteins of \textit{P. sabinae} T27, \textit{P. forsythia} T98 and \textit{P. zanthoxyli} JH29 could not resume the nitrogenase activity of \textit{P. polymyx}a \textit{ΔnifB} mutant (Additional file 5: 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 can support any type of nitrogenase activity. However, expression analysis showed that \textit{nifB1} exhibited the greatest increase in expression under Mo-dependent conditions and \textit{nifB2} is even more induced under alternative fixation conditions. This implies that that the \textit{nifB1} and \textit{nifB2} genes are specifically expressed under different metal conditions to support synthesis of Mo- and alternative nitrogenases in original host cell, respectively. As in \textit{Anabaena variabilis} ATCC 29413, two \textit{nifB} genes are specifically expressed in heterocysts or vegetative cells [20]. It is reported that \textit{P. sabinae} T27, \textit{P. zanthoxyli} JH29 and \textit{P. forsythia} T98 exhibited high nitrogenase activities compared to \textit{P. polymyx}a WLY78 [31]. Previous studies showed that 3 \textit{nifH} genes of \textit{P. sabinae} T27 are functional by complementing \textit{K. oxytoca} \textit{ΔnifH} mutant [32]. Our present work demonstrated that \textit{nifB2}
restored the nitrogenase activity of *P. polymyxa* WLY78 Δ*nifB* mutant. Thus, the higher nitrogenase activity exhibited by these species may be due to their additional *nif* genes.

The *nifB3* and *nifB4* were not expressed under nitrogen fixing conditions, nor functionally complementing the most common and active *nifB1* copy, and in some cases, displaying sequence divergence in regions of the protein already described as critical for NifB activity. This suggested that these *nifB* genes may have lost its capability of fixing nitrogen. They could be related to pseudogenization. Taking into account that the product of *nifB3* and *nifB4* showed the sequence similarity and conservation to NifB1 and NifB2, their inactivation seems to be caused by mutations in their regulatory sequence, leading to prevent their expression.

Moreover, we extended the studies to reconstruct gene requirements for the alternative nitrogenase. Our current study has demonstrated that the reconstituted *anf* system composed of 8 genes (*nifBanfHDGK* and *nifXhesAnifV*) can support synthesis of Fe-nitrogenase to fix nitrogen in *P. polymyxa*. This is consistent with previous report that the *nifEN* is not required for the reconstruction Fe-nitrogenase in *Escherichia coli* [33]. In contrast, synthesis of V-nitrogenase is dependent on either *nifEN* or *vnfEN*. In *A. vinelandii*, NifEN can substitute for VnfEN in vnfEN mutants for the biosynthesis of VFe-co, but the VnfEN not NifEN 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 *Methanocaldococcus 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 *N*₂ 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 fixing plants.

**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* sp. EAN1pec, *Nostoc* sp. PCC7120, *Bradyrhizobium japonicum* USDA 6, *Kyrpidia spormannii* CVV65, *Clostridium kluveri* DSM 555, *Dehalobacter* sp. CF, *A. vinelandii* DJ, *K. oxytoca* KONIH1, *Methanococcus maripaludis* S2 and *Methanosarcina acetivorans* 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 6: Table S2). *Paenibacillus* strains were routinely grown in LD medium (per liter contains: 2.5 g NaCl, 5 g yeast and 10 g tryptone) at 30°C with shaking under aerobic condition. For nitrogen fixation, *Paenibacillus* strains were grown in nitrogen-limited medium (2 mM glutamate) under anaerobic condition. Nitrogen-limited medium used in this study contains 10.4 g/L of Na₂HPO₄, 3.4 g/L of KH₂PO₄, 26 mg/L of CaCl₂·2H₂O, 30 mg/L of MgSO₄, 0.3 mg/L of MnSO₄, 36 mg/L of ferric citrate, 7.6 mg Na₂MoO₄·2H₂O, 10 mg/L of p-aminobenzoic acid, 5 mg/L of biotin, and 2% (wt/vol) glucose, with 2 mM glutamate as the nitrogen source. *Escherichia coli* JM109 was used as routine cloning host. Thermo-sensitive vector pRN5101 [27, 28] was used for gene disruption and complementation experiment in *P. polymyxa* WLY78. When appropriate, antibiotics were added in the following concentrations: 100 µg/mLampicillin and 5µg/mL erythromycin for maintenance of plasmids.

For diazotrophic growth, *Paenibacillus* strains and complementary strains were initially grown overnight in LD medium at 30°C. Cells were collected, washed, and resuspended in nitrogen-free medium (nitrogen-limited medium without glutamate) under N₂ atmosphere, with initial OD₆₀₀ of 0.3. After 48 h, OD₆₀₀ 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 their 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 OD₆₀₀ of 0.3 to 0.5. The headspace in the tube was then evacuated and replaced with argon gas. After C₂H₂ (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 in nmol C₂H₄/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 Na₃VO₄ was added to the nitrogen-limited medium to take place of Na₂MoO₄. 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). *P. sabinae* T27 was grown in nitrogen-limited medium containing Mo (Na₂MoO₄), 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) 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. Removal 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 7: 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 $2^{-\Delta\Delta Ct}$ method [42]. Each experiment was performed in triplicate.

Construction of the nifB, nifBHDK and nifBHDKEN deletion mutants

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 PCR amplified from the genomic DNA of P. polymyxa WLY78, respectively. The two fragments flanking coding region of nifB or nifBHDK or nifBHDKEN were then fused with BamH digest pRN5101 vector using Gibson assembly master mix (New England Biolabs), 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) ΔnifB, ΔnifBHDK and ΔnifBHDKEN were selected from the initial Emf transformants after several rounds of nonselective 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 7: Table S3.

Construction of plasmids for complementation of the P. polymyxa ΔnifB mutant

Here, 9 nifB genes from P. sabinae T27, P. forsythia T98 and P. zanthoxyli JH29 were used to complement the P. polymyxa ΔnifB 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 nifBHDKENXhesAnifV 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 7: Table S3.

Construction of the recombinant plasmids for complementation of the P. polymyxa ΔnifBHDK or ΔnifBHDKEN 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 nifBHDKENXhesAnifV operon of 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 nifB1vnfHDGK or nifB2vnfHDGK or nifE2N2anfHDGK was under control of the P. polymyxa nifB promoter. Finally, these plasmids were individually transformed into ΔnifBHDK or ΔnifBHDKEN 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 nifBHDKENXhesAnifV operon of 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 ΔnifBHDK mutant or ΔnifBHDKEN of P. polymyxa WLY78.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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.

Competing interests

The authors declare no competing interests.

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Author 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.

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References


Genetic organization of the nifB loci and other nif, anf, vnf genes in N2-fixing Paenibacillus strains. The compact nif gene cluster comprising contiguous 9-10 genes nifBHDKENX(orf1)hesAnifV. The anf genes are marked with yellow color and the vnf genes are marked with apricot yellow. The nifB genes are shown
in magenta. The nifX-like genes whose predicted products show high sequence similarity with the C-terminal domain of NifB are shown in pink.

Figure 2

Maximum likelihood phylogenetic tree and architectures of NifB proteins from N2-fixing Paenibacillus strains. All the NifB1 proteins in N2-fixing Paenibacillus strains clustered together and were not shown. The SAM-radical is shown in red and the NifX-like domain in blue. Color dots represent conserved motifs in the NifB proteins. The NifB has only a stand-alone SAM-radical domain marked blue triangle.
Figure 3

Transcription profile of the multiple nifB genes from P. sabinae T27(a), P. forsythia T98(b) and P. zanthoxyli JH29(c). RT-qPCR analysis of the relative transcript levels of the nifB genes in these Paenibacillus species grown in Mo-dependent, Fe-dependent and V-dependent nitrogen fixation conditions, with non-nitrogen fixing conditions of N-rich (LD medium) cultures as negative controls. The data are the mean of three biological replicates.

Figure 4

The nitrogenase activities of the P. polymyxa ΔnifB mutant and its complementary strains in Mo-dependent nitrogen fixation conditions. The nitrogenase activity was measured by acetylene reduction
assay when bacterial cells were grown anaerobically in nitrogen limited medium containing Mo. Error bars indicate the SD observed from at least three independent experiments.

Figure 5

Schematic map and nitrogenase activity of the ΔnifBHDK and ΔnifBHDKEN mutants of P. polymyxa and the complementary strains carrying nifB1anfHDGK, nifB2anfBHDGK, nifB2E2N2anfBHDGK of P. forsythia T98, respectively and the complementary strains carrying nifB1vnfHDGK, nifB2vnfHDGK,
nifB2vnfHDGKEN of P. zanthoxyli JH29, respectively. a Schematic map of the P. polymyxa ΔnifBHDK and P. polymyxa ΔnifBHDKEN mutants and the complementary strains. b The nitrogenase activity of the P. polymyxa ΔnifBHDK and P. polymyxa ΔnifBHDKEN mutants and the complementary strains. Activity was measured by acetylene reduction assay. The complementary strains carrying nifB1anfHDGK, nifB2anfBHDGK and nifB2E2N2anfBHDGK were cultivated in Fe-dependent conditions. The complementary strains carrying nifB1vnfHDGK, nifB2vnfHDGK and nifB2vnfHDGKEN were cultivated in V-dependent conditions. Error bars indicate the SD observed from at least three independent experiments.

**Supplementary Files**

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