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

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Research

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

Posted Date: April 22nd, 2021

DOI: https://doi.org/10.21203/rs.3.rs-444251/v1

Version of Record: A version of this preprint was published at Microbial Cell Factories on July 19th, 2021.
See the published version at https://doi.org/10.1186/s12934-021-01629-9.

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 *nifB*3 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 N_2-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 N_2-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 $\alpha_2\beta_2$ 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 N_2 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 *Paenibacullus* strains have revealed that a compact *nif* gene cluster comprising 9–10 genes (*nifB nifH nifD nifK nifE nifN nifX* (*orf1*) *hesA nifV*) encoding Mo-nitrogenase is conserved in the N_2-fixing *Paenibacillus* genus [24]. The 9 genes (*nifBHDKENXhesAnifV*) in *Paenibacillus polymyxa* WLY78 are organized as an operon under control of a σ^{70} 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.*

azotofixans) 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 *nifENXorforf* genes preceding *vnfHDGKEN* or *orforf* 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 (*nifBHDKENXhesAnifV*) 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 kluyveri, 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, Cx_3Cx_2C motif, ExRP motif, AGPG motif, TxTxN motif and Cx_2CRxDAxG (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. forsythiae* 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 (Δ*nifB*) of *P. polymyxa* WLY78 was here constructed by using recombination method as described in materials and methods. The *P. polymyxa* Δ*nifB* mutant nearly completely lost the nitrogenase activity and its *nifB* gene carried in plasmid can restore the nitrogenase activity (Fig. 4). Thus, *P. polymyxa* Δ*nifB* 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* Δ*nifB* 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* Δ*nifB* 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 Δ*nifBHDK* 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 (Δ*nifBHDK/nifBHDK*) and (Δ*nifBHDKEN/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* Δ*nifBHDK* 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 Δ*nifBHDKEN* mutant of *P. polymyxa* WLY78. As shown in Fig. 5, either *nifB2E2N2anfHDGK* or *nifB2anfHDGK* can support Δ*nifBHDKEN* 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 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* Δ*nifBHDK* 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 Δ*nifBHDKEN* mutant of *P. polymyxa* WLY78. The operon *nifB2vnfHDGKEN* can effectively enable Δ*nifBHDKEN* 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 Δ*nifBHDKEN* 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 Δ*nifBHDKEN*/*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 *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 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 *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 N_2-fixing *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 *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 operon *nifHEN* in the order of *nifH* *nifX*-like *nifEN* and is significantly transcribed under nitrogen-fixing conditions (Additional file 5: Figure S4a). This could be *nifX*-like and *nifH* are organized as one operon and the previous reports that the transcription of *nifH* genes was up-regulated under nitrogen fixation condition [26, 30]. However, the *nifX*-like gene is linked together with *gldA* gene in *P. forsythia* T98 and *P. zanthoxyli* JH29 and both *nifX*-like genes were nearly not expressed (Additional file 5: 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* Δ*nifB* 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 *nifB1* exhibited the greatest increase in expression under Mo-dependent conditions and *nifB2* is even more induced under alternative fixation conditions. This implies that that the *nifB1* and *nifB2* genes are specifically expressed under different metal conditions to support synthesis of Mo- and alternative nitrogenases in original host cell, respectively. As in *Anabaena variabilis* ATCC 29413, two *nifB* genes are specifically expressed in heterocysts or vegetative cells [20]. It is reported that *P. sabinae* T27, *P. zanthoxyli* JH29 and *P. forsythia* T98 exhibited high nitrogenase activities compared to *P. polymyxa* WLY78 [31]. Previous studies showed that 3 *nifH* genes of *P. sabinae* T27 are functional by complementing *K. oxytoca* Δ*nifH* mutant [32]. Our present work demonstrated that *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 N_2-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 kluyveri* 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℃ 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 Na_2HPO4, 3.4 g/L of KH_2PO_4, 26 mg/L of $CaCl_2{\cdot}2H_2O$, 30 mg/L of $MgSO_4$, 0.3 mg/L of $MnSO_4$, 36 mg/L of ferric citrate, 7.6 mg $Na_2MoO_4{\cdot}2H_2O$, 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℃. 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.

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℃ 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_2H_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 in nmol C_2H_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_3VO_4 was added to the nitrogen-limited medium to take place of Na_2MoO_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). *P. sabinae* T27 was grown in nitrogen-limited medium containing Mo (Na_2MoO4), 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℃ 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. 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 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℃ for 15 min, 1 cycle; 95℃ for 10 s and 65℃ 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* Ⅰ digested 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) Δ*nifB*, Δ*nifBHDK* and Δ*nifBHDKEN* were selected from the initial Em^r 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* 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 *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* 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 *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 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 *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 Δ*nifBHDK* mutant or Δ*nifBHDKEN* 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.

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).

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.

Acknowledgements

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

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Figures

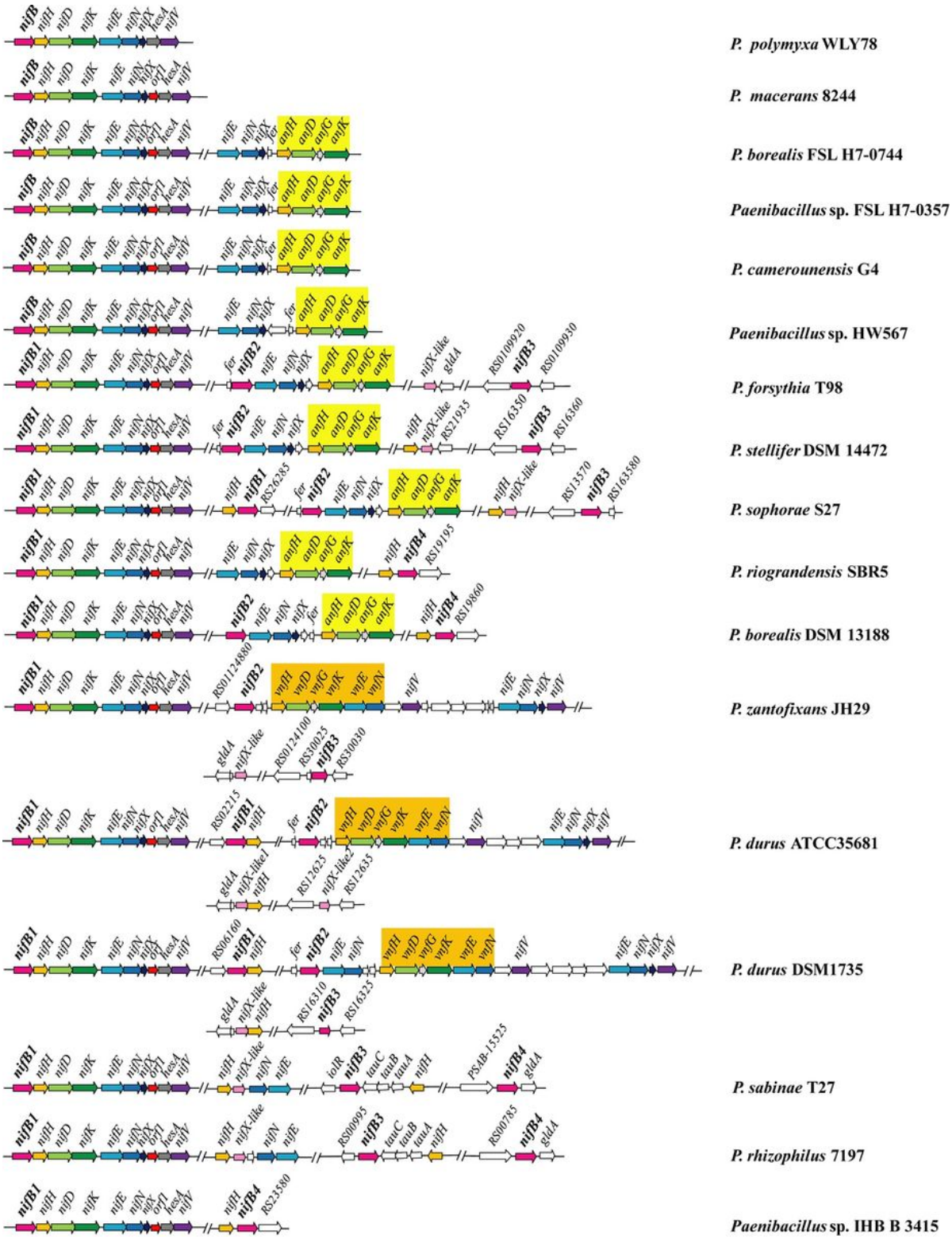

Figure 1

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.

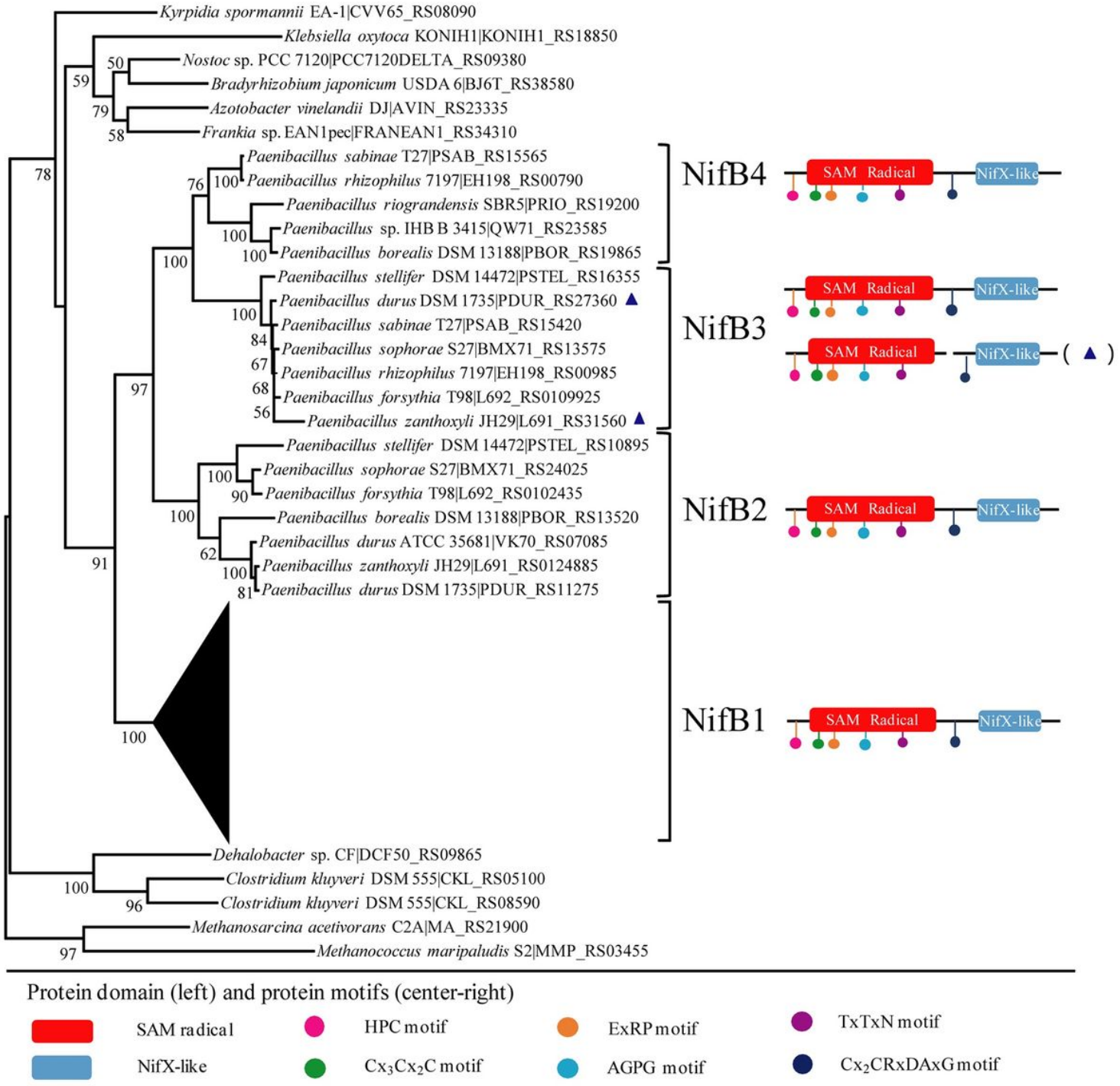

Figure 2

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.

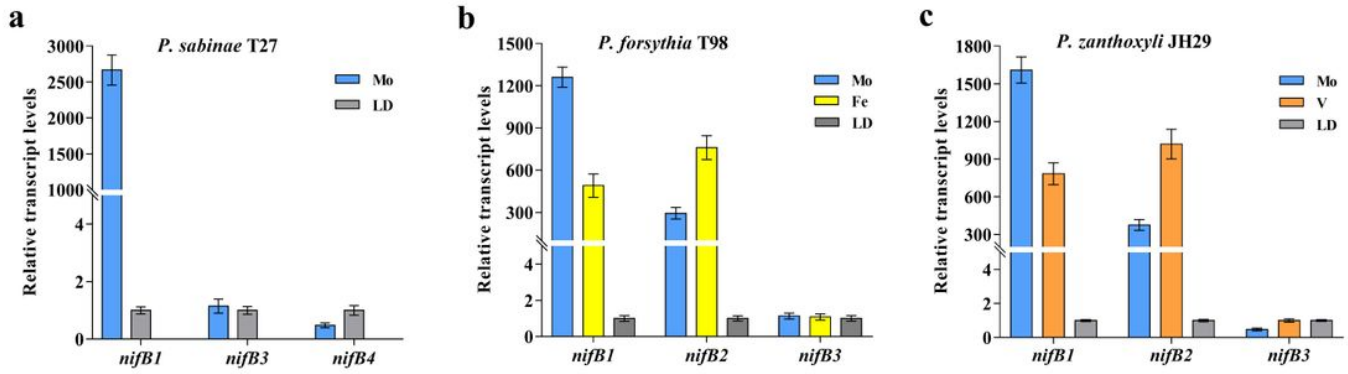

Figure 3

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.

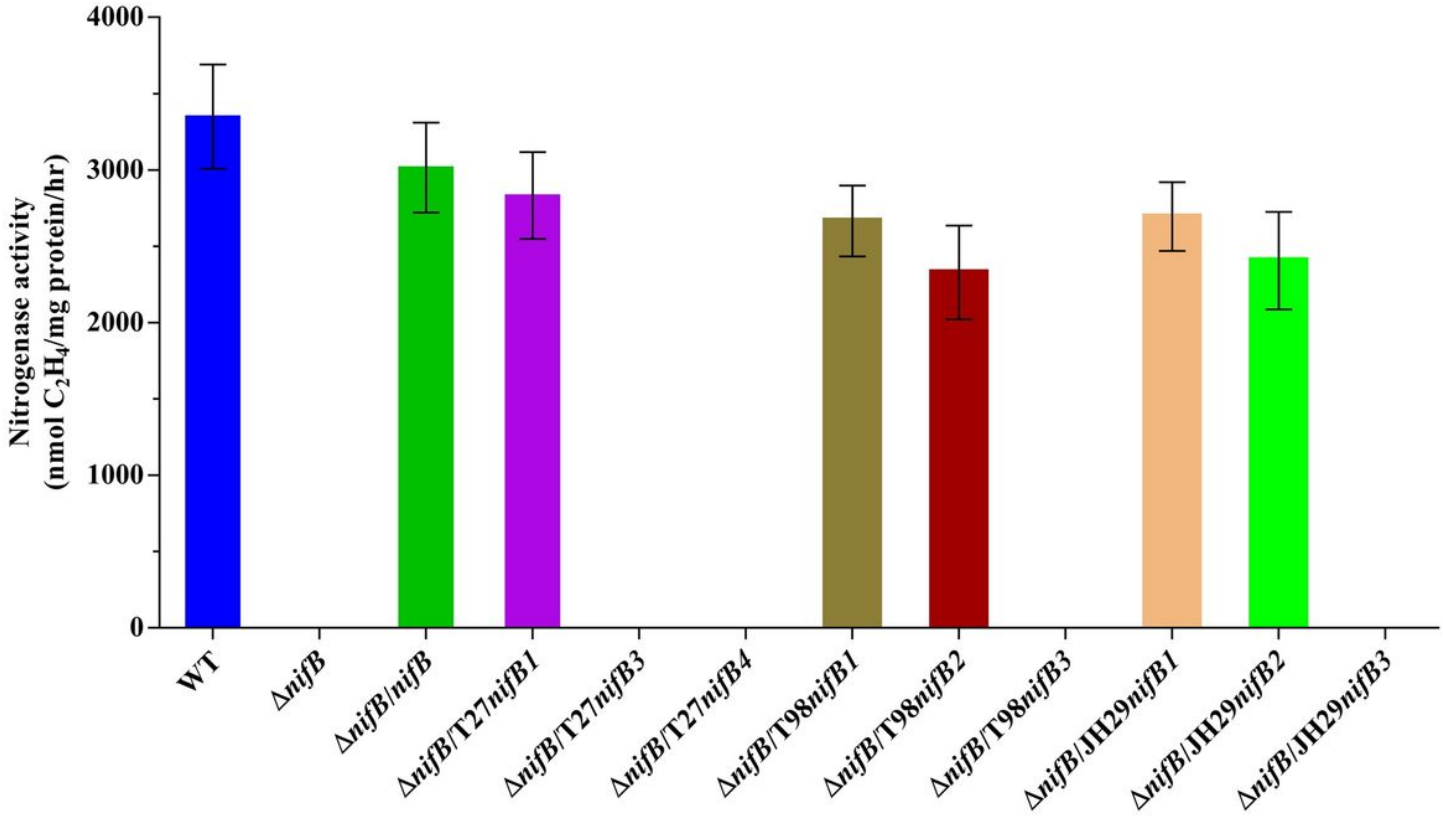

Figure 4

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.

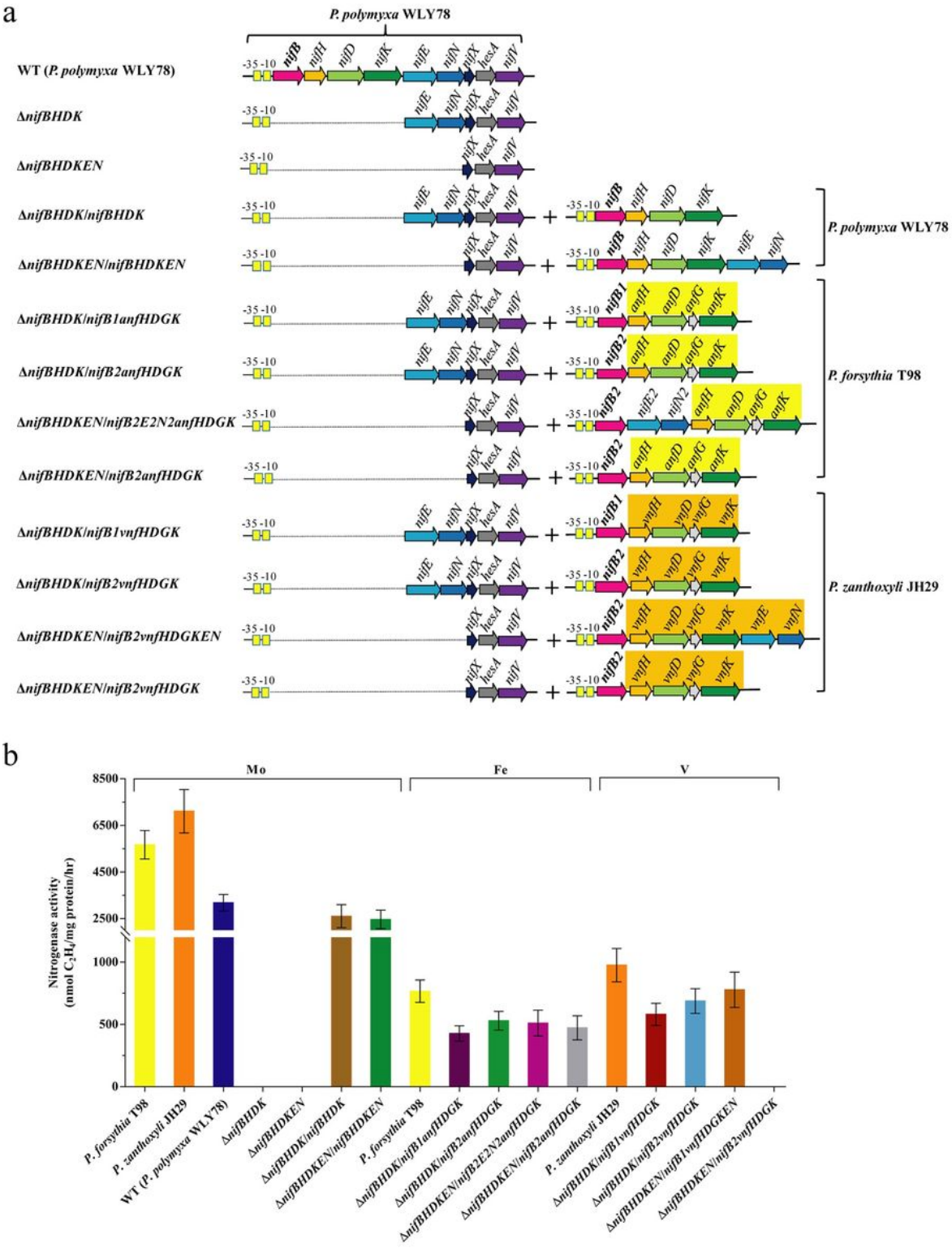

Figure 5

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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