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

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Abstract

Most biological nitrogen fixation is catalyzed by molybdenum-dependent nitrogenase, an enzyme complex comprising two 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*, and *hesA* and *nifV* and that the *nif* cluster is under the control of a σ70 (σα)-dependent promoter located upstream of *nifB*. To investigate genetic requirements for diazotrophy, we transferred the *Paenibacillus* *nif* cluster to *Escherichia coli*. The minimal *nif* 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 for 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 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. 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* and *nifZ* play an important role in the 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 *Eklebiella 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 nifP, 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 a single σ70 (σ^70)-like promoter that functions in E. coli, suggesting that nif transcription in Paenibacillus is negatively regulated in response to these effectors.
Results

genome sequencing of *Paenibacillus* sp. WLY78 identifies a minimal nitrogen fixation (*nif*) gene cluster

*Paenibacillus* sp. WLY78 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 organism and identified a nitrogen fixation gene cluster since it is able to fix nitrogen and also produces antimicrobial endospore-forming bacterium is isolated from the rhizosphere of *Paenibacillus*.

Characterization of the *Paenibacillus* sp. WLY78 *nif* promoter and transcription unit

The transcriptional start site (TSS) of the *nif* gene cluster in *Paenibacillus* sp. WLY78 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 −35 (TTGACT) and −10 (TAAGAT) sequences in the *nifB* promoter were similar to the corresponding consensus sequences (TTGACA and TATAAT respectively) of E. coli σ70-dependent promoters. Unlike other members of the Bacillales, the *Paenibacillus* sp. WLY78 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. WLY78 genome (data not shown). Downstream of *nifB*, 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. WLY78 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. WLY78, which was overexpressed and purified from E. coli (Figure 3C). EMSA experiments revealed that both purified σ70 from *Paenibacillus* sp. WLY78 and *E. coli* σ70-RNAP holozyme bind to the 50 bp *nifB* promoter fragment. Competition experiments with non-labelled σ70 DNA indicated that the *E. coli* RNAP holozyme binds more tightly to this DNA fragment, since higher concentrations of competitor were apparently required to dissociate the *E. coli* σ70-RNAP (Figure 3, 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. WLY78 *nifB* promoter, we made substitutions in the −35 (TTGACT to GCTGACT) and −10 (TAAGAT to GGAGAC) 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

Figure 2. The *nif* genes of *Paenibacillus* sp. WLY78 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. WLY78 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 σ70-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 σ70 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 σ70-dependent and thus distinct from the typical σ54-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 Paenibacillus sp. WLY78 nifB promoter was 6σ70-dependent and thus distinct from the typical σ24-dependent −24/−12 promoters found upstream of nif genes in gram-negative diazotrophs.

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 gene 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

**Figure 3. Characterization of the nif promoter of Paenibacillus sp. WLY78.** (A) Schematic representation of the Paenibacillus sp. WLY78 nif operon. (B) Nucleotide sequence of the nifB promoter and the putative terminator sequence flanking the 3' end of nifV. The asterisks below TAA indicate the nifV stop codon. (C) Overexpression and purification of σ70 from Paenibacillus sp. WLY78. Lane 1: protein marker; lane 2: uninduced protein; lane 3: induced protein; lanes 4: purified σ70 factor. (D) Electrophoretic mobility shift assays (EMSA) demonstrating binding of Paenibacillus σ70 to the 50 bp nifB promoter DNA fragment (final concentration 0.03 pmol). The protein concentration is indicated in pmol above each lane (left hand panel). In the right hand panel, the protein concentration was maintained at 2.4 pmol and unlabeled nifB promoter fragment was added as competitor (concentration indicated above each lane). (E) EMSA experiments demonstrating binding of E. coli σ70-RNAP to the 50 bp nifB promoter DNA fragment (final concentration 0.03 pmol). The protein concentration is indicated in pmol above each lane (left hand panel). In the right hand panel, the protein concentration was maintained at 0.2 pmol and unlabeled nifB promoter fragment was added as competitor (concentration indicated above each lane).

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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 nifBHDRENXhesAnifV 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
bacillus sp. WLY78 exhibits both acetylene reduction and $^{15}\text{N}_2$ 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 $^{15}\text{N}_2$. 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 $^{15}\text{N}_2$, 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 $^{15}\text{N}_2$ incorporation was detectable in the nifB deletion, supporting the original observation that nifB is essential for synthesis of nitrogenase [5]. When nifV was deleted, $^{15}\text{N}_2$ 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 $^{15}\text{N}_2$ 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 (nifXhesAnifV) 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 $^{\text{70}}$-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 $^{\text{70}}$-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 $^{\text{70}}$-RNAP with the nifB promoter using an automated capillary sequencer. The top lane is an electropherogram obtained in the presence of $^{\text{70}}$-RNAP with the sequence protected from cleavage shown below. A control electropherogram obtained from a reaction containing BSA is shown in the bottom lane.

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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 NH₄Cl (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.

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that all nine *Paenibacillus* genes (*nif*BHDKENXhesAnifV) 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$_4^+$ and O$_2$ on *nif* gene transcription in the native *Paenibacillus* sp. WLY78 strain with that of engineered *E. coli* strain 78-7 (Figure 7). Both *Paenibacillus* sp. WLY78 and the engineered *E. coli* strain 78-7 strain did not exhibit nitrogenase activity at O$_2$ concentrations above 5% (Figure 7A). In addition, acetylene reduction by *Paenibacillus* sp. WLY78 was not observed at NH$_4^+$ concentrations above 1 mM. In contrast, the engineered *E. coli* strain 78-7 exhibited nitrogenase activity even in the presence of 200 mM NH$_4$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$_4^+$ 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*.
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 elsewhere in the genome. Alternatively, the large nif gene clusters found primarily in Proteobacteria may have evolved from more simple clusters in which assembly, processing and maintenance of nitrogenase activity is less well optimized.

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 15N2 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 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 nifM [36]. The Paenibacillus NifM sequence contains the seven conserved proline residues identified in other NifH sequences that are considered to be potential substrates for NifM [6]. However, it is possible that other amino acid substitutions in NifH may enable assembly of Fe protein in the absence of NifM. 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

![Figure 7. Effects of O2 and NH4+ on nitrogenase activity and nif gene transcription.](image)

(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 "NH4+,O2" indicate that both 2 mM ammonium and 21% oxygen were present. Lanes labeled "+" indicate positive controls in which genomic DNA was used as template in the RT-PCR. Lanes labeled "−" 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).

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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. oxytoca* 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. oxytoca* *nifS* is apparently required for the biosynthesis of the P cluster in the MoFe protein, when NiFe polyhaptides 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-termius 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 adenylylation by the activating enzyme [42]. Intriguingly, both *NifB* and *NifN* from *Paenibacillus* contain C-terminal Gly-Gly motifs and therefore are potential targets for adenylylation by HesA. Given the potential role of HesA as an activating enzyme for sulphur transfer, it is tempting to speculate that HesA may play a role in metallocluster biosynthesis.

In the Proteobacteria, *nif* genes are generally transcribed from σ**54**-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**54**-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 GhnR 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 diazotrophic 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. polyspora* 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 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: 40 μg/ml chloramphenicol, 100 μg/ml ampicillin, and 20 μg/ml tetracycline for maintenance of plasmids.

Nitrogen-free, nitrogen-deficient and nitrogen-excess media were used in this study. Nitrogen-free medium contained (per liter) 10.4 g Na2HPO4, 3.4 g KH2PO4, 26 mg CaCl2·2H2O, 30 mg MgSO4·7H2O, 0.3 mg MnSO4, 36 mg Ferri 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. Nitrogen-excess medium contained 100 mM NH4Cl 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 LB 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 and IPTG 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 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 NH4Cl 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.

15N2 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 OD600 of 0.4 in a 120 ml serum bottle. The serum bottles were filled with N2 gas, and then 8 ml gas was removed and 5 ml 15N2 (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 δ15N whose values are a linear transform of the isotope ratios 15N/15N, representing the per mille difference between the isotope ratios in a sample and in atmospheric N2 [26].
Genome sequencing, assembly and annotation

Total DNA was extracted from *Paenibacillus* sp. WLY78. DNA sequencing was performed using Illumina technologies. A total length of 600,000,120 base pairs of reads was obtained, to enable the assembly of all tags using SOAP denovov. 1.04 assembler [44]. Finally, 87 scaffolds were assembled, giving 101.3-fold coverage of the genome. Glimmer 3 (version 3.0.2) was used for gene finding [45]. Transfer RNA genes were identified by the program rRNAscan-SE [46]. Genes coding for proteins with known functions were annotated by searches against KEGG Genes, Pfam, and SWISSPROT. The complete genome sequence of *Paenibacillus* sp. WLY78 has been deposited at DDBJ/EMBL/Genbank under the accession ALJY0000000. The version described in this paper is version ALJY01000000.

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 σ20 from *Paenibacillus* sp. WLY78 in *E. coli*

A 1154 bp DNA fragment carrying the *spaD* gene (encoding σ20 of *Paenibacillus* sp. WLY78) was PCR amplified with primers sigma A-F and sigma A-R (Table S2). The PCR product was ligated to the pET-28b expression vector, yielding plasmid pET28-σ20. *E. coli* strain BL21 (DE3) was transformed with this plasmid and utilized for protein expression. The bacterial cells were grown in LB medium to the end of log phase and then a final concentration of 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) was added to the culture and the cells were harvested after incubation for another 4 h at 16°C. The cells were then harvested and disrupted by sonication on ice. The protein was purified from the supernatant with Ni2+-NTA agarose (Qiagen) according to the manufacturer’s instructions.

Electrophoretic mobility shift assay (EMSA)

For the electrophoretic mobility shift assay (EMSA), a 50 bp *nifB* promoter fragment (from −47 to +3 relative to the transcription start site of *nifB* in *Paenibacillus* sp. WLY78) was synthesized by Sangon Biotech Co., Ltd (Shanghai). To do this, two DNA fragments corresponding to the sequences of the first strand (5'-GGAGAAGTGAATTGGACTTATTGTGTCCCGTCTCTTACTAGTTGATATATATAT-3') and the complementary DNA strand (5'-ATATAATTCATCTTAGAGACAGGGACAAATACAG-TCAATTCGTTAGCACAATCTCCTGG-3') were synthesized. The two strands were annealed and then labeled with digoxin using the DIG Gel Shift Kit (Roche). The binding of *E. coli* σ20-RNAP (RNA polymerase) (Epizyme) or σ20 of *Paenibacillus* sp. WLY78 to the *nifB* promoter was carried out using a gel shift kit (Roche). A scrambled 39 bp DNA fragment formed by annealing the following complementary oligonucleotides (5'-GTACGGAGTATCTCAGCTCCGATAGCATAATCTCCTGG-3') and (5'-CCAGTATTTCGCTACGGACTGCGAGGATCGATCCGTTGG-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 GTGACT) and −10 (TAAAG 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 Zhao et al. [47]. A 365 bp *nifB* promoter fragment (from −315 to +50 relative to the transcription start site) was PCR amplified from *Paenibacillus* sp. WLY78 with primer pfoot-up whose terminal base was fluorescent 6-carboxyfluorescein (FAM)-labeled and primer pfoot-down (Table S2). The 5'-FAM-labeled DNA fragment (400 ng) was incubated with the *E. coli* σ20-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 (PnifB 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-PnifB-lacZ. For β-galactosidase activity assays, *E. coli* JM109/pPR9TT and *E. coli*/pPR9TT-PnifB-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 OD600 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 oxygen concentration was adjusted to the initial concentration indicated and cultures were then incubated for 20 h at 30°C with shaking under anaerobic conditions. For measuring the effect of oxygen on nitrogenase activity, the test tubes were capped and filled with argon, and the oxygen concentration was adjusted to the initial concentration indicated and cultures were then incubated for 20 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 chloroform 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 Na2CO3 solution. The mixture was then centrifuged for 15 min at 12000 rpm and the supernatant was used to measure the OD420 and OD500 values. 1 unit of β-galactosidase = [1000×(OD420−1.7 OD500)]/[Time (min)×vol (ml)×OD600].

RT-PCR

For RT-PCR, *Paenibacillus* sp. WLY78 and the recombinant *E. coli* strains were grown in N2-fixing conditions (without NH4Cl 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). 0.8 μg of cDNA was used for RT-PCR. The nifH and nifK transcripts were detected by using an RT-PCR Kit with 16S rRNA as a control. Primers for nifH, nifK and 16S rRNA used for PCR are listed in Table S2.

Western blot assays for NifH and NifDK expression

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 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. Antibsera 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 antisemur.

Supporting Information

Figure S1 Immunological detection of nitrogenase MoFe protein and Fe protein in Paenibacillus sp. WLY78, the engineered E. coli strain 78-7 and nif gene deletion mutants. (A) Cultures grown in N₂-fixing conditions (2 mM glutamate and in the absence of O₂). (B) Cultures grown under non-N₂-fixing conditions (LD medium and 21% O₂). Antiseras against K. oxytoca MoFe and Fe proteins, respectively, were used as probes. WT indicates Paenibacillus sp. WLY78. Vector indicates E. coli JM109 carrying empty vector pHY300PLK.

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Author Contributions

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