A Minimal Nitrogen Fixation Gene Cluster from Paenibacillus sp. WLY78 Enables Expression of Active Nitrogenase in Escherichia coli

Liyong Wang1*, Lihong Zhang12*, Zhangzhi Liu1, Dehua Zhao2, Xiaomeng Liu1, Bo Zhang1, Jianbo Xie1, Yuanyuan Hong1, Pengfei Li1, Sanfeng Chen1*, Ray Dixon4*, Jilun Li1

1 State Key Laboratory for Agrobiotechnology and College of Biological Sciences, China Agricultural University, Beijing, P. R. China, 2 College of Life Science, Shanxi Normal University, Linfen, P. R. China, 3 Synthetic Biology Center, Department of Biological Engineering, Massachusetts Institute of Technology, Cambridge, Massachusetts, United States of America, 4 Department of Molecular Microbiology, John Innes Centre, Norwich, United Kingdom

Abstract
Most biological nitrogen fixation is catalyzed by molybdenum-dependent nitrogenase, an enzyme complex comprising three component proteins that contain three different metalloclusters. Diazotrophs contain a common core of nitrogen fixation nif genes that encode the structural subunits of the enzyme and components required to synthesize the metalloclusters. However, the complement of nif genes required to enable diazotrophic growth varies significantly amongst nitrogen fixing bacteria and archaea. In this study, we identified a minimal nif gene cluster consisting of nine nif genes in the genome of Paenibacillus sp. WLY78, a gram-positive, facultative anaerobe isolated from the rhizosphere of bamboo. We demonstrate that the nif genes in this organism are organized as an operon comprising nifB, nifH, nifD, nifK, nifE, nifN, nifS, hesA and nifV and that the nif cluster is under the control of a σ70 (σ7) dependent promoter located upstream of nifB. To investigate genetic requirements for diazotrophy, we transferred the Paenibacillus nif cluster to Escherichia coli. The minimal nifS gene cluster enables synthesis of catalytically active nitrogenase in this host, when expressed either from the native nifB promoter or from the T7 promoter. Deletion analysis indicates that in addition to the core nif genes, hesA plays an important role in nitrogen fixation and is responsive to the availability of molybdenum. Whereas nif transcription in Paenibacillus is regulated in response to nitrogen availability and by the external oxygen concentration, transcription from the nifB promoter is constitutive in E. coli, indicating that negative regulation of nif transcription is bypassed in the heterologous host. This study demonstrates the potential for engineering nitrogen fixation in a non-nitrogen fixing organism with a minimum set of nine nif genes.

Introduction
Although fixed nitrogen plays a critical role in the global food supply, overuse of chemical nitrogen fertilizers has led to increased costs for farmers and harmful consequences for the environment and human health. Biological nitrogen fixation, the conversion of atmospheric N2 to NH3 offers a natural means of providing nitrogen to plants [1]. There has been a long-standing interest in reducing dependence on fertilizers through engineering non-legume crops that “fix” nitrogen but maintain growth yields [2], [3]. Achieving this goal will require elucidating the minimal number of genes required to sustain biological nitrogen fixation. Most biological nitrogen fixation is catalyzed by molybdenum-dependent nitrogenase, which is distributed within bacteria and archaea. This enzyme is composed of two component proteins, MoFe protein and Fe protein. The MoFe protein component is an α2β2 heterotetramer (encoded by nifD and nifK) that contains two metalloclusters; FeMo-co, a [Mo-7Fe-9S-C-homocitrate] cluster which serves as the active site of substrate binding and reduction and the P-cluster, a [8Fe-7S] cluster that 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. The assembly pathway for the biosynthesis of nitrogenase is complex. Apart from the structural subunits encoded by nifH, nifD and nifK, several genes are required for the biosynthesis of the metalloclusters, in addition to other gene products necessary to produce a fully functional enzyme. It is now well established from genetic and biochemical analysis that nifE, nifN, nifX, nifB, nifQ, nifY, nifJ and nifH contribute to the synthesis and insertion of FeMo-co into nitrogenase, that nifU, nifS and nifZ play an important role in synthesis of metalloclusters and that nifM is required for proper folding of nitrogenase Fe protein [4–7].

The inventory of genes required for diazotrophy varies greatly amongst species, dependent upon the environmental niche and physiology of the host. For example, in Klebsiella oxytoca, twenty nif
genes are co-located within a ~24 kb cluster [8], whereas in *Azotobacter vinelandii* the nif genes are more dispersed and distributed as two clusters in the genome [9] (Figure 1). However, in contrast to these paradigm diazotrophs, other nitrogen fixing organisms possess a more restricted nif gene set, for example the archean, *Methanococcus maripaludis*, contains only 9 nif genes (Figure 1), two of which nifH and nifP2, are not essential for nitrogen fixation, but serve a regulatory function [10]. Analysis of the distribution of nif gene sequences within microbial genomes indicates that nearly all diazotrophs have a minimal gene set consisting of six conserved genes nifH, nifD, nifK, nifE, nifN, and nifB [11]. This concurs with the minimal catalytic core required to assemble FeMo-co *in vivo* [12].

One of the difficulties in determining the precise genetic requirements for nitrogen fixation in diazotrophs arises from the presence of “housekeeping” counterparts in the genome that may substitute for the function of known nif genes. This may be particularly important in the case of diazotrophs that possess minimal nif gene clusters. One approach to investigate the inventory of genes required for diazotrophy in such cases is to transfer the nif cluster to a distantly related organism that does not have the capacity to fix nitrogen. *Escherichia coli* provides an important model organism for such studies as physiology and gene function is extremely well understood. Since transfer of the complete cluster of 20 nif genes from *K. oxytoca* to *E. coli* confers the ability to fix nitrogen [13], we were interested to determine whether a more evolutionary distant nif gene cluster would also enable nitrogenase activity in *E. coli*. In this study, we identified a minimal nif cluster consisting of nine genes, in the genome of *Paenibacillus* sp. WLY78 (Figure 1). The cluster is apparently transcribed from an *σ70*-like promoter that functions in *E. coli* to express active nitrogenase, which is competent to reduce both acetylene and dinitrogen as substrates of the enzyme. Environmental regulation of nif gene expression in *Paenibacillus*, in response to either oxygen or fixed nitrogen, is circumvented when the nif operon is expressed from its native promoter in *E. coli*, suggesting that nif transcription in *Paenibacillus* is negatively regulated in response to these effectors.

**Author Summary**

Biological nitrogen fixation plays an essential role in the nitrogen cycle, sustaining agricultural productivity by providing a source of fixed nitrogen for plants and ultimately animals. The enzyme nitrogenase that catalyses the reduction of atmospheric dinitrogen to ammonia contains one of the most complex heterometal cofactors found in biology. Biosynthesis of nitrogenase and provision of support for its activity requires a large number of nitrogen fixation (nif) genes, which vary according to the physiological lifestyle of the host organism. In this study, we identified a nif cluster with reduced genetic complexity, consisting of nine genes organized as a single operon in the genome of *Paenibacillus* sp. WLY78. When transferred to *Escherichia coli*, the *Paenibacillus* nif cluster enables synthesis of catalytically active nitrogenase, which is competent to reduce both acetylene and dinitrogen as substrates of the enzyme. Environmental regulation of nif gene expression in *Paenibacillus*, in response to either oxygen or fixed nitrogen, is circumvented when the nif operon is expressed from its native promoter in *E. coli*, suggesting that nif transcription in *Paenibacillus* is negatively regulated in response to these effectors.
Results

Genome sequencing of Paenibacillus sp. WL78 identifies a minimal nitrogen fixation (nif) gene cluster

Paenibacillus sp. WL78 is a gram-positive, facultative anaerobic, endospore-forming bacterium isolated from the rhizosphere of bamboo [14]. This bacterium has potential use in agriculture, since it is able to fix nitrogen and also produces antimicrobial substances. We therefore determined the genome sequence of this organism and identified a nitrogen fixation gene cluster consisting of nine genes arranged within a 10.5 kb region in the genome (Figure 1). The nif cluster is flanked by genes coding for a hypothetical protein upstream and an ABC transporter downstream. The G+C content of this nif cluster was higher than the average of the entire genome (52.8% vs. 45.1%), suggesting that it may have been acquired by horizontal gene transfer. The Paenibacillus sp. WL78 nif cluster is one of the most compact compared with other diazotrophs described to date (Figure 1). Similar nif gene arrangements and neighborhoods are observed in other Paenibacillus strains, including Paenibacillus terrae HPL-003. Multiple alignments revealed that the predicted protein products of the Paenibacillus nif genes showed 67–80% identity to the corresponding nif gene products of their gram-positive counterparts [15], but showed only 35–69% identity to the corresponding nif genes of K. oxytoca and A. vinelandii (Table S1). The gene designated as hesA, which is located between nifX and nifV is found in other nif clusters (Figure 1) and the predicted product shares ~45% identity with the putative molybdenum cofactor biosynthesis protein HesA of Frankia abisi ACN14a [16] and Cyanothecae sp. ATCC 51142 [17]. HesA is a member of the ThiF-MoeB-HesA family and contains an N-terminal nucleotide binding domain and a C-terminal MoeZ/MoeB-like domain.

RT-PCR experiments using primers designed to span across intergenic regions indicated that the nine genes within the nif cluster are organized in a single operon (Figure 2). Single operon nif clusters have been reported in gram-positive prokaryotes and in the archaea, e.g. Helobacterium chlorum [18] and Methanococcus maripaludis [19]. However, in contrast to these nif clusters Paenibacillus sp. WL78 does not contain the negative regulatory genes nifII and nif2 (homologues of glbB), which are involved in post-translational regulation of nitrogenase activity in response to fixed nitrogen [10].

Characterization of the Paenibacillus sp. WL78 nif promoter and transcription unit

The transcriptional start site (TSS) of the nif gene cluster in Paenibacillus sp. WL78 was determined by using the 5′-RACE (Rapid Amplification of cDNA Ends) method. The TSS was located 59 bp upstream of the translational start site of nifB and a putative promoter was identified 6 nucleotides preceding the TSS (Figure 3). The −33 (TTGACT) and −10 (TAAGAT) sequences in the nifB promoter were similar to the corresponding consensus sequences (TTGACA and TATAA respectively) of E. coli σ70-dependent promoters. Unlike other members of the Bacillales, the Paenibacillus sp. WL78 genome does not contain a homolog of rpoN and consequently σ24-dependent −24/−12 promoter sequences were not observed either upstream of the nif cluster or in the 5′ regions of other genes in the Paenibacillus sp. WL78 genome (data not shown). Downstream of nifV, a potential transcriptional termination site was identified, containing two potential stem loops followed by a T-rich region (Figure 3B). These findings indicate that the nif genes in Paenibacillus sp. WL78 are organized as a single operon containing 9 genes, which is transcribed from an rpoD-dependent promoter.

To analyze the σ70-dependency of the nifB promoter, electrophoretic mobility shift assays (EMSA) were carried out using either E. coli σ70-RNAP (RNA polymerase) or σ70 from Paenibacillus sp. WL78, which was overexpressed and purified from E. coli (Figure 5C). EMSA experiments revealed that both purified σ70 from Paenibacillus sp. WL78 and E. coli σ70-RNAP holoenzyme bind to the 50 bp nifB promoter fragment. Competition experiments with non-labelled σ70 DNA indicated that the E. coli RNAP holoenzyme binds much more tightly to this DNA fragment, since higher concentrations of competitor were apparently required to dissociate the E. coli σ70-RNAP (Figure 5, panels D and E). EMSA experiments with a scrambled double-stranded oligonucleotide did not reveal binding of either protein (data not shown). These results are consistent with the ability of σ70 of Bacillus subtilis to bind to promoters independent of core RNAP [20,21].

To further examine the specificity of binding of E. coli σ70-RNAP to the Paenibacillus sp. WL78 nifB promoter, we made substitutions in the −35 (TTGACT to GGCTAC) and −10 (TAAGAT to GGAGGC) regions of the promoter (Figure 4A). Binding of E. coli σ70-RNAP to the nifB promoter fragment was weakened considerably by the presence of the −35 and −10 substitutions in the promoter; (−), negative control in which no reverse transcriptase was added to the RT reaction; (+), positive control in which genomic DNA was used as template in the RT-PCR.

Figure 2. The nif genes of Paenibacillus sp. WL78 are organized in an operon as determined by RT-PCR. (A) Outline of the strategy. Primers used and amplified products (numbered) are given below the schematic representation of the genes. (B) Result of RT-PCR reactions with RNA from Paenibacillus sp. WL78 grown under N2-fixing conditions. The numbering on the top of the gels corresponds to the product numbers drawn schematically in the outline given above. RT, standard RT-PCR reaction; (−), negative control in which no reverse transcriptase was added to the RT reaction; (+), positive control in which genomic DNA was used as template in the RT-PCR.

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substitutions (compare Figure 4, panels B and C), suggesting that E. coli s70-RNAP specifically interacts with the nifB promoter from Paenibacillus sp. WLY78. In order to confirm this, we performed DNAse I footprinting with a fluorescently labeled 319 bp DNA target carrying the nifB promoter and analyzed the digested DNA fragments using a capillary sequencer. As expected, the region protected from DNAse I digestion corresponded to the nifB promoter, confirming that E. coli s70-RNAP specifically binds to the 235 and 210 regions upstream of the transcription start site. (Figure 4D). Our studies thus demonstrate that the nifB promoter of Paenibacillus sp. WLY78 is s70-dependent and thus distinct from the typical s54-dependent 224/212 promoters found upstream of nif genes in gram-negative diazotrophs.

To verify if the nifB promoter of Paenibacillus sp. WLY78 is functional in E. coli, it was fused to the lacZ reporter gene. The level of β-galactosidase activity expressed from the PnifB::lacZ fusion in E. coli strain JM109 was not influenced either by the concentration of fixed nitrogen in the culture medium or by the external oxygen concentration (Figure 5). Hence, the Paenibacillus sp. WLY78 nifB promoter is apparently recognized by E. coli s70 RNA polymerase in vivo. These data concur with previous studies where promoters of gram-positive bacteria, for example, Bacillus stereothermophilus [22] and Corynebacterium glutamicum [23], were shown to be functional in E. coli.

The Paenibacillus nif gene cluster enables nitrogen fixation by E. coli

To transfer the Paenibacillus nif gene cluster to E. coli, we cloned a 10.5-kb DNA fragment (containing the sequence from the ATG start codon of nifB to the TAA stop codon of nifV) in the expression vector pET-28b bringing the nif genes under control of the T7 promoter. This construct was then transformed into E. coli BL21 (DE3), yielding the engineered E. coli strain 78-32. We further cloned the 11-kb full-length nif cluster containing its own nif promoter and the contiguous nine genes nifBHDKENXhesAnifV into the multicopy plasmid pHY300PLK and transformed this into E. coli JM109, yielding the engineered E. coli strain 78-7 (Figure 6A). To determine whether the Paenibacillus nif cluster functions in E. coli, we employed two independent methods to assess nitrogenase activity; firstly, reduction of the alternative substrate acetylene to ethylene, which can be readily quantified by gas chromatography [24], [25] and secondly, a 15N2 enrichment assay to directly measure the incorporation of this tracer into organic nitrogen [26]. When grown anaerobically in nitrogen-deficient medium, Paenibacillus sp. WLY78 Minimal nif Gene Cluster

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bacillus sp. WLY78 exhibits both acetylene reduction and $^{15}$N$_2$ incorporation (Figure 6, panels B and C). The engineered E. coli strain 78-7, which expresses the nif genes from the native promoter showed approximately 10% of the specific activity for acetylene reduction when compared with Paenibacillus and was competent to assimilate $^{15}$N$_2$. In contrast, when expressed from the T7 promoter and induced with 2 mM IPTG the Paenibacillus nif cluster exhibited relatively low levels of nitrogenase activity in the recombinant E. coli strain 78-32 (Figure 6). Therefore, the engineered E. coli strain 78-7 was used for most of the studies reported here. When compared with the recipient E. coli strain JM109, the engineered strain 78-7 had an identical cellular phenotype when analyzed by Biolog phenotypic microarrays [27] (data not shown). In comparison with the Paenibacillus sp. WLY78 strain, which is capable of diazotrophic growth, the engineered E. coli strain 78-7 grew poorly in liquid media with dinitrogen as the sole nitrogen source (data not shown). Therefore, although the recombinant strain expresses active nitrogenase and assimilates $^{15}$N$_2$, this does not enable the engineered E. coli strain to grow as a diazotroph.

Minimal Paenibacillus nif genes required for nitrogenase activity

To further determine the minimal nif genes required for nitrogen fixation, we constructed a series of nif gene deletions (Figure 6). Neither acetylene nor $^{15}$N$_2$ incorporation was detectable in the nifB deletion, supporting the original observation that nifB is essential for synthesis of nitrogenase [5]. When nifV was deleted, $^{15}$N$_2$ assimilation decreased more significantly than acetylene reduction, in agreement with the substrate reduction properties of nifV mutants [28], which are unable to synthesize the homocitrate moiety of FeMo-co [29]. Deletion of hesA also influenced $^{15}$N$_2$ incorporation more significantly than acetylene reduction, suggesting that hesA is required for nitrogen fixation. In contrast, deleting nifX gave rise to a similar decrease (~50%) in the reduction of both substrates. In the ΔnifXhesA double deletion, nitrogenase activity was similar to that in the single hesA mutant, whereas in the double ΔhesAnifV deletion, activities were similar to those exhibited by the single nifV mutant. Deletion of three (nifXhesAnifV) or four genes (nifXNhesAnifV) ablated nitrogenase activity. In all cases the phenotypic defects exhibited by the deletions could be reversed by complementation with plasmids bearing the missing genes (data not shown). These results suggest

Figure 4. E. coli σ$^{70}$-RNAP binds preferentially to the −35 region and −10 region of the nifB promoter of Paenibacillus sp. WLY78. (A) Substitutions introduced in the nifB promoter sequence. The sequences of the −35 and −10 regions of the nifB promoter are underlined (Wt indicates the wild-type sequence). Base substitutions in the mutant promoter are indicated in red. (B) and (C) EMSA experiments comparing the binding of E. coli σ$^{70}$-RNAP to the wild-type nifB promoter fragment (panel B) with the mutant promoter fragment (panel C). The protein concentration is indicated above each lane. (D) DNase I footprinting of the interaction of E. coli σ$^{70}$-RNAP with the nifB promoter using an automated capillary sequencer. The top lane is an electropherogram obtained in the presence of σ$^{70}$-RNAP with the sequence protected from cleavage shown below. A control electropherogram obtained from a reaction containing BSA is shown in the bottom lane. doi:10.1371/journal.pген.1003865.g004

Figure 5. Expression of the Paenibacillus sp. WLY78 nifB::lacZ promoter fusion is constitutive in E. coli. Black bars indicate expression of β-galactosidase driven by the nifB promoter; grey bars indicate the level of β-galactosidase activity exhibited by the vector plasmid (pPR9TT) alone. Cultures were grown in nitrogen deficient medium, with 2 mM glutamate as nitrogen source, either anaerobically with the indicated concentrations of NH$_4$Cl (left panel) or with the indicated initial oxygen concentrations shown in the right-hand panel. Error bars indicate the standard deviation observed from at least two independent experiments. doi:10.1371/journal.pген.1003865.g005
that all nine *Paenibacillus* genes (nifBHDKENXhesAnifV) are necessary for optimal nitrogenase activity in *E. coli*.

Effects of fixed nitrogen and oxygen on nif transcription

In many diazotrophs such as *K. oxytoca* and *A. vinelandii*, expression of the *nif* genes is tightly controlled at the transcriptional level in response to the concentration of fixed nitrogen and the oxygen [30]. In addition, the activity of nitrogenase itself can be regulated at the post-translational level in response to environmental effectors [31]. To examine whether the *Paenibacillus* *nif* cluster is subject to similar regulation, we compared the effects of NH\textsubscript{4}\textsuperscript{+} and O\textsubscript{2} on in vivo nitrogenase activity and *nif* gene transcription in the native *Paenibacillus* sp. WLY78 strain with that of engineered *E. coli* 78-7 (Figure 7). Both *Paenibacillus* sp. WLY78 and the engineered *E. coli* 78-7 strain did not exhibit nitrogenase activity at O\textsubscript{2} concentrations above 5% (Figure 7A). In addition, acetylene reduction by *Paenibacillus* sp. WLY78 was not observed at NH\textsubscript{4}\textsuperscript{+} concentrations above 1 mM. In contrast, the engineered *E. coli* strain 78-7 exhibited nitrogenase activity even in the presence of 200 mM NH\textsubscript{4}Cl (Figure 7B). The latter observation suggests that the *Paenibacillus* *nif* cluster is not subject to regulation by fixed nitrogen in *E. coli*. In agreement with the acetylene reduction data, the α and β subunits of the MoFe protein and the Fe protein component of nitrogenase were only detectable by Western blotting in *Paenibacillus* sp. WLY78 grown under nitrogen fixation conditions, whereas nitrogenase components were detectable in the engineered *E. coli* strain even in the presence of oxygen (Figure S1).

The influence of oxygen and fixed nitrogen on transcription was assessed by RT-PCR using *nifH* and *nifK* probes. Converstant with the acetylene reduction data, *nif* transcription in *Paenibacillus* sp. WLY78 was inhibited by NH\textsubscript{4}\textsuperscript{+} concentrations above 1 mM and by the presence of 21% oxygen (Figure 7C). In contrast, *nifH* and *nifK* transcription in *E. coli* 78-7 was insensitive to the presence of oxygen and fixed nitrogen (Figure 7D). Thus the *Paenibacillus* *nif* genes are constitutively transcribed in the engineered strain indicating that the transcriptional regulation observed in the native host does not occur in *E. coli*.
In contrast with earlier studies in which transfer of the complete complement of 20 nif genes from *K. oxytoca* enabled *E. coli* to fix nitrogen [12], our results with *Paenibacillus* sp. WLY78 demonstrate that only nine nif genes are needed to synthesize active nitrogenase in *E. coli*. The specific activity of the enzyme expressed in *E. coli* was approximately 10% of that observed in *Paenibacillus*, but nevertheless sufficient to provide \(^{15}\)N\(_2\) assimilation. However, synthesis of active nitrogenase in the recombinant *E. coli* strain did not enable diazotrophic growth. This implies that this level of enzyme activity is insufficient to support growth on dinitrogen as sole nitrogen source. However, we cannot rule out the possibility that other physiological factors in *E. coli*, for example the ability to synthesis high levels of nitrogenase proteins under conditions of nitrogen starvation, limit the capacity for diazotrophic growth. Considering the physiological background of *E. coli*, one of the notable absences in the minimal *Paenibacillus* nif gene cluster is the presence of *nifF* and *nifJ*, which provide the electron transport chain to nitrogenase in some diazotrophs [34], [35]. The activity of *Paenibacillus* nitrogenase is therefore likely to be reductant limited in *E. coli* in the absence of this electron transport chain. Another notable absence is *nifM*, which encodes a cis-trans peptidyl prolyl isomerase required for proper folding of nitrogenase Fe protein in diazotrophic proteobacteria [6]. Potentially this function is provided by a counterpart enzyme encoded elsewhere in the genome in other diazotrophs such as *Paenibacillus*. However, a functional equivalent of *nifM* is not present in *E. coli*, since assembly of active *K. oxytoca* Fe protein in this background requires the presence of both *nifH* and *nifJ* [36]. The *Paenibacillus* NirM sequence contains the seven conserved proline residues identified in other NirH sequences that are considered to be potential substrates for NirM [6]. However, it is possible that other amino acid substitutions in NirH may enable assembly of Fe protein in the absence of NirM. The *Paenibacillus* sp. WLY78 nif gene operon does not contain homologs of the nitrogen fixation-specific iron-sulphur cluster assembly pathway encoded by *nifU* and *nifS*. As in

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**Figure 7. Effects of O\(_2\) and NH\(_4^+\) on nitrogenase activity and nif gene transcription.** (A) and (B) Comparison of the acetylene reduction activities of *Paenibacillus* sp. WLY78 (panel A) and the engineered *E. coli* 78-7 strain (panel B), when cultures are grown in the presence of either oxygen or ammonium (at the initial concentrations shown on the y axis). Error bars indicate the standard deviation observed from at least two independent experiments. (C) and (D) Comparison of transcription of *nifH* and *nifK* as determined by RT-PCR in *Paenibacillus* sp. WLY78 (panel C) and *E. coli* 78-7 (panel D). Initial concentrations of ammonium and oxygen are indicated above relevant lanes. Lanes labeled "NH\(_4^+\),O\(_2\)" indicate negative controls in which no reverse transcriptase was added to the RT-PCR reaction. In each case a parallel RT-PCR reaction was performed to detect the level of 16S rRNA, to provide a loading control (shown beneath relevant lanes). doi:10.1371/journal.pgen.1003865.g007

**Discussion**

Although the biochemical properties and structure of molybdenum nitrogenases are remarkably similar when purified from diverse bacteria and archaea, genetic requirements for the synthesis and assembly of the enzyme and maintenance of its activity differ widely amongst diazotrophs [11], [32], [33]. Some of this diversity is undoubtedly determined by the environmental lifestyle of each diazotroph, the need to protect the enzyme from damage by oxygen and the requirement to provide sufficient ATP and reductant to support enzyme activity under different physiological conditions. Although the conserved nature of the structural genes and the assembly pathway for FeMoco biosynthesis dictates the presence of a common core of structural genes and the assembly pathway for FeMoco biosynthesis, the conservation of this diversity is undoubtedly determined by the environmental conditions. The specific activities of the enzyme expressed in *E. coli* and *K. oxytoca* are approximately 10% of that observed in *Paenibacillus*, but nevertheless sufficient to provide \(^{15}\)N\(_2\) assimilation. However, synthesis of active nitrogenase in the recombinant *E. coli* strain did not enable diazotrophic growth. This implies that this level of enzyme activity is insufficient to support growth on dinitrogen as sole nitrogen source. However, we cannot rule out the possibility that other physiological factors in *E. coli*, for example the ability to synthesis high levels of nitrogenase proteins under conditions of nitrogen starvation, limit the capacity for diazotrophic growth. Considering the physiological background of *E. coli*, one of the notable absences in the minimal *Paenibacillus* nif gene cluster is the presence of *nifF* and *nifJ*, which provide the electron transport chain to nitrogenase in some diazotrophs [34], [35]. The activity of *Paenibacillus* nitrogenase is therefore likely to be reductant limited in *E. coli* in the absence of this electron transport chain. Another notable absence is *nifM*, which encodes a cis-trans peptidyl prolyl isomerase required for proper folding of nitrogenase Fe protein in diazotrophic proteobacteria [6]. Potentially this function is provided by a counterpart enzyme encoded elsewhere in the genome in other diazotrophs such as *Paenibacillus*. However, a functional equivalent of *nifM* is not present in *E. coli*, since assembly of active *K. oxytoca* Fe protein in this background requires the presence of both *nifH* and *nifJ* [36]. The *Paenibacillus* NirM sequence contains the seven conserved proline residues identified in other NirH sequences that are considered to be potential substrates for NirM [6]. However, it is possible that other amino acid substitutions in NirH may enable assembly of Fe protein in the absence of NirM. The *Paenibacillus* sp. WLY78 nif gene operon does not contain homologs of the nitrogen fixation-specific iron-sulphur cluster assembly pathway encoded by *nifU* and *nifS*. As in
the case of other diazotrophs, this function may be provided by the Suf system, encoded elsewhere in the *Paenibacillus* genome. When *nifH* and *nifM* are expressed in *E. coli*, assembly of the 4Fe-4S cluster in the *E. coli* Fe protein does not require *nifU* and *nifS* [36], [37]. This function is probably provided by the general Isc, Csd or Suf machineries for iron-sulphur cluster biosynthesis in *E. coli*. However, *E. coli* *nifS* is apparently required for the biosynthesis of the P cluster in the MoFe protein, when NiF polypeptides are expressed in *E. coli* [38]. Although *nifU* and *nifS* also participate in FeMo-co biosynthesis [37], the requirement for these genes is not absolute, particularly if *nifB* is strongly expressed [38].

Systematic deletion of genes in the *Paenibacillus nif* gene cluster suggests they have functions similar to those of other diazotrophs. As anticipated, *nifB* is essential for nitrogen fixation in *E. coli* and the substrate reduction profile of the *nifV* deletion is expected for a mutant lacking homocitrate synthase and therefore unable to make the homocitrate moiety of FeMo-co [39]. The co-localisation of *hesA* within the *nif* operon is an interesting feature of *Paenibacillus* and other minimal *nif* clusters such as those of cyanobacteria and Frankia (Figure 1). Our deletion analysis demonstrates that *hesA* is important for nitrogenase activity, but the function of *hesC* in nitrogen fixation has not so far been determined. Well-characterised homologs belonging to the ThiF-MoeB-HesA family engage in an ATP-dependent process that activates the C-terminus of partner ubiquitin-like proteins by forming an acyl adenylate complex that facilitates sulfur transfer [40], [41]. Ubiquitin-like proteins contain a conserved C-terminal Gly-Gly motif that is the target for adenyllylation by the activating enzyme [42]. Intriguingly, both NiB and NiN from *Paenibacillus* contain C-terminal Gly-Gly motifs and therefore are potential targets for adenyllylation by HesA. Given the potential role of HesA as an activating enzyme for sulphur transfer, it is tempting to speculate that HesA may perform a role in metallocluster biosynthesis.

In the Proteobacteria, *nif* genes are generally transcribed from σ^70-dependent promoters that are subject to transcriptional activation by the enhancer binding protein NifA and are regulated in response to fixed nitrogen and oxygen [30]. However, much less is known about *nif* gene regulation in other diazotrophs where this paradigm is absent. Our results demonstrate that the *nif* cluster of *Paenibacillus* sp. WLY78 is transcribed from a σ^70*-dependent promoter, most likely as a single operon, and that transcription of the *nif* genes is subject to regulation in response to the extracellular concentration of oxygen and fixed nitrogen in *Paenibacillus*. As no transcriptional regulation by either oxygen or fixed nitrogen was detectable when the *Paenibacillus* sp. WLY78 *nif* cluster was expressed from the native *nifB* promoter in *E. coli*, it seems likely that the transcriptional regulation of the *nif* system in *Paenibacillus* involves repression mechanisms. Potential candidates for repression of transcription in response to the nitrogen source are the global nitrogen regulators GlnR and TnrA, which are present in *Paenibacillus* [43].

In summary our results demonstrate that a minimal *nif* gene cluster derived from a gram-positive bacterium can function to synthesize active nitrogenase when expressed in the very different host environment of *E. coli*. This raises various questions concerning the repertoire of genes required for nitrogen fixation and may have important biotechnological implications for engineering diazotopic eukaryotes.

**Materials and Methods**

**Strains and media**

*Paenibacillus* sp. WLY78 was isolated from the rhizosphere of bamboo in Beijing, China by enrichment in nitrogen-free medium after heating at 100°C for 10 min [14]. Strain WLY78 is similar to *P. polymyxa* based on 16S rDNA phylogeny and whole genome sequencing. *E. coli* strains JM109 and BL21 were used as the recipient strains for constructing the engineered *E. coli* strains carrying nitrogen fixation genes.

*Paenibacillus* sp. WLY78 and the engineered *E. coli* strains were routinely grown in LB or LD medium (per liter: 10.4 g Na_2HPO_4, 3.4 g KH_2PO_4, 26 mg CaCl_2, 2H_2O, 30 mg MgSO_4, 0.3 mg MnSO_4, 36 mg Ferric citrate, 7.6 mg Na_2MoO_4.2H_2O, 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. Nitrogen-excess medium contained 100 mM NH_4Cl in nitrogen-free medium [14].

**Acetylene reduction assays**

For nitrogenase activity assays, *Paenibacillus* sp.WLY78 and the engineered *E. coli* strains were grown in 5 ml of LD media (supplemented with antibiotics) in 50 ml flask 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 and IPTG when necessary) to a final OD_600 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, C_2H_2 (10% of the headspace volume) was injected into the test tubes. After incubating the cultures for a further 3 h, 100 μl 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.

For measuring the effect of ammonium on nitrogenase activity, nitrogen-deficient medium was supplemented with NH_4Cl at the concentrations indicated and the cultures were also grown under anaerobic conditions. For measuring the effect of oxygen on nitrogenase activity, nitrogen-deficient medium containing 2 mM glutamate as nitrogen source was used, and oxygen was adjusted to the initial concentration indicated at the start of the incubation.

**15N_2 incorporation assay**

*Paenibacillus* sp.WLY78 and the engineered *E. coli* strains were grown overnight in LD medium. The cultures were collected and resuspended in 70 ml nitrogen-deficient medium containing 2 mM glutamate as nitrogen source, to an OD_600 of 0.4 in a 120 ml serum bottle. The serum bottles were filled with N_2 gas, and then 8 ml gas was removed and 5 ml 15N_2 (99%+, Shanghai Engineering Research Center for Stable Isotope) gas was injected. After 72 h of incubation at 30°C, the cultures were collected, and were freeze dried, ground, weighed and sealed into tin capsules. Isotope ratios are expressed as δ^15N whose values are a linear transform of the isotope ratios 15N/14N, representing the per mille difference between the isotope ratios in a sample and in atmospheric N_2 [26].
Construction of recombinant plasmids and recombinant E. coli strains

Genomic DNA of Paenibacillus sp. WLY78 was used as template for cloning nif genes. Primers used for construction of the engineered E. coli strains are listed in Table S2. Recombinant plasmids and strains are listed in Table S3.

Transcription start site identification

The 5'-RACE method was used to determine the transcription start site (TSS) using the SMARTer RACE cDNA Amplification Kit (Clontech). Gene-specific primers are listed in Table S2. The PCR product was cloned into the pMD18-T Vector and then sequenced.

Overexpression and purification of σ\(^70\) from Paenibacillus sp. WLY78 in E. coli

A 1154 bp DNA fragment carrying the spoD gene (encoding σ\(^70\) of Paenibacillus sp. WLY78) was PCR amplified with primers σA-F and sigma A-R (Table S2). The PCR product was ligated to the pET-28b expression vector, yielding plasmid pET28-σ\(^70\). E. coli strain BL21 (DE3) was transformed with expression plasmid pET28-σ\(^70\) (DGAGGATTTGCATGCTACGGAGCTGGATACTCCGTAC - 3') and the complementary DNA strand (5'-GGAGAAGTGAATT GACTGTATTTGTCCCTGTCTCTA-3') was used to assay non-specific binding.

To examine the specificity of binding to the promoter sequence per σ, primers designed with substitutions in the −35 (TTGACT to GCTACT) and −10 (TAAGAT to GCAGAC) regions of the nifB promoter were utilized and were annealed and labeled as described above.

DNase I footprinting

The DNase I footprinting assay was performed as described by Ziani et al. [47]. A 365 bp nif promoter fragment (from −315 to +50 relative to the transcription start site) was PCR amplified from Paenibacillus sp. WLY78 with primer footprint-up whose terminal base was fluorescent 6-carboxyfluorescein (FAM)-labeled and primer footprint-down (Table S2). The 5'-FAM-labeled DNA fragment (400 ng) was incubated with the E. coli σ\(^70\)-RNAP (10 pmol) for 30 min at 25°C. Bovine serum albumin (BSA) was used for the control experiment. After incubation, the mixtures were digested with DNase I for 40 seconds at 37°C and then the reactions were stopped by adding 0.2 M EDTA (pH 8.0). The digested DNA fragments were extracted with phenol-chloroform, precipitated with ethanol, and the pellets dissolved in Mini-Q water. The samples were sequenced with the ABI 3730 DNA analyzer by Genolab Co. and the data were analyzed with GeneMarker software.

Construction of a nifB promoter::lacZ fusion

A 100 bp DNA fragment (P\(_{\text{nif}}\) from −97 to +3 relative to the nifB transcription start codon) containing the nifB promoter was amplified from total DNA of Paenibacillus sp. WLY78 using primers (Table S2). The fragment was cloned into the promoterless plasmid pPR9TT yielding plasmid pPR9TT-Pnif. The plasmid was then transformed into E. coli JM109, yielding E. coli/pPr9TT-Pnif::lacZ.

For β-galactosidase activity assays, E. coli JM109/pPR9TT and E. coli/pPr9TT-Pnif::lacZ were grown overnight in LB medium at 30°C with shaking. 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 to a final OD\(_{600}\) of 0.2–0.4. For measuring the effect of oxygen on nitrogenase activity, the test tubes were capped and filled with argon, and the cells were harvested after incubation for 2 h at 30°C with shaking under anaerobic conditions. The mixture was incubated for 2 h at 30°C with shaking and then the cells were harvested by centrifugation, washed three times with sterilized water and then resuspended in nitrogen-deficient medium containing 2 mM glutamate as nitrogen source to a final OD\(_{600}\) of 0.2–0.4. For measuring the effect of oxygen on nitrogenase activity, the test tubes were capped and filled with argon, and the cells were harvested after incubation for 2 h at 30°C with shaking.

β-galactosidase activity was assayed according to the method described by Miller [48]. A 100 μl sample was taken and then mixed with 900 μl Z buffer containing β-mercaptoethanol, 40 μl chlorofom and 20 μl 10% SDS and then shaken for 20 sec. Then 200 μl o-nitrophenyl-β-D-galactopyranoside (ONPG) (4 mg/ml) was added to the mixture and incubated in a water bath for 20 min at 28°C. The reaction was stopped with 500 μl 1M Na\(_2\)CO\(_3\) solution. The mixture was then centrifuged for 15 min at 12000 rpm and the supernatant was used to measure the OD\(_{420}\) and OD\(_{590}\) values. 1 unit of β-galactosidase is = [1000×OD\(_{420}\)−1.7 OD\(_{590}\)]/[Time (min)×vol (ml)×OD\(_{600}\)].

RT-PCR

For RT-PCR, Paenibacillus sp. WLY78 and the recombinant E. coli strains were grown in N\(_2\)-fixing conditions (without NH\(_4\)Cl and
O₂, non-N₂-fixing conditions (100 mM NH₄Cl and 21% O₂) or at different concentrations of NH₄Cl in the absence of O₂ or at different concentration of O₂ in the absence of NH₄Cl. The cultures were harvested by centrifugation at 4 °C, and total RNA was isolated using the PrimeScript RT reagent Kit with gDNA Eraser (Takara Bio) according to the manufacturer’s instructions. The possibility of contamination of genomic DNA was eliminated by digestion with RNase-free DNAse I (Takara Bio). The integrity and size distribution of the RNA was verified by agarose gel electrophoresis, and the concentration was determined spectrophotometrically. Synthesis of cDNA was carried out using RT-Prime Mix according to the manufacturer’s specifications (Takara Bio) with an acrylamide:bis-acrylamide ratio of 17:2:1. Antisera against dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) proteins were separated by sodium electrophoresis, and the concentration was determined spectrophotometrically.

For Western blotting, cultures of *Paenibacillus* sp. WLY78 and the engineered *E. coli* strains were grown either in non-N₂-fixing conditions (LD medium and 21% O₂) and harvested after 6–8 h of incubation or grown in N₂-fixing conditions (2 mM glutamate and without O₂) and harvested after 20 h of incubation, respectively. The cell pellet collected from 4 ml cultures at OD₆₀₀ = 1 was dissolved in 200 μl sodium dodecyl sulfate (SDS) gel-loading buffer, boiled for 5 min and then 20 μl was loaded onto the stacking gel. Proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with an acrylamide:bis-acrylamide ratio of 17:2:1. Antisera raised against MoFe protein and Fe protein of *K. oxytoca* M5al were used as probes for Western blotting. The MoFe protein and Fe protein components of nitrogenase were purified from *K. oxytoca* M5al under anaerobic conditions and then used to make rabbit antisera.

**Supporting Information**

**Table S1** Identity of *Paenibacillus* sp. WLY78 Nif polypeptides to those of other diazotrophs.

**Table S2** Primers used in this study.

**Table S3** Bacterial strains and plasmids used in this study.

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**Author Contributions**

Conceived and designed the experiments: SC RD. Performed the experiments: LW LZ JL. Wrote the paper: SC RD. Designed the figures: PL.

**References**

21. Escherichia coli ATCC 13032.1


