Using synthetic biology to increase nitrogenase activity

Xin-Xin Li, Qi Liu, Xiao-Meng Liu, Hao-Wen Shi and San-Feng Chen*

Abstract

Background: Nitrogen fixation has been established in protokaryotic model Escherichia coli by transferring a minimal nif gene cluster composed of 9 genes (nifB, nifH, nifD, nifK, nifE, nifN, nifX, hesA and nifV) from Paenibacillus sp. WLY78. However, the nitrogenase activity in the recombinant E. coli 78-7 is only 10% of that observed in wild-type Paenibacillus. Thus, it is necessary to increase nitrogenase activity through synthetic biology.

Results: In order to increase nitrogenase activity in heterologous host, a total of 28 selected genes from Paenibacillus sp. WLY78 and Klebsiella oxytoca were placed under the control of Paenibacillus nif promoter in two different vectors and then they are separately or combinatorially transferred to the recombinant E. coli 78-7. Our results demonstrate that Paenibacillus suf operon (Fe–S cluster assembly) and the potential electron transport genes pfoAB, fldA and fer can increase nitrogenase activity. Also, K. oxytoca nifSU (Fe–S cluster assembly) and nifFJ (electron transport specific for nitrogenase) can increase nitrogenase activity. Especially, the combined assembly of the potential Paenibacillus electron transporter genes (pfoABfldA) with K. oxytoca nifSU recovers 50.1% of wild-type (Paenibacillus) activity. However, K. oxytoca nifWZM and nifQ can not increase activity.

Conclusion: The combined assembly of the potential Paenibacillus electron transporter genes (pfoABfldA) with K. oxytoca nifSU recovers 50.1% of wild-type (Paenibacillus) activity in the recombinant E. coli 78-7. Our results will provide valuable insights for the enhancement of nitrogenase activity in heterogeneous host and will provide guidance for engineering cereal plants with minimal nif genes.

Keywords: Nitrogenase, Paenibacillus, Fe–S cluster assembly, Electron transporter

Background

Most biological nitrogen fixation is catalyzed by the molybdenum nitrogenase enzyme. The molybdenum nitrogenase is composed of two proteins, MoFe protein (NifDK) and Fe protein (NifH). The MoFe protein is an α2β2 heterotetramer that contains the iron–molybdenum cofactors (FeMo-co) and P clusters. The FeMo-co is a [Mo–7Fe–9S–C-homocitrate] cluster which serves as the active site of nitrogen binding and reduction. The P-cluster is a [8Fe–7S] cluster which shuttles electrons to the FeMo-co. The Fe protein is a γ2 homodimer bridged by an intersubunit [4Fe–4S] cluster that serves as the obligate electron donor to the MoFe protein [1–5].

Although the biochemical properties and structure of molybdenum nitrogenases are remarkably similar when purified from diverse bacteria and archaea, the organization and numbers of nif genes required for the synthesis and assembly of the enzyme varies greatly among these nitrogen-fixing species [6–8]. For example, in K. oxytoca (previously called K. pneumoniae), 20 nif genes, nifHDKTYENXUSVWZMFLABQ, organized in 7 transcriptional units are co-located within a 24 kb cluster [4], while Paenibacillus sp. WLY78 possesses a minimal and compact nif gene cluster consisting of 9 genes (nifBnifHnifDnifKnifEnifNnifXnifV) (Fig. 1) [9, 10]. This variability in nif genes content is undoubtedly determined by the environmental lifestyle of each diazotroph on one hand, the minimal nif gene sets are probably...
complemented by housekeeping counterparts located elsewhere in the genome on the other hand.

Genetic and biochemical studies on the two model diazotrophs K. oxytoca and A. vinelandii revealed that 16 nif genes (nifH,D,K,Y,T,E,N,X,U,S,V,Z,W,M,B,Q) products are probably essential for efficient biosynthesis of nitrogenase [11]. It has been demonstrated that nifH, nifD and nifK genes encodes the structural subunits, the nifE, nifN, nifX, nifB, nifQ, nifV, nifY and nifH contribute to the synthesis and insertion of FeMo-co into nitrogenase, nifUL, nifS and nifZ play an important role in synthesis of metalloclusters and nifM is required for properfolder of nitrogenase Fe protein [1–5]. However, mutations in some of these genes (notably nifY, nifT, nifX, nifU, nifS, nifV, nifW, nifM and nifQ) do not completely eliminate nitrogenase activity, and there is evidence that homologues elsewhere on the genome may at least par
eliminate nitrogenase activity, and there is evidence that nifD nifK nifE nifN nifX hesA nifV

Nitrogen fixation plays an important role in agriculture, and there has been a goal to engineer nitrogen fixation into cereals crops to reduce the use of chemically derived fertilizer. The complex nature of the FeMo-co assembly pathway and the large number of genes required for nitrogenase biosynthesis and maintenance of its activity represent a daunting engineering task, even in the age of systems biology. Thus, it is necessary to increase nitrogenase activity through synthetic biology.

In this study, two cloning and expression vectors with Paenibacillus nif promoter and ribosome binding site are constructed for transferring foreign genes to the recombinant E. coli 78-7 which carrying the Paenibacillus nif gene operon. A total of 28 selected genes from Paenibacillus and K. oxytoca were placed under the control of Paenibacillus nif promoter in these vectors and then are transferred to E. coli 78-7. Our results demonstrate that Fe–S cluster assembly system and electron transport system from Paenibacillus or K. oxytoca can increase E. coli nitrogenase activity mediated by the minimal nif gene cluster composed of 9 genes (nifBHDKENXhesAnifV). But K. oxytoca nifWZM and nifQ which are required in synthesis and maturation of nitrogenase in K. oxytoca can not increase any activity. Here is the first time to demonstrate that the potential electron transport genes (pfoAB, fer and fldA) are involved in nitrogen fixation of Paenibacillus. Also, it is the first time to demonstrate that Paenibacillus suf and K. oxytoca nifJ and nifSU can significantly increase nitrogenase activity in E. coli mediated by the Paenibacillus nif gene operon (nifBHDKENXhesAnifV). Our results will provide valuable information for the incoming hot research that engineer nitrogen fixation pathway into cereal crops.

Results
Design of combinatorial assembly of the nif and nif-related genes

E. coli 78-7 is a recombinant strain carrying a Paenibacillus nif gene operon (nifBHDKENXhesAnifV) in vector pH300PLK [9, 16] (Fig. 2a). As described in methods, two vectors carrying Paenibacillus nif

---

**Fig. 1** Schematic representation of the Paenibacillus nif gene cluster compared with syntenic nif clusters of K. oxytoca M5a1. a Paenibacillus sp. WLY78. b K. oxytoca M5a1
promoter, ribosome-binding site and the multiple cloning site (MCS) are constructed (Fig. 2b, c) for expressing other \( nif \) or \( nif \)-related genes in \( E. coli \) 78-7. The two vectors can coexist in \( E. coli \) cells with plasmid pHY300PLK. Then the two vectors carrying foreign genes from \textit{Paenibacillus} sp. WLY78 or \textit{K. oxytoca} were separately or combinationally transferred to \( E. coli \) 78-7 (Additional file 1: Table S1, Additional file 2: Table S2).

\textit{Paenibacillus} Suf system can increase nitrogenase activity of the recombinant \( E. coli \) 78-7

Nitrogenase is a complex [Fe–S] enzyme. Many diazotrophs, such as \textit{K. oxytoca} and \textit{A. vinelandii}, contain \( nifU \) and \( nifS \) whose products were involved in the assembly of [Fe–S] clusters of nitrogenase [2–5]. NifU and NifS separately provide the Fe and S required for nitrogenase maturation. The genome of \textit{Paenibacillus} sp. WLY78 does not have \( nifSU \), but contains iron-sulfur cluster assembly systems: a complete \( suf \) (\( sufCBSUD \)) operon and a partial \( isc \) system (\( iscSR \)). Similarly, there are no \( nifS \) and \( nifU \) in \( E. coli \), but \( E. coli \) has two iron-sulfur cluster assembly systems: the \( sufABCDSE \) operon and the \( isc \) system composed of \( iscR, iscS, iscU, iscA, hscB, hscA, fdx \), and \( orf3 \) [17]. The \( nif \) gene operon from \textit{Paenibacillus} sp. WLY78 could enable \( E. coli \) to fix nitrogen, suggesting that the assembly of Fe–S clusters for the nitrogenase was provided by \( E. coli \) iron-sulfur cluster assembly systems.

In this study, the \( suf \) (\( sufCBSUD \)) operon and \( iscSR \) system from \textit{Paenibacillus} sp. WLY78 are placed under the control of \textit{Paenibacillus} \( nif \) promoter, respectively, and then are separately transferred into the recombinant \( E. coli \) 78-7. As shown in Fig. 3, the \( suf \) (\( sufCBSUD \)) operon can increase nitrogenase activity of \( E. coli \) 78-7 from 10 to 20 %, while \( iscSR \) system cannot increase any activity. The data suggest that the \( suf \) (\( sufCBSUD \)) operon plays an important role in Fe–S cluster assembly in nitrogenase synthesis of \textit{Paenibacillus}.

\textit{Klebsiella oxytoca} \( nifSU \) can increase nitrogenase activity of the recombinant \( E. coli \) 78-7

As described in methods, \textit{K. oxytoca} \( nifSU \) gene cluster was placed under the control of \textit{Paenibacillus} \( nif \) promoter and then was transferred to \( E. coli \) 78-7. As shown in Fig. 4, \textit{K. oxytoca} \( nifSU \) can increase activity of \( E. coli \) 78-7 from 10 to 19.5 %. Our data that \textit{K. oxytoca} \( nifSU \) or \textit{Paenibacillus} \( sufCBSUD \) can increase nitrogenase activity of \( E. coli \) 78-7 is consistent with the fact that nitrogenase is a complex [Fe–S] enzyme which contains 38 Fe atoms, 40 S atoms and 2 Mo atoms.

The potential electron transporters from \textit{Paenibacillus} can increase nitrogenase activity of the recombinant \( E. coli \) 78-7

Nitrogen fixation is carried out by the enzyme nitrogenase, which transfers electrons originating from
low-potential electron carriers, such as flavodoxin or ferredoxin molecules, to molecular N\textsubscript{2} [18]. In \textit{K. oxytoca}, the physiological electron flow to nitrogenase involves specifically the products of the \textit{nifF} and \textit{nifJ} genes [19]. The \textit{nifF} gene product, a flavodoxin, mediates electron transfer from the \textit{nifJ} gene product, a pyruvate: flavodoxin oxidoreductase, to the Fe protein of nitrogenase [20–23]. Unlike \textit{K. oxytoca} \textit{nif} gene cluster, \textit{Paenibacillus} \textit{nif} gene cluster does not have \textit{nifF} and \textit{nifJ}. Genome sequence analysis revealed that there are several genes encoding ferredoxin, flavodoxin and flavodoxin oxidoreductase in the genome of \textit{Paenibacillus sp. WLY78}. For example, \textit{fer} and \textit{COG3411} encode ferredoxin, \textit{fldA} and \textit{fldB} encode flavodoxin, and \textit{fpr} encodes ferredoxin-NADP reductase, \textit{nfrA} encodes NAD(P)H-flavin oxidoreductase, and \textit{pfoAB} separately encode pyruvate: ferredoxin oxidoreductase gamma subunit and alpha subunit. Of these genes, \textit{fldA} and \textit{fldB} shows 30 % identity with \textit{K. oxytoca} \textit{nifF}, and \textit{pfoAB} exhibit 33 % identity with \textit{K. oxytoca} \textit{nifJ}, but other genes do not show identity with \textit{K. oxytoca} \textit{nifF} or \textit{nifJ}.

Fig. 3 Assembly and functional analysis of the \textit{K. oxytoca} Fe–S cluster assembly system (\textit{nifUS}) in \textit{E. coli} 78-7. \textbf{a} Linear view of the Fe–S cluster assembly gene region in pBluescript SK (+)-derived plasmid. \textbf{b} Relative nitrogenase activity of wild-type \textit{Paenibacillus sp. WLY78}, \textit{E. coli} 78-7 (\textit{nifUS}), \textit{E. coli} 78-7 (pBluescript SK (+)) was used as a control. Each experiment was repeated at least three times, and the error bars represent standard error.

Fig. 4 Assembly and functional analysis of the \textit{Paenibacillus} Fe–S cluster assembly systems (\textit{suf} and \textit{isc}) in \textit{E. coli}. \textbf{a} Linear view of the Fe–S cluster assembly gene region in pBluescript SK (+)-derived plasmid. \textbf{b} Relative nitrogenase activity of wild-type \textit{Paenibacillus sp. WLY78}, \textit{E. coli} 78-7 (pBluescript SK (+)), \textit{E. coli} 78-7 (suf), \textit{E. coli} 78-7 (isc). \textit{E. coli} 78-7 (pBluescript SK (+)) was used as a control. Each experiment was repeated at least three times, and the error bars represent standard error.
into the recombinant *E. coli* 78-7. As shown in Fig. 5, each of *fer* and *fldA* can increase nitrogenase of *E. coli* 78-7 from 10 to 20.1%, while *fldB* and COG3411 cannot increase any activity. The data suggest that *fer* (ferredoxin) and *fldA* (flavodoxin) might be an electron transporter of nitrogenase. Our data are consistent with the previous report that either flavodoxins or ferredoxins are the direct electron donor to nitrogenase in diazotrophic bacteria [21].

Furthermore, *nfrA, fpr* and *pfoAB*, the orthlogs of *K. oxytoca nifF*, were separately transferred into the recombinant *E. coli* 78-7. As shown in Fig. 5, *pfoAB* increase nitrogenase activity from 10 to 15%, while *nfrA* and *fpr* do not increase any activity. The data suggest that *pfoAB* play a role in nitrogen fixation. Notably, the nitrogenase activity of *E. coli* 78-7 is increased to 35.1 and 40.1%, respectively, when *pfoAB* is combined with *Paenibacillus fer* gene (*Paenibacillus-ferproAB*) or *Paenibacillus fldA* gene (*Paenibacillus-fldAproAB*). We deduce that in *Paenibacillus*, *pfoAB* (pyruvate: ferredoxin oxidoreductase) might be involved in the pyruvate breakdown to yield electrons, and then *fldA* and *fer* mediate electron to nitrogenase.

**Klebsiella oxytoca nifF and nifU can increase nitrogenase activity of the recombinant *E. coli* 78-7**

As shown in Fig. 6, *K. oxytoca nifF* and *nifJ*, whose products are electron transporters, can increase nitrogenase of *E. coli* 78-7 from 10 to 20.4 and 12.1%, respectively. When *nifF* and *nifJ* were carried in two different vectors and co-transferred into *E. coli* 78-7, the activity was increased from 10 to 32%. However, nitrogenase activity could not be increased when *nifF* and *nifJ* were assembled as an operon. Our results are consistent with the report that coordinated and balanced expression of *nifF* and *nifJ* genes is important for nitrogenase activity in *E. coli* carrying *K. oxytoca nif* clusters [15].

**Combination of Fe–S cluster synthesis system and electron transporters can significantly increase nitrogenase activity**

As described above, the (potential) electron transporters and iron-sulfur cluster assembly systems from *Paenibacillus* sp. WLY78 or *K. oxytoca* can increase activity of *E. coli* 78-7. Here, combination of the (potential) electron transporters and iron-sulfur cluster assembly system was transferred to *E. coli* 78-7. Considering that *K. oxytoca nifSU* genes are much shorter and easier to operate...
in gene cloning than Paenibacillus suf system, K. oxytoca nifSU genes were used in this combined assembly with the (potential) electron transporters from Paenibacillus sp. WLY78 or K. oxytoca. As shown in Fig. 7, the combined Kp-nifJnifEanifUS, WLY78-ferpfoAB-Kp-nifUS and WLY78- fldApfoAB-Kp-nifUS increase activity from 10 to 39.1, 45.1 and 50.1 %, respectively. The highest activity obtained by WLY78-fldApfoAB-Kp-nifUS suggests that fldApfoAB are the electron transport of nitrogenase in Paenibacillus.

Klebsiella oxytoca nifWZM and nifQ can not increase nitrogenase activity

It was reported that the nifW and nifZ genes seem to be involved in MoFe protein maturation, while nifM is required for proper folding of nitrogenase Fe protein [1, 3]. nifM mutants of K.oxytoca and A. vinelandii were unable to synthesise action Fe protein [24–26].Unlike in K. oxytoca, Paenibacillus has not the nifWZM genes. And E. coli has also not the nifWZM genes. In this study, the K. oxytoca nifWZM genes were transferred to E. coli 78-7, but the nitrogenase activity was not enhanced by these genes. The data suggest that the requirement of nifWZM genes on maturation of nitrogenase vary greatly among diazotrophs.

NifQ has been implicated in the processing of molybdenum specifically for the biosynthesis of FeMo-co [1, 2]. Unlike in K. oxytoca, Paenibacillus dose not have nifQ gene. In this study, the K. oxytoca nifQ gene was transferred to E. coli 78-7, but the nitrogenase activity was not enhanced by nifQ gene. The result indicates that K. oxytoca nifQ is not involved in the processing of molybdenum specifically for the biosynthesis of FeMo-co of nitrogenase encoded by Paenibacillus nif genes (Fig. 8).

Discussion

Our recent studies have revealed that the genome of Paenibacillus sp. WLY78 contains a minimal nif gene cluster composed of nine genes nifBHDKENXhesAnifV and the nif operon under the control of its own nif promoter enabled E. coli to synthesize the active nitrogenase [9]. However, the specific activity of the enzyme expressed in E. coli was approximately 10 % of that observed in Paenibacillus. In this study, synthetic biology was used to determine whether 28 selected genes from Paenibacillus sp. WLY78 and K. oxytoca can increase nitrogenase activity of the recombinant E. coli 78-7.

Compared with K. oxytoca nif clusters, one of the notable absences in the minimal Paenibacillus nif gene cluster
is the two genes nifS and nifU, which provide the nitrogen fixation-specific iron-sulfur cluster assembly. The genome of Paenibacillus sp. WLY78 does not have nifSU, but contains iron-sulfur cluster assembly systems: a complete suf (sufCBSUD) operon and a partial isc system (iscSR). In this study, we demonstrate that Paenibacillus suf (sufCBSUD) operon can increase the nitrogenase activity of the recombinant E. coli 78-7, and K. oxytoca nifSU also can increase activity. The results reveal that iron-sulfur cluster assembly system specific for Fe–S cluster of nitrogenase is very important to nitrogen fixation. The results also imply that although E. coli iron-sulfur cluster assembly system can support the synthesis of active nitrogenase, it cannot fully support the requirement for synthesis of Fe–S cluster.

It was reported that pyruvate is a major source of electrons in diazotrophic Clostridium pasteurianum and Bacillus polymyxa (now called as Paenibacillus polymyxa) [22]. In K. oxytoca, the pyruvate oxidoreductase (nifJ gene product) was responsible for the pyruvate breakdown to yield electrons, and then the flavodoxin
(the *nifF* gene product) mediates electron transfer to the Fe protein of nitrogenase [23]. The *Paenibacillus* sp. WLY78 *nif* gene operon does not contain homologs of *nifF* (encoding a flavodoxin) and *nifJ* (pyruvate: flavodoxin oxidoreductase) which provide the electron transport chain to nitrogenase in some diazotrophs [22, 23]. In this study, we search and find that the *fer* (ferredoxin), COG3411 (ferredoxin), *fldA* (flavodoxin), *fldB* (flavodoxin), *fpr* (ferredoxin-NAD(P)H reductase), *nfrA* (NAD(P)H-flavin oxidoreductase) and *pfoAB* (pyruvate: ferredoxin oxidoreductase) are scared on *Paenibacillus* genomic regions outside of *nif* genes cluster. When each of these genes is separately transferred to *E. coli* 78-7, only *fer*, *fldA* and *pfoAB* can increase activity. Combinational assembly of *fer* or *fldA* with *pfoAB* can significantly increase activity. We deduce that *pfoAB* gene product (pyruvate: ferredoxin oxidoreductase) might be involved in the pyruvate breakdown to yield electrons, and then *fldA* and *fer* mediate electron to nitrogenase. Here is the first time to reveal that *pfoAB*, *fer* and *fldA* genes are involved in nitrogen fixation mediated by *Paenibacillus nif* genes. Notably, *PfoAB* shows 33 % identity with *K. oxytoca Nif*. But the *K. oxytoca nif* gene product is a single subunit, while *Paenibacillus pfoAB* gene products are two subunits. Here, we show that both *fldA* and *fer* can enhance nitrogenase activity, suggesting that the both genes can transfer electron to Fe protein. *fldA* also exists in *E. coli* and *K. oxytoca* [27]. Whether *fldA* is involved in transferring electron to Fe protein of nitrogenase in *K. oxytoca* is not known. In *E. coli*, *FldA* and *Fpr* (the NADPH-dependent flavin adenine dinucleotide (FAD) containing flavodoxin/ferredoxin reductase) are required for the activation of key enzymes in the synthesis of methionine, biotin, pyruvate and deoxyribonucleotides [28–30]. Remarkably, the *Fpr-FldA* redox system can effectively deliver electrons to non-physiological partners, which include a variety of P450 enzymes [31]. Thus, we deduce that the *Fpr-FldA* redox system might be responsible for electron transport to nitrogenase in *E. coli*.

Also, we demonstrate that each of *K. oxytoca nifJ* and *nifF* genes can increase nitrogenase activity of *E. coli* 78-7. The higher activity is obtained when *K. oxytoca nifJ* and *nifF* genes were carried in different vectors. However, nitrogenase could not be increased when *nifF* and *nifJ* were assembled as an operon. Our results are consistent with the report that coordinated and balanced expression of *nifF* and *nifJ* genes is important for nitrogenase activity in *E. coli* carrying *K. oxytoca nif* clusters.

Furthermore, in order to increase nitrogenase activity of *E. coli* 78-7, we design to assemble electron transport genes from *Paenibacillus* or *K. oxytoca* with Fe–S cluster synthesis genes from *Paenibacillus* or *K. oxytoca*. Considering *K. oxytoca nifSU* are much shorter and easier to operate in gene cloning than *Paenibacillus suf* operon, *K. oxytoca nifSU* are used in the combinational assembly with electron transport genes. The combinational assembly of *Paenibacillus fer-pfoAB* with *K. oxytoca nifSU*, *Paenibacillus fldA-pfoAB* with *K. oxytoca nifSU*, *K. oxytoca nifF* and *nifJ* with *nifSU* was constructed. Our results demonstrated that these combinational assemblies can significantly increase activity. Especially, *Paenibacillus fldA-pfoAB* with *K. oxytoca nifSU* can recover 50 % activity of wild-type *Paenibacillus*. Our results provide valuable information for engineering nitrogen fixation pathway into cereal crops.

The *nifW* and *nifZ* genes seem to be involved in MoFe protein maturation [1, 3], while *nifM* is required for proper folding of nitrogenase Fe protein in *K. oxytoca* [24–26]. The *nifWZM* genes are not only absent in the *Paenibacillus nif* cluster, but also in the *nif* clusters of the Gram-positive *Clostridium*, *Helio bacterium chlorum* and archaeal *Methanococcus maripaludis* [32]. Also, *nifM* is absent in *Rhizobia*, such as *Azorhizobium caulinodans*, *Sinorhizobium meliloti* and *Rhizobium leguminosarum* [7]. Our current studies demonstrate that *K. oxytoca nifwZM* can not increase nitrogenase activity of *E. coli* 78-7. These data support that the *nifWZM* genes are not required for nitrogen fixation in *Paenibacillus* sp. WLY78. Whether the functions of the *nifWZ* genes are replaced by other components scared in the genome of *Paenibacillus* sp. WLY78 and *E. coli* is not known.

It was reported that *nifM* encodes a cis–trans peptidyl prolyl isomerase and are involved in proper folding of nitrogenase Fe protein in *Azotobacter vinelandii* [26]. When the conserved Pro258 located in the C-terminal region of Fe protein (NifH) of *A. vinelandii*, which wraps around the other subunit in the NifH dimer, is replaced by serine, the correct folding of Fe protein (NifH) can acquire NifM independence [26, 33]. We compare the *Paenibacillus Nif*H sequence with other NifH sequences and find that *Paenibacillus* contains the conserved proline residues identified in other NifH sequences that are considered to be potential substrates for NifM (Additional file 3: Figure S1). It is possible that other amino acid substitutions in NifH may enable assembly of Fe protein in the absence of NifM.

It has been demonstrated that *nifQ* is required for nitrogen fixation in *K. oxytoca* and *A. vine llandii*. Recent results show that NifQ is an iron-sulfur protein with a redox-responsive [Fe–S] cluster and NifQ is also a molybdoprotein that serves as a direct molybdenum donor for FeMo-co synthesis, replacing molybdate in the in vitro FeMo-co synthesis assay [34]. Electron paramagnetic resonance (EPR) spectroscopic studies indicated that NifQ carries a [Mo-Fe3-S4] cluster, and that the presence
of this metal cluster in NifQ correlates with its ability to support in vitro FeMo-co synthesis [1]. However, there is no nifQ in diazotrophic Paenibacillus, Clostridium, cyanobacteria and Frankia [35, 36]. This study demonstrates that K. oxytoca nifQ did not enhance the activity of E. coli 78-7. Interestingly, there is a hesA gene located within the nif clusters of diazotrophic Paenibacillus, cyanobacteria and Frankia [35, 36]. Our deletion analysis demonstrates that hesA is important for nitrogenase activity, but the function of hesA in nitrogen fixation has not so far been determined. HesA belongs to the ThiF-MoeB-HesA family which engages in an ATP-dependent process that activates the C-terminus of partner ubiquitin-like proteins by forming an acyladenylate complex that facilitates sulfur transfer [37, 38]. It is to speculate that HesA may perform a role in metalcluster biosynthesis. These data suggest that synthesis and maturation of nitrogenase exhibit some different features between nif gene clusters of Gram-negative K. oxytoca/A. vinelandii and Gram-positive Paenibacillus.

Conclusion
A total of 28 selected genes from Paenibacillus sp. WLY78 and K. oxytoca are separately or combinationally transferred into the recombinant E. coli 78-7. Of these 28 genes, 8 genes (pfoAB, fldA, fldB, fer, fpr, nfrA and COG3411) encoding the potential electron transport and 2 gene clusters (suf and isc) encoding Fe–S cluster synthesis are from Paenibacillus sp. WLY78, and 8 genes (nifF, nifJ, nifSU, nifWZM and nifQ) specific for electron transport, Fe–S cluster synthesis and maturation of nitrogenase are from K. oxytoca. Our results demonstrate that Paenibacillus suf operon and the potential electron transporter genes (pfoAB, fldA and fer) can increase nitrogenase activity. Also, K. oxytoca nifSU and nifFJ can increase nitrogenase activity. Especially, combined assembly of the potential electron transporter genes (pfoABfldA) with K. oxytoca nifSU recovers 50.1 % of wild-type activity. Also, we demonstrate that nifWZM and nifQ can not increase activity, suggest ing that the requirement of nifWZM and nifQ genes on maturation of nitrogenase vary greatly among diazotrophs. This study will provide valuable insights for the enhancement of nitrogenase activity in heterogeneous host and will provide guidance for engineering cereal plants with minimal nif genes.

Methods
Strains and medium
Paenibacillus sp. WLY78, a nitrogen-fixers, was isolated by our lab [9]. The recombinant E. coli 78-7 which carries an 11 kb nif genes cluster from Paenibacillus sp. WLY78 was constructed by our lab [9]. Paenibacillus sp. WLY78 and E. coli strains were routinely grown in LB or LD medium (per liter contains: 2.5 g NaCl, 5 g yeast and 10 g tryptone) at 30°C with shaking. When appropriate, antibiotics were added in the following concentrations: 50 m g/m kanamycine, 100 m g/ml ampiceline, and 12.5 m g/ml tetracycline for maintenance of plasmids. Nitrogen-free and nitrogen-deficient media were used for assay of nitrogenase activity. Nitrogen-free medium contained (per liter) 10.4 g NaHPO₄, 3.4 g KH₂PO₄, 26 mg CaCl₂·2H₂O, 30 mg MgSO₄·7H₂O, 0.3 mg MnSO₄, 36 mg Ferric citrate, 7.6 mg Na₂MoO₄·2H₂O, 10 μg p-aminobenzoic acid, 5 μg biotin and 4 g glucose as carbon source. Nitrogen-deficient medium contained 2 mM glutamate as nitrogen source in nitrogen-free medium [9].

Nitrogenase activity assays
For nitrogenase activity assays, Paenibacillus sp.WLY78 and the recombinant E. coli strains were grown in 5 ml of LD media (supplemented with antibiotics) in 50 ml flasks shaken at 250 rpm for 16 h at 30 °C. The cultures were collected by centrifugation, washed three times with sterilized water and then resuspended in nitrogen-deficient medium containing 2 mM glutamate as nitrogen source (supplemented with antibiotics for the engineered E. coli strains when necessary) to a final OD600 of 0.2–0.4. Then, 1 ml of the culture was transferred to a 25-ml test tube and the test tube was sealed with rubber stopper. The headspace in the tube was then evacuated and replaced with argon gas [14]. After incubating the cultures for 6–8 h at 30°C with shaking at 250 rpm, C₂H₂ (10 % of the headspace volume) was injected into the test tubes. After incubating the cultures for a further 3 h, 100 ml of culture headspace was withdrawn through the rubber stopper with a gas tight syringe and manually injected into a HP6890 gas chromatograph to quantify ethylene production. All treatments were in three replicates and all the experiments were repeated three or more times.

Construction of cloning and expression vectors
Since E. coli 78-7 carries a Paenibacillus nif gene operon in vector pHY300PLK which is a shuttle vector with two replication origins: one is p15A which can be reproduced in E. coli and the other is a PAMα1 replicon from a plasmid pAMα1 of Streptococcus faecalis which can be reproduced in Gram-positive Bacillus [16]. The p15A replicon allows itself to coexist in E. coli cells with plasmids of the CoE1 compatibility group (e.g., pBR322, pUC19, pBluescript II SK (+)). Thus, two cloning and expression vectors carrying a Paenibacillus nif promoter and a ribosome binding site are here constructed in order to express foreign genes in the recombinant E. coli 78-7. The first vector (here called pBC) contains the backbone...
derived from pBluescript II SK (+), including the \textit{E. coli} origin ColE1, ampicillic resistance marker amp and the multiple cloning sites (MCS). A 307 bp \textit{Paenibacillus nif} promoter region (carrying Xhol and HindIII restriction sites at both ends) from the genomic DNA of \textit{Paenibacillus} sp. WLY78 and a 1.2 kb chromophenicol resistance gene fragment (carrying KpnI restriction sites at both ends) from the plasmid pPR9TT were PCR amplified and then ligated to the ampicillic-resistant plasmid pBluescript II SK (+), resulting vector pBC. The second vector (here named as pCK) contains the \textit{E. coli} origin ColE1, kanamycine resistance marker kan and the multiple cloning site (MCS) from plasmid pCAMBIA1301 and a 307 bp \textit{nif} promoter region (carrying Xhol and HindIII restriction sites at both ends) from the genomic DNA of \textit{Paenibacillus} sp. WLY78.

Construction of recombinant plasmids and recombinant \textit{E. coli} strains

Here, a total of fourteen DNA fragments including 28 genes were PCR amplified from \textit{Paenibacillus} sp. WLY78 and \textit{K. oxytoca}. First, nine DNA fragments (488 bp, 246 bp, 899 bp, 553 bp, 345 bp, 827 bp, 1045 bp, 3306 bp, 6455 bp and 1345 bp) which contain \textit{fldA}, \textit{fer}, \textit{fldB}, \textit{COG3411}, \textit{nfrA}, \textit{fpr}, \textit{pfoAB} genes, \textit{suf} and \textit{isc} genes cluster of \textit{Paenibacillus} sp. WLY78, respectively, were PCR amplified. Five DNA fragments (564, 3543, 2090, 1534 and 533 bp) containing \textit{nifF}, \textit{nifL}, \textit{nifUS}, \textit{nifWZM} and \textit{nifQ} genes, respectively, were PCR amplified from \textit{K. oxytoca} M5a1. The \textit{fldA}, \textit{fer}, \textit{fldB}, \textit{COG3411}, \textit{nifF}, \textit{nifWZM} and \textit{nifQ} gene fragments carried BamHI and Xbal target sites flanking the coding region. The \textit{nfrA}, \textit{fpr}, \textit{pfoAB} and \textit{nif} gene fragments carried HindIII and BamHI target sites flanking the coding region. The \textit{suf} and \textit{isc} cluster and \textit{nifUS} genes fragment carried Xbal and SacI target sites at both ends. Each of these gene or genes cluster was cloned to the plasmid pBC and was placed under the control of \textit{nif} promoter. The \textit{nifF}, \textit{fldA}, \textit{fer}, \textit{nifUS} genes were cloned into the vector pCK, respectively. Primers for PCR, recombinant plasmids and strains are listed in Additional file 1: Tables S1, Additional file 2: Table S2, Additional file 3: Table S3 and Additional file 4: Table S4.

Additional files

- Additional file 1: Table S1. The vectors constructed in this study.
- Additional file 2: Table S2. Bacterial strains and plasmids used in this study.
- Additional file 3: Figure S1. Comparison of different NifH proteins showing the Proline258 is conserved in \textit{Paenibacillus}.
- Additional file 4: Table S3. The recombinant strains constructed in this study.
- Additional file 5: Table S4. Primers used in this study.

**Authors’ contributions**

XXL performed all experiments, prepared Figures and Tables. QL and XML performed partial experiments. HWS analyzed partial results. SC conceived the study, guided its coordination and wrote the manuscript. All authors read and approved the final manuscript.

**Acknowledgements**

This work was supported by the National Nature Science Foundation of China (Grant No. 31470189) and by Key Laboratory for Agrobiotechnology, China Agricultural University (the Innovative Project of 2015SKLAB02-02).

**Competing interests**

The authors declare that they have no competing interests.

Received: 11 November 2015 Accepted: 8 February 2016
Published online: 20 February 2016

**References**

1. Rubio LM, Ludden PIW. Biosynthesis of the iron-molybdenum cofactor of nitrogenase. Annu Rev Microbiol. 2008;62:93–111.
2. Hu Y, Ribbe MW. Biosynthesis of Nitrogenase FeMoco. Coord Chem Rev. 2011;255:1218–24.
3. Roberts GP, MacNeil T, MacNeil D, Brill WJ. Regulation and characterization of protein products coded by the \textit{nif} (nitrogen fixation) genes of \textit{Klebsiella pneumoniae}. J Bacteriol. 1979;136:267–79.
4. Arnold W, Rump A, Klipp W, Pifferer UB, Puheer A. Nucleotide sequence of a 24,206-base-pair DNA fragment carrying the entire nitrogen fixation gene cluster of \textit{Klebsiella pneumoniae}. J Mol Biol. 1988;203:715–38.
5. Setubal JC, dos Santos P, Goldman BS, Ertesvag H, Espin G, Rubio LM, Valla S, Almeida NF, Balasubramanian D, Cronies L, Curatti L, Du Z, Godsy E, Goodner B, Hellner-Burri K, Hernandez JA, Houmell E, Imperial J, Kennedy C, Lason TJ, Latreille P, Ligon LS, Lu J, Maek P, Miller NM, Norton S, O’Carroll JP, Paulsen I, Raaf EC, Roemer R, Rossier J, Segura D, Slater S, Stricklin SL, Studdholme DJ, Sun J, Viana CJ, Wallin E, Wang B, Wheeler C, Zhu H, Dean DR, Dixon R, Wood D. Genome sequence of \textit{Azotobacter vinelandii}, an obligate aerobe specialized to support diverse anaerobic metabolic processes. J Bacteriol. 2009;191:4534–45.
6. Dos SP, Fang Z, Mason SW, Setubal JC, Dixon R. Distribution of nitrogen fixation and nitrogenase-like sequences amongst microbial genomes. BMC Genom. 2012;13:162.
7. Masson-Boivin C, Giraud E, Perret X, Batut J. Establishing nitrogen-fixing symbiosis with legumes: how many rhizobium recipes? Trends Microbiol. 2009;17:458–66.
8. Boyd ES, Anbar AD, Miller S, Hamilton TL, Lavin M, Peters JW. A late methanogen origin for molybdenum-dependent nitrogenase. Geobiol. 2011;9:221–32.
9. Wang L, Zhang L, Liu Z, Zhao D, Liu X, Zhang B, Xie J, Hong Y, Li P, Chen S, Dixon R, Li J. A minimal nitrogen fixation gene cluster from \textit{Paenibacillus} sp. WLY78 enables expression of active nitrogenase in \textit{Escherichia coli}. PLoS Genet. 2013;9:e1003865.
10. Xie JB, Du Z, Bai L, Tian C, Zhang Y, Xie JY, Wang T, Liu X, Chen X, Cheng Q, Chen S, Li J. Comparative genomic analysis of \textit{N2}-fixing and non-\textit{N2}-fixing \textit{Paenibacillus} spp. organization, evolution and expression of the nitrogen fixing genes. PLoS Genet. 2014;10:e1004231.
11. Dixon R, Cheng Q, Shen GF, Day A, Dawson-Day M. Nif gene transfer and expression in chloroplasts: prospects and problems. Netherlands: Springer; 1997. p. 193–203.
12. Dixon RA, Postgate JR. Genetic transfer of nitrogen fixation from \textit{Klebsiella pneumoniae} to \textit{Escherichia coli}. Nature. 1972;237:102–3.
13. Temme K, Zhao D, Voigt CA. Refactoring the nitrogen fixation gene cluster from \textit{Klebsiella oxytoca}. Proc Natl Acad Sci. 2012;109:7085–90.
14. Smanski MJ, Bhata S, Zhao D, Park Y, Goodner B, Hellner-Burri K, Hernandez JA, Houmell E, Imperial J, Kennedy C, Lason TJ, Latreille P, Ligon LS, Lu J, Maek P, Miller NM, Norton S, O’Carroll JP, Paulsen I, Raaf EC, Roemer R, Rossier J, Segura D, Slater S, Stricklin SL, Studholme DJ, Sun J, Viana CJ, Wallin E, Wang B, Wheeler C, Zhu H, Dean DR, Dixon R, Wood D. Genome sequence of \textit{Azotobacter vinelandii}, an obligate aerobe specialized to support diverse anaerobic metabolic processes. J Bacteriol. 2009;191:4534–45.
15. Wang X, Yang JG, Chen L, Wang JL, Cheng Q, Dixon R, Wang YP. Using synthetic biology to distinguish and overcome regulatory and functional barriers related to nitrogen fixation. PLoS One. 2013;8:e68677.
16. Ishiwa H, Shibahara H. New shuttle vectors for *Escherichia coli* and *Bacillus subtilis*. IV. The nucleotide sequence of pYH300PLK and some properties in relation to transformation. Jpn. J Genet. 1986;61:515–28.
17. Johnson DC, Dean DR, Smith AD, Johnson MK. Structure, function, and formation of biological iron-sulfur clusters. Annu Rev Biochem. 2005;74:247–81.
18. Dixon R, Kahn D. Genetic regulation of biological nitrogen fixation. Nat Rev Microbiol. 2004;2:621–31.
19. Nieva-Gomez D, Roberts GP, Klevickis S, Brill WJ. Electron transport to nitrogenase. Eur J Biochem. 1982;123:563–9.
20. Hill S, McLean PA, Hansen FB, Lemley PV, Kobljan KS, Orme-Johnson WH. *Klebsiella pneumoniae* *nifM* gene product is required for stabilization and activation of nitrogenase iron protein in *Escherichia coli*. J Biol Chem. 1986;261:772–8.
21. Ludden PW. Energetics and sources of energy for biological nitrogen fixation. Curr Topics Bioenerg. 1991;16:369–90.
22. Yoch DC. Electron-transport systems coupled to nitrogenase. A treatise on dinitrogen fixation. New York: Wiley; 1979. p. 605–52.
23. Deistung J, Cannon FC, Cannon MC, Hill S, Thorneley RN. Electron transfer to nitrogenase in *Klebsiella pneumoniae*. Proc Natl Acad Sci. 1980;77:2555–8.
24. Roberts GP, MacNeil T, MacNeil D, Brill WJ. Regulation and characterization of protein products coded by the *nif* (nitrogen fixation) genes of *Klebsiella pneumoniae*. J Bacteriol. 1978;136:267–79.
25. MacNeil T, MacNeil D, Roberts GP, Supiano MA, Brill WJ. Fine-structure mapping and complementation analysis of *nif* (nitrogen fixation) genes in *Klebsiella pneumoniae*. J Bacteriol. 1978;136:253–66.
26. Gavini N, Tungtur S, Pulakat L. Peptidyl-prolyl cis/trans isomerase-independent functional NifH mutant of *Azotobacter vinelandii*. J Bacteriol. 2006;188:6020–5.
27. Achenbach LA, Genova EG. Transcriptional regulation of a second flavodoxin gene from *Klebsiella pneumoniae*. Gene. 1997;194:235–40.
28. Bakkes PJ, Biemann S, Rokel A, Eickholt M, Girhard M, Urlacher VB. Design and improvement of artificial redox modules by molecular fusion of flavodoxin and flavodoxin reductase from *Escherichia coli*. Sci Rep. 2015;5:12158.
29. Blaschkowski HP, Neuer G, Ludwig-Festl M, Knappe J. Routes of flavodoxin and ferredoxin reduction in *Escherichia coli*. CoA-acylating pyruvate:flavodoxin and NADPH:flavodoxin oxireductases participating in the activation of pyruvate formate-lyase. Eur J Biochem. 1982;123:563–9.
30. Bianchi V, Reichard P, Eliasson R, Pontis E, Krook M, Jonvall H, Haggard-Ljungquist E. *Escherichia coli* ferredoxin NADP + reductase: activation of *E. coli* anaerobic ribonucleotide reduction, cloning of the gene (*fpr*), and overexpression of the protein. J Bacteriol. 1993;175:1590–5.
31. McRae L, Leadbeater C, Campopiano DJ, Baxter RL, Daff SN, Chapman SK, Munro AW. Characterisation of flavodoxin NADP + oxireductase and flavodoxin, key components of electron transfer in *Escherichia coli*. Eur J Biochem. 1998;257:577–85.
32. Enkh-Aimagal J, Kawasaki H, Seki T. Molecular evolution of the *nif* gene cluster carrying *nifH* and *nifD* genes in the Gram-positive photosynthetic bacterium Helobacterium chlorum. Int J Syst Evol Microbiol. 2005;56:665–74.
33. Howard KS, McLean PA, Hansen FB, Lemley PV, Kobljan KS, Orme-Johnson WH. *Klebsiella pneumoniae* *nifM* gene product is required for stabilization and activation of nitrogenase iron protein in *Escherichia coli*. J Bacteriol. 1986;261:772–8.
34. Imperial J, Ugalde RA, Shah VK, Brill WJ. Role of the *nifQ* gene product in the incorporation of molybdenum into nitrogenase in *Klebsiella pneumoniae*. J Bacteriol. 1984;158:187–94.
35. Oh CJ, Kim HB, Kim J, Kim WJ, Lee H, An CS. Organization of *nif* gene cluster in *Frankia* sp. EuK1 strain, a symbiont of *Eleagnus umbellata*. Arch Microbiol. 2012;194:29–34.
36. Welsh EA, Liberton M, Stockel J, Loh T, Evtitaga T, Wang C, Wollam A, Fulton RS, Clifton SW, Jacobs JM, Aurora R, Gosh BK, Sherman LA, Smith RD, Wilson RK, Pakrasi HB. The genome of *Cyanothecae* S1 142, a unicellular diazotrophic cyanobacterium important in the marine nitrogen cycle. Proc Natl Acad Sci USA. 2008;105:15094–9.
37. Lake MW, Wuebbens MM, Rajagopalan KV, Schindelin H. Mechanism of ubiquitin activation revealed by the structure of a bacterial MoeB-MoaD complex. Nature. 2003;421:235–9.
38. Lehmann C, Begley TP, Ealick SE. Structure of the *Escherichia coli* THS-ThIF complex, a key component of the sulfur transfer system in thiamin biosynthesis. Biochemistry. 2006;45:11–9.

Submit your next manuscript to BioMed Central and we will help you at every step:
- We accept pre-submission inquiries
- Our selector tool helps you to find the most relevant journal
- We provide round the clock customer support
- Convenient online submission
- Thorough peer review
- Inclusion in PubMed and all major indexing services
- Maximum visibility for your research

Submit your manuscript at www.biomedcentral.com/submit