J. Microbiol. Biotechnol. (2015), 25(8), 1339–1348
http://dx.doi.org/10.4014/jmb.1502.02027

jmb

Interspecies Transfer and Regulation of *Pseudomonas stutzeri* A1501 Nitrogen Fixation Island in *Escherichia coli* S

Yunlei Han[1,2], Na Lu[3], Qinghua Chen[2,4], Yuhua Zhan[2], Wei Liu[2,5], Wei Lu[2], Baoli Zhu[3], Min Lin[2], Zhirong Yang[1], and Yongliang Yan[2*]

[1]*Key Laboratory of Bio-resources and Eco-environment Ministry of Education, College of Life Sciences, Sichuan University, Chengdu, Sichuan 610064, P.R. China*

[2]*Biotechnology Research Institute, Chinese Academy of Agricultural Sciences, Beijing 100081, P.R. China*

[3]*CAS Key Laboratory of Pathogenic Microbiology and Immunology, Institute of Microbiology, Chinese Academy of Sciences, Beijing 100101, P.R. China*

[4]*CAS Key Laboratory for Biological Effects of Nanomaterials and Nanosafety, National Center for Nanoscience and Technology, Beijing 100190, P.R. China*

[5]*Institute of Plant Protection and Microbiology, Zhejiang Academy of Agricultural Sciences, Zhejiang 310021, P.R. China*

Received: February 11, 2015
Accepted: March 30, 2015

First published online
March 31, 2015

**Corresponding author*
Phone: +86-10-82109868;
Fax: +86-10-82106106;
E-mail: yanyongliang@caas.cn

Supplementary data for this paper are available on-line only at http://jmb.or.kr.

pISSN 1017-7825, eISSN 1738-8872

Copyright© 2015 by
The Korean Society for Microbiology and Biotechnology

Until now, considerable effort has been made to engineer novel nitrogen-fixing organisms through the transfer of *nif* genes from various diazotrophs to non-nitrogen fixers; however, regulatory coupling of the heterologous *nif* genes with the regulatory system of the new host is still not well understood. In this work, a 49 kb nitrogen fixation island from *P. stutzeri* A1501 was transferred into *E. coli* using a novel and efficient transformation strategy, and a series of recombinant nitrogen-fixing *E. coli* strains were obtained. We found that the nitrogenase activity of the recombinant *E. coli* strain EN-01, similar to the parent strain *P. stutzeri* A1501, was dependent on external ammonia concentration, oxygen tension, and temperature. We further found that there existed a regulatory coupling between the *E. coli* general nitrogen regulatory system and the heterologous *P. stutzeri nif* island in the recombinant *E. coli* strain. We also provided evidence that the *E. coli* general nitrogen regulator GlnG protein was involved in the activation of the *nif*-specific regulator NifA *via* a direct interaction with the NifA promoter. To the best of our knowledge, this work plays a groundbreaking role in increasing understanding of the regulatory coupling of the heterologous nitrogen fixation system with the regulatory system of the recipient host. Furthermore, it will shed light on the structure and functional integrity of the *nif* island and will be useful for the construction of novel and more robust nitrogen-fixing organisms through biosynthetic engineering.

Keywords: *Pseudomonas stutzeri* A1501, *Escherichia coli*, nitrogen fixation island, biological nitrogen fixation, horizontal gene transfer

Introduction

The biological nitrogen fixation performed by a limited number of bacterial and archaeal species directly or indirectly supports all plant growth on Earth [14]. The well-known Mo-Fe-containing protein catalyst Mo-nitrogenase carries out the reduction process of converting dinitrogen to ammonia. Highly conserved among diazotrophs and typically located contiguously in the genome, nitrogen fixation systems may originate from the most recent common ancestor, providing the possibility that the capability for nitrogen fixation could be acquired through horizontal gene transfer in natural environments or under laboratory conditions [20].

The ability to fix nitrogen is widely distributed among bacteria and archaea but is an extremely rare feature in the genus *Escherichia*. Over the past decades, considerable effort has been made to engineer novel nitrogen-fixing organisms *via* transfer of *nif* genes from various diazotrophs [11, 18, 21, 27], and the genetic transfer of nitrogen fixation genes to enteric bacteria has received much attention. In the early 1970s, pioneering work proved that a functional nitrogenase could be transferred between two *Enterobacter* strains; typically, *E. coli* was shown to express an active nitrogenase from *Klebsiella pneumoniae* [5, 6]. Most recently, a *nif* cluster consisting of nine genes from *Paenibacillus* sp. WLY78 was transferred to *E. coli*, and the *nif* cluster enabled the synthesis of catalytically active nitrogenase [21]. However, such a horizontally acquired ability is not sufficient to enable diazotrophic growth on nitrogen-free medium of *E. coli*. *Klebsiella* and *Azotobacter* can enable associative nitrogen fixation with non-legume plants such as rice, corn, and sorghum, thereby enhancing the growth of these plants. Unlike most other strains, the nitrogen-fixing *P. stutzeri* A1501, isolated from the root of rice, harbors a presumptive nitrogen fixation island (NFI) [26]. Inoculation of *Arabidopsis*, alfalfa, tall fescue, and maize with *Pseudomonas protegens* Pf-5 carrying the NFI from *P. stutzeri* A1501 increased both the ammonium concentration in the soil and plant productivity under nitrogen-deficient conditions [18].

As described above, the physiological roles of the transferred *nif* clusters/systems in the recipient bacteria are clear; however, the regulatory coupling of the heterologous *nif* genes with the regulatory system of the new host is still not well understood. In this work, the NFI of *P. stutzeri* A1501 was transferred into *E. coli via* a novel and efficient transformation strategy, and a series of recombinant nitrogen-fixing *E. coli* strains were obtained. Subsequently, expression of the *nif* genes in the recombinant *E. coli* strain EN-01 was characterized under different growth conditions, and the regulatory mechanisms of the heterologous NFI in *E. coli* were also investigated.

To the best of our knowledge, this work plays a groundbreaking role in increasing understanding of horizontal gene transfer and the regulatory coupling of the heterologous nitrogen fixation system with the regulatory system of the recipient host. Furthermore, gene transfer of the nitrogen fixation pathway into well-developed hosts such as *E. coli* represents not only an attractive alternative to the production of the natural parent product itself but also a useful tool in the construction of novel and more robust nitrogen-fixing organisms through biosynthetic engineering.

Materials and Methods

Bacterial Strains and Growth Conditions

P. stutzeri A1501 and its mutant derivatives were grown at 30°C in LB medium or in minimal lactate-containing medium (medium K) as described previously [9]. *E. coli* DH10B T1-phage resistant cells (Invitrogen, China) were used as the recipient strain for constructing the engineered *E. coli* strains that carry nitrogen fixation genes. *E. coli* and the recombinant strains were grown in LB medium or SOC medium at 37°C with shaking.

Construction of the Δ*cobS/gshP* Double Mutant

The strategy for constructing a Δ*cobS/gshP* mutant was to knock out *cobS* with a tetracycline (Tc) resistance gene and insert a hygromycin (Hyg) resistance gene in the middle of the *gshP* region (see Table S1 for a list of the primers used and their sequences). In-frame deletion mutants were generated *via* homologous recombination using the pKnockout vector pK18mob and previously described methods [23]. Crossover events were first screened using Minimal K medium containing Tc, followed by screening using the same medium containing kanamycin (Km). Single crossover events confer resistance to both Tc and Km. Double crossover events confer resistance to tetracycline only, which enabled the identification of the desired *P. stutzeri* A1501 mutant, named Δ*cobS*, with successful conjugal transfer. Recombination at the correct location was confirmed by PCR. The success rate of transfer was approximately 1 out of 55 mutants.

The same procedure was used to transfer Hyg resistance to the Δ*cobS* mutant *via* inactivation of *gshP* by the insertion of a hygromycin resistance gene. The resulting *P. stutzeri* mutant was named A1501B. The resulting double-resistant mutant strain that lacked a reduction in nitrogen-fixing ability was named A1501R2.

Transfer of NIF to *E. coli* Using BAC Resources.

The BAC library was constructed using protocols similar to those found in Osoegawa *et al*. [16]. *Eco*RI and *Eco*RI methylase were used to partially digest the DNA of A1501R2 and to ligate the partially digested DNA into the vector pTARBAC2.1. Vectors were transferred to electrocompetent *E. coli* DH10B T1 phage-resistant cells (Invitrogen, USA) for expression. The library was designated EN and comprised approximately 6,000 clones arrayed in 624 384-well microtiter dishes. High-density replica filters were prepared as previously described [16].

High Molecular Weight Genomic DNA Preparation.

Isolating chromosomal DNA is a critical step in the construction of a genomic DNA library. To construct a large-insert (>50 kb) library, high molecular weight DNA must be isolated from cells. Detailed procedures for the preparation of high molecular weight DNA have been described previously [16]. A1501R2 cells were embedded in agarose plugs at a final concentration of 3×10^8 cells/ml, followed by a sequence of treatments. DNA used in the construction of the library was partially digested with *Eco*RI in the

presence of *Eco*RI methylase and size-fractionated by pulsed-field gel electrophoresis using a CHEF apparatus (BioRad, USA). The size-fractionated agarose gels were stored in 0.5 M EDTA until use. Electroelution procedures were used to obtain the partially digested DNA from the gel slices [16].

BAC Vector Preparation

The pTARBAC2.1 plasmid DNA was isolated using cesium chloride gradient purification, digested with *Eco*RI and treated with calf intestine phosphatase (New England Biolabs, USA), and then separated on a 1.0% agarose CHEF gel. The vector fragment was purified from the gel as previously described [16].

Construction of the BAC Library

The BAC library was constructed following standard protocols using the pTARBAC2.1 vector [16]. The ligation products were transformed into electrocompetent *E. coli* DH10B T1 phage-resistant cells (Invitrogen, USA). High-density replica filters were prepared.

Single Colony Isolation of Recombinant *E. coli* Bearing the Nitrogen Fixation Island

Nitrogen-fixing strains of recombinant *E. coli* DH10B were selected on LB agar plates containing sucrose and the antibiotics 20 μg/ml chloramphenicol (Cm), 100 μg/ml Hyg, and 10 μg/ml Tc.

PCR Verification of Positive Clones

PCR primers were constructed based on the genomic sequence of A1501R2 (see Table S1 for a list of the primers used and their sequences). LB plates contained 100 μg/ml Hyg and 10 μg/ml Tc, and positive clones were transferred to a new tube containing the PCR mix. PCRs were carried out in a total volume of 20 μl. After thermocycling, 2 μl of each PCR was analyzed on a 1.5% agarose gel (ReadyGel, Amersham Biosciences, USA).

BAC End Sequence Analysis

All acquired clones were end-sequenced with a pair of universal primers, the T7 primer and SP6 primer (see Table S1 for a list of the primers used and their sequences).

Nitrogenase Activity Assays

Nitrogenase activity is measured by the reduction of acetylene relative to the increase in ethylene production using gas chromatography analysis. The nitrogenase activity of *P. stutzeri* was determined according to the protocol described by Desnoues *et al*. [9]. The nitrogenase activity of *E. coli* was determined according to the protocol described by Cannon *et al*. [6]. Nitrogenase-specific activity is expressed as nmol ethylene/mg protein/h. Each experiment was repeated at least three times.

Biolog Phenotype Profiling

Colonies from the test strains were inoculated from pre-growth LB agar plates into Biolog inoculating fluid (Biolog, USA), and the optical density of the inoculating suspension was adjusted to 60% transmittance using a turbidimeter. Cell suspensions were inoculated (150 μl of suspension per well) into 96-well Biolog gram-negative (GN2) MicroPlates, which were then incubated at 37°C for 24 h. Each strain was assayed in duplicate plates. The OD_{595} was measured with a microtiter plate reader (model Vmax; Molecular Devices Corp., USA) after 6 and 24 h of incubation.

Quantitative Real-Time PCR

Gene expression was examined by quantitative real-time PCR. For RT-PCR, EN-01 strains were grown under nitrogen-fixing conditions (without $(NH_4)_2SO_4$ and O_2) or excess-nitrogen conditions (10 mM $(NH_4)_2SO_4$ without O_2). The cultures were harvested by centrifugation at 4°C, and total RNA was isolated using the SV Total RNA Isolation System (Promega, USA) according to the manufacturer's instructions. RT-PCR experiments were performed with three independent RNA preparations using the ABI 7500 Real-Time PCR System (Applied Biosystems, USA) and SYBR Green PCR Master Mix (Applied Biosystems, USA) according to the manufacturer's recommendations. First-strand cDNAs were synthesized from 2 μg of total RNA in a 20 μl reaction volume, using the ProtoScript First Strand cDNA Synthesis Kit (New England Biolabs, USA). The 16S RNA gene was used as a reference for the normalization of samples. Primers were generated in a previous study [26].

Phage Transduction

To move the *glnG* mutation into the *EN-01* background for characterization, phage transduction using the P1 phage was used. The *glnG* mutation from Baba *et al*. [2] was used as a donor to prepare the P1 phage lysate that was subsequently used to transduce *EN-01* as per the Court Lab Protocol [19]. Selection for transductants was performed on LB agar plates containing kanamycin. The resulting EN-01 mutant was named EN-01M.

Construction of the GlnG-P Expression Vector

The D54E,S160F mutation was generated by overlap extension PCR as described previously [4]. The final PCR products were double-digested with *Nde*I and *Eco*RI and cloned into a pTWIN1 vector that had been digested with the same enzymes to yield pTW-GlnG-P (see Table S1 for a list of the primers used and their sequences).

Optimization of Protein Expression and Purification

Protein expression and purification were performed as described by Xie *et al.* [24]. Briefly, a 2 L culture of *E. coli* BL21 harboring pTW-GlnG-P was grown to an optical density at OD_{600} of 0.3 to 0.5 at 37°C and then transferred to a 30°C shaker. After 20 min, isopropyl-β-D-thiogalactoside was added to a final concentration of 0.1 mM, and the culture was incubated at 30°C for 3 h. Cells were then harvested by centrifugation, resuspended in 10 ml of E-buffer (5 mM Tris-HCl, pH 7.8, 1 mM EDTA, and 1 mM dithiothreitol (DTT)), and broken by sonication. After centrifugation at 5,000 ×*g* for 30 min at 4°C, the supernatant was loaded onto a

2 ml chitin (NEB, USA) column equilibrated with Buffer B1 (20 mM Tris-HCl, pH 8.5, 500 mM NaCl, and 1 mM EDTA). Nonspecifically bound proteins were removed by washing with 20 ml of washing buffer (20 mM Tris-HCl, pH 8.0, 500 mM NaCl, and 1 mM EDTA). The cleavage of GlnG-P-intein was induced with 5 ml cleavage buffer B3 (20 mM Tris-HCl pH 8.5, 500 mM NaCl, 40 mM DTT, and 1 mM EDTA), and the solution was subsequently incubated at 4°C overnight. Protein concentration was determined by the Bradford protein assay and is expressed in nM. Protein samples were stored at −80°C.

Electrophoretic Mobility Shift Assays (EMSAs)

EMSAs were carried out according to previously described methods [28]. Using a pGEM-T bearing the promoter regions of *nifA* or *nifH* as a template, the promoter regions of *nifA* and *nifH* were successively amplified by PCR with IRDye-800 fluorescent DNA primers (Li-Cor Biosciences, USA). Labeled DNA fragments were independently mixed with purified GlnG-P proteins using the Odyssey Infrared EMSA Kit (Li-Cor Biosciences, USA) according to the manufacturer's instructions (see Table S1 for a list of the primers used and their sequences). For competitive inhibition of

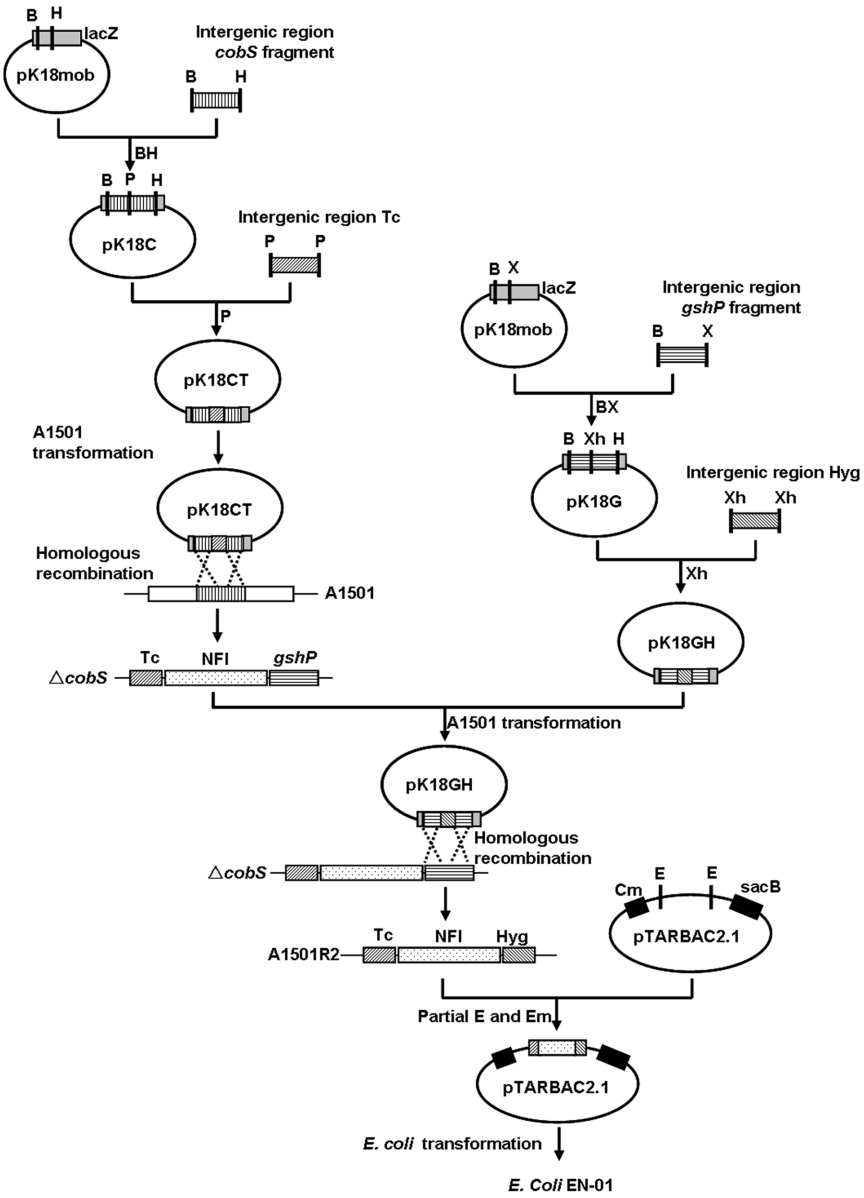

Fig. 1. Overview of the protocol developed to obtain recombinant *E .coli* bearing the NFI derived from *P. stutzeri* A1501.
Abbreviation: A1501, *P. stutzeri* A1501; A1501R2, *acdS* and *gshP* double mutant of *P. stutzeri* A1501; NFI, nitrogen fixation island; Tc, tetracycline resistance gene; Hyg, hygromycin resistance gene; Cm, chloramphenicol resistance gene; B, *Bam*HI; H, *Hin*dIII; P, *Pst*I; X, *Xba*I; Xh, *Xho*I; E, *Eco*RI, and Em, *Eco*RI methylase.

the binding reaction, 50- or 100-fold unlabeled DNA fragments were added to that reaction system. After incubation on ice for 10 min, the reactants were run on a 5% TBE polyacrylamide gel (Bio-Rad, USA) at 110 V for 1 h, with 0.5× TBE as the running buffer.

Results

Construction of Recombinant *E. coli* Clones Containing the Entire *P. stutzeri* A1501 NFI

The complete nucleotide sequence of the *P. stutzeri* A1501 genome has been determined, which led to the identification of a 59-gene, 49 kb island that comprises the largest group of *nif* genes identified to date. This discovery was also confirmed by transcriptomic analysis showing the characteristics of an "expression island" under nitrogen-fixing conditions [26]. In this study, we used a novel and efficient transformation strategy to transfer the 49 kb island into *E. coli*.

First, the A1501R2 with two antibiotic resistance genes to tetracycline and hygromycin inserted into both sides of the NFI was constructed. Under nitrogen-fixing conditions, A1501R2 had the same nitrogenase activity as the wild type (Fig. 2). Growth was also identical to the parent while grown aerobically in LB. This confirmed that the genes *cobS* and *gshP* were not required for the survival and nitrogen-fixing capability of *P. stutzeri* A1501.

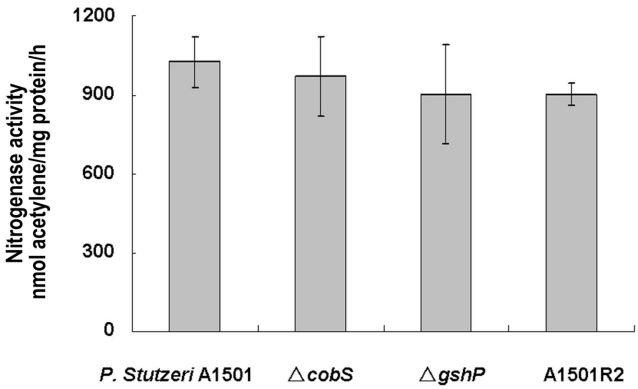

Fig. 2. Nitrogenase activity of *P. stutzeri* A1501, Δ*cobS*, Δ*gshP*, and A1501R2.

Second, the BAC library was constructed based on the genomic DNA of A1501R2. High molecular weight DNA fragments (about 50−100 kb) of A1501R2 were cloned into the pTARBAC2.1 vector and then introduced into the non-restricting *E. coli* DH10B strain. BAC clones containing the complete *P. stutzeri* A1501 NFI could then be isolated easily by selecting for both resistance genes. Altogether, 17 recombinants carrying the complete NFI were obtained by plating on LB agar containing 100 μg/ml Hyg and 10 μg/ml Tc. BAC plasmids were then end-sequenced to determine whether the sequences and sizes of the inserts were correct.

Table 1. Nitrogenase activity of the recombinant *E. coli* strains.

Recombinant *E. coli* strain	Start position in *P. stutzeri* A1501 genome	Insertion size (bp)	Nitrogenase activity (nmol ethylene/mg protein/h)
EN-01	1403078	59,336	104.9 ± 15.85
EN-02	1401104	84,941	49 ± 4.68
EN-03	1401104	87,487	69.9 ± 5.90
EN-04	1377614	89,528	42.4 ± 5.73
EN-05	1403094	91,354	12.2 ± 2.58
EN-06	1401104	92,838	84.4 ± 9.96
EN-07	1401098	98,168	91.8 ± 9.14
EN-08	1362307	100,107	56.5 ± 4.14
EN-09	1365856	101,275	50.6 ± 4.65
EN-10	1362309	104,822	73.4 ± 7.23
EN-11	1362307	104,835	33.1 ± 7.23
EN-12	1377616	110,358	30.4 ± 5.6
EN-13	1377614	116,328	54.5 ± 4.89
EN-14	1350541	116,590	15.2 ± 2.16
EN-15	1346634	120,508	33.1 ± 6.32
EN-16	1376178	120,871	52.3 ± 9.76
EN-17	1362307	125,669	60.8 ± 8.93

Among the 17 strains, the insert sizes ranged from 59–124 kb; the insert sites were also slightly different. We tested various growth conditions using a series of carbon sources and found that optimal conditions consisted of anaerobic conditions with a supply of glucose. Therefore, for the 17 recombinant strains, all of the nitrogenase activity assays were carried out under anaerobic conditions using glucose as the sole carbon source. The nitrogenase activity assays proved that all 17 recombinants could reduce acetylene to ethylene (Table 1). Compared with *P. stutzeri* A1501, the recombinant *E. coli* strains showed approximately 10% of the nitrogenase activity for acetylene reduction. The recombinant strain with the highest nitrogenase activity and the smallest insert size (59.3 kb) was used for further study and named *E. coli* EN-01.

Physiological Alteration of *E. coli* Transformed with the Entire *P. stutzeri* NFI

As a large fragment of foreign DNA carried by a BAC vector, the entire NFI would presumably affect host gene replication, energy distribution, *etc*., especially because the NFI represents a "functional entity" as a nitrogen-fixing unit and likely reconstructs a global host network for metabolism and energy utilization. We compared the growth curves of wild-type *E. coli* and *E. coli* EN-01 under rich medium (LB) conditions; the results indicated a reduced growth rate (Fig. 3A). We also compared them in a Biolog GN2 carbon metabolism characterization system using 95 common carbon sources. The results showed rapid changes in the utilization of 35 of the carbon sources in EN-01. Decreases of over 2-fold were found for 19 of the carbon sources, including maltose, D-sorbitol, glycerol, D,L-lactose, and L-alanine; however, an increase of 1-fold was found for L-arabinose (data not shown). Therefore, it seems that the metabolism of the host *E. coli* bearing the introduced NFI was altered, indicating a coupling of the networks for nitrogen and carbon metabolism. Such coupling may have strong effects on the reconstruction of

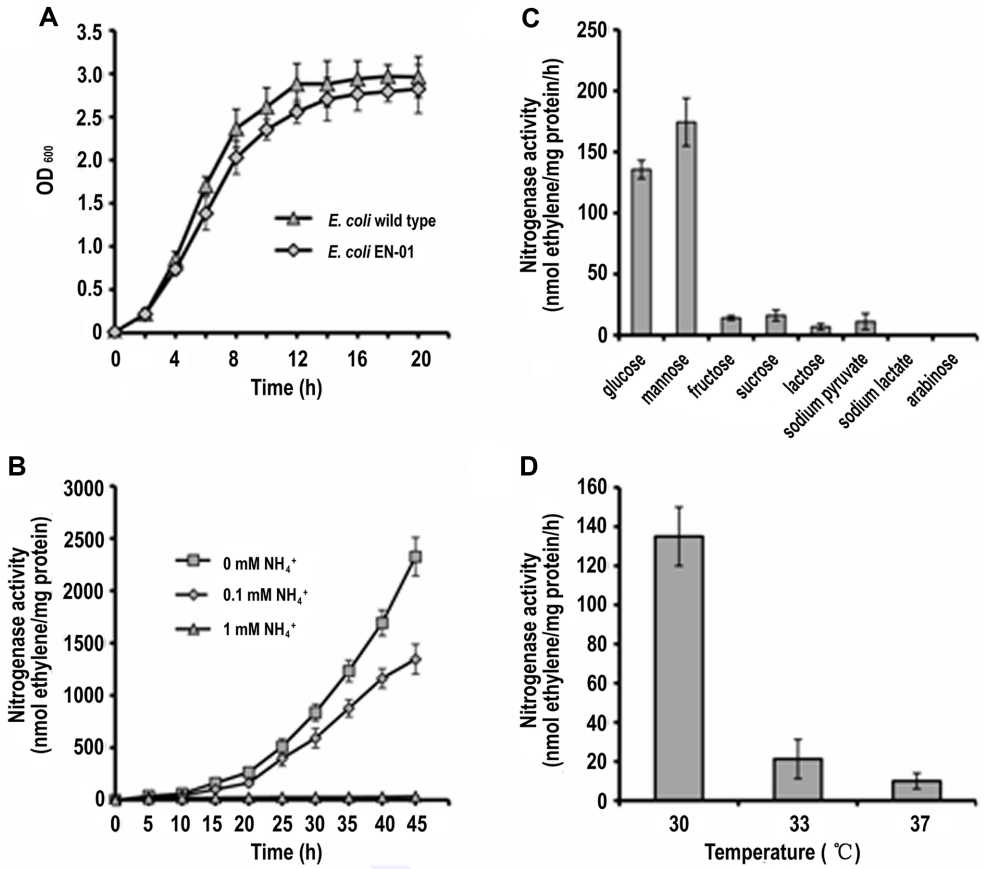

Fig. 3. Phenotypes of the recombinant *E. coli* EN-01 under different conditions.
(**A**) Growth curve of *E. coli* EN-01 in LB medium; (**B**) time course of the nitrogenase activity of EN-01 under different ammonium concentrations; (**C**) nitrogenase-specific activity of strain EN-01 in NFDM medium with different carbon resources; (**D**) nitrogenase activity of EN-01 at different temperatures.

metabolic flux in the recombinant strain EN-01, which displayed a novel physiological phenotype compared with the wild-type *E. coli* strain.

The Expression of NFI in Recombinant *E. coli* EN-01 is Regulated by Ammonia, Carbon, Oxygen, and Temperature

Although both the host *P. stutseri* A1501 and the recipient *E. coli* belong to γ-Proteobacteria, the two species have obvious differences in taxonomic status and in the microenvironments in which they live. As members of Enterobacteriaceae, *E. coli* can generate energy through anaerobic fermentation. Their optimum growth temperature was 37°C, whereas for *P. stutzeri* A1501 isolated from rice soil, the optimum growth temperature was 30°C, and the optimum nitrogen-fixing condition was a nitrogen-free, microaerobic environment. Overall, nitrogen fixation activity was dependent on ammonia, carbon, and oxygen availability and on temperature [9].

In many diazotrophs, the addition of excess ammonium to the growth medium results in immediate repression of *nif* gene transcription. To examine whether the recombinant strain EN-01 is subject to this regulation, we investigated the effects of ammonium on nitrogenase activity. As shown in Fig. 3B, $(NH_4)_2SO_4$ was added to yield final ammonium concentrations of 0, 0.1 and 1 mmol in the medium, and the nitrogenase activity of *E. coli* EN-01 was highest in the nitrogen-free medium. In addition, nitrogenase activity was inhibited in the medium containing 0.1 mmol ammonium ion, whereas the medium containing 1 mmol ammonium ion completely inhibited the nitrogenase activity of EN-01.

Carbon and nitrogen metabolism are two major cellular processes, and they are usually coupled to reach optimal growth *via* various strategies. Microbes utilize different carbon sources at various rates; this is reflected by the variation in ultimate energy production that affects nitrogen fixation efficiency. The experiments demonstrated that under anaerobic and nitrogen-deficient conditions, the recombinant *E. coli* EN-01 strain exhibits the greatest nitrogenase activity by using glucose or mannose as the sole carbon source. Furthermore, it exhibits rapidly decreased activity when using fructose, sucrose, lactose, and sodium pyruvate, and it has almost no detectable activity when using sodium lactate or arabinose (Fig. 3C).

Nitrogenase is highly sensitive to oxygen, which can cause it to be irreversibly inactivated. For *P. stutzeri* A1501, nitrogenase activity was detected at a range of initial oxygen concentrations (from 0.5% to 4%), suggesting that nitrogen fixation occurs at low oxygen tension [9]. To evaluate the effect of oxygen on the expression of nitrogenase in *E. coli* EN-01, we determined nitrogenase activity in air and under microaerobic and anaerobic conditions. The results showed that EN-01 nitrogenase activity can only be detected through acetylene reduction in the anaerobic environment when using the nitrogen-free medium NFDM in the presence of various carbon sources, including glucose, mannose, fructose, sucrose, lactose, and pyruvate. Among the various carbon sources, glucose was optimal for nitrogen fixation. Furthermore, no activity was detected when the cells grew on lactate, but *P. stutzeri* A1501 was able to fix nitrogen and grow on lactate as the sole carbon source. The results also showed that, in the EN-01 strain, nitrogenase activity was regulated by the concentration of oxygen in the environment, which was also the case for *P. stutzeri* A1501.

Temperature could be one of the factors affecting nitrogenase activity. It has been reported that NifA, a positive regulator of nitrogenase activity, is very sensitive to temperature [12, 22]. On the other hand, nitrogenase is also temperature-sensitive; higher temperatures can decrease its activity or denature it. The optimal temperature for the growth of *P. stutzeri* A1501 is 30°C, whereas for *E. coli*, the optimal temperature is 37°C. Therefore, we assayed the nitrogenase activities of EN-01 at 30°C, 33°C, and 37°C. The results showed that the recombinant EN-01 could grow normally at both 37°C and 30°C, but it had higher nitrogenase activity at 30°C. Increasing the growth temperature to 33°C or 37°C reduced the nitrogenase activity 6- and 14-fold, respectively (Fig. 3D).

The above results indicate that expression of the NFI in the recombinant strain *E. coli* EN-01 is very similar to its expression in *P. stutzeri* A1501, suggesting that the expression and regulation of nitrogen fixation genes may be affected by the nitrogen and oxygen signaling networks of the recipient bacteria *E. coli.* Variations in the nitrogen, oxygen, and temperature conditions of the external environment may signal through the induction system of *E. coli* and somehow be transferred to *nifLA* of the NFI, regulating the expression of nitrogen fixation genes.

Expression of NFI Genes in *E. coli* EN-01

As mentioned above, the recombinant strain EN-01 displays nitrogenase activity, indicating the heterologous production of a functional nitrogenase complex encoded by the *nifHDK* genes. The nitrogenase activity of EN-01 is regulated by ammonia, oxygen, and temperature, strongly suggesting that the *nif*-specific transcriptional regulator *nifLA* is active in the heterologous host strain EN-01. To further test the heterologous expression of *nifLA* and *nifHDK*,

Table 2. Expression of the *nif* genes in *E. coli* EN-01 under nitrogen-fixing conditions compared with excess-nitrogen conditions.

Gene ID	Gene name	Up-regulation folds
PST1313	*nifA*	7.53 ± 0.56
PST1314	*nifL*	5.58 ± 0.31
PST1326	*nifH*	69.51 ± 8.95
PST1327	*nifD*	32.36 ± 1.79
PST1328	*nifK*	43.88 ± 2.37

we determined the induction ratio of these genes when strain EN-01 was grown anaerobically under nitrogen-fixing conditions compared with when it was grown under excess-nitrogen conditions using quantitative real-time RT-PCR (Table 2). The results showed that, compared with conditions of excess nitrogen, under nitrogen-fixing conditions, the five genes were significantly up-regulated (Table 2), and the expression patterns were very similar to those in *P. stutzeri* A1501 [25]. This finding indicates that in the *E. coli* EN-01 strain, the expression levels of *nifLA* and *nifHDK* are controlled by the *E. coli* nitrogen metabolism regulatory system.

Transcriptional Regulation of the *P. stutzeri* NFI by *E. coli* GlnG Protein *via* NifA

Both *E. coli* and *P. stutzeri* belong to the γ-Proteobacteria and have highly similar general nitrogen regulatory systems. The nitrogen regulatory protein NRI plays an essential role in nitrogen regulation in different bacteria [17]. It has been shown that in *P. stutzeri* A1501, NtrC controls the expression of the alternative PII protein GlnK, as well as of the *nif*-specific activator NifA [13, 25]. Mutation of *ntrC* leads to the loss of nitrogenase activity in *P. stutzeri* A1501 [9]. In *E. coli*, the NRI protein is encoded by *glnG*, which has 65.4% homology to the *P. stutzeri* NtrC protein. Moreover, qRT-PCR analysis showed that *glnG* was significantly up-regulated (over 10-fold) in EN-01 under nitrogen-fixing conditions compared with excess-nitrogen conditions. To investigate the role of *E. coli* GlnG on the expression of the *P. stutzeri* A1501 NFI, a *glnG* mutant derived from the recombinant *E. coli* EN-01 was constructed and named *E. coli* EN-01M. No nitrogenase activity was detected in *E. coli* EN-01M, indicating that GlnG may be involved in the expression of the heterologous *P. stutzeri* A1501 NFI.

A phosphorylated form of the *E. coli* GlnG protein was purified and used to investigate the direct interaction of the GlnG protein with the *P. stutzeri nifLA* promoter using EMSA. As shown in Fig. 4A, a putative GlnG-binding site

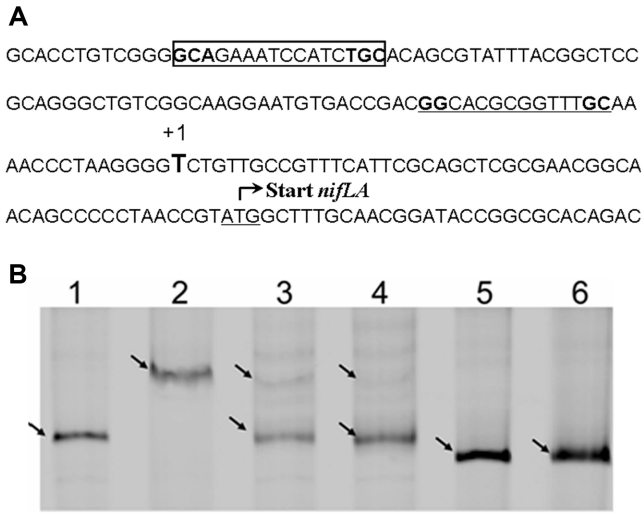

Fig. 4. Electrophoretic mobility shift assays (EMSAs) of the promoter regions of *nifLA* from *P. stutzeri* A1501 and purified phosphorylated *E. coli* GlnG protein.

(**A**) Sequence of the *nifLA* promoter region, showing the putative GlnG-binding sites (open boxes). The σ^{54} promoter (sequence underlined with GG GC in bold), the transcription initiation site (T in bold), and the translation initiation site (underlined ATG) are also highlighted for comparison; (**B**) EMSA experiments demonstrating the binding of *E. coli* phosphorylated GlnG to the *nifLA* promoter region. Lane 1: labeled *nifA* promoter DNA fragment; lane 2: labeled *nifA* promoter DNA fragment incubated with phosphorylated GlnG protein; lanes 3 and 4: phosphorylated GlnG protein incubated with labeled *nifA* promoter DNA fragment containing 50- and 100-fold non-labeled *nifA* promoter DNA, respectively; lane 5: labeled *nifH* promoter DNA fragment; and lane 6: labeled *nifH* promoter DNA fragment incubated with GlnG-P protein.

(containing GCA and TGC) was located in the *nifLA* promoter region, which is also a σ^{54}-dependent promoter bearing the consensus GG-N10-GC motif of the RpoN protein. These motifs are similar to the corresponding consensus sequences found in the promoter regions of the *E. coli glnKamtB*, *glnALG*, and *gltIJKL* gene cluster [3]. The DNA fragment P_{nifA}, which completely covers the *nifLA* promoter region from positions -327 to 141 relative to the transcription start site, was cloned and fluorescently labeled. The promoter region of the *nifHDK* operon (P_{nifH}) was used as a negative control. The DNA fragment was mixed with the purified GlnG, and after that, the mixtures containing the DNA/GlnG complex and the free DNA were resolved by native PAGE. As shown in Fig. 4B, the addition of purified phosphorylated GlnG protein to P_{nifA} caused a band shift of P_{nifA}. As the amount of non-labeled P_{nifA} increased, the shifted band disappeared, indicating that the

phosphorylated GlnG protein binds specifically to DNA fragments containing the *nifLA* operon promoter region. This observation provides evidence for a direct interaction of the *E. coli* phosphorylated GlnG with the heterologous *nifLA* promoter region, suggesting a regulatory coupling of these two different evolutionary systems through a direct, activating interaction.

Discussion

The ability to fix nitrogen is found exclusively among bacteria. Although there is considerable biodiversity among diazotrophic microorganisms, nitrogen fixation is not found in eukaryotes. Eventually, a successfully engineered N_2-fixing crop may significantly reduce the need for chemical fertilizers, resulting in a cleaner environment and higher yield [7, 8, 22]. Currently, understanding the physiological changes that take place in an engineered cell after the insertion of a full set of large fragments of foreign DNA, especially from a distant species, may provide the knowledge necessary to pave the way towards the goal of nitrogen fixation engineering.

Biological nitrogen fixation is an energy-dependent and oxygen-sensitive process that requires ATP and a supply of Fe-S clusters, not only for the nitrogenase component but also for the many other proteins involved in electron transfer, including redox and non-redox catalysis and the sensing of regulatory processes [10]. Genetically, one may succeed in transferring 1–2 functional *nif* genes into foreign organisms, including eukaryotes; it remains a daunting task to insert an entire nitrogen fixation system, because, for it to be functional in a typical "alien" environment, many biochemical and physiological changes may be required.

An important corollary to the acquisition of new genes is the problem of how to best integrate them into the recipient's existing gene regulatory circuits so that fitness is not initially compromised and can be enhanced in the future through optimal expression of the new genes. A recent report found that the transfer to *E. coli* of a nine-*nif*-gene operon derived from *Paenibacillus* sp. WLY78 and transcribed from the σ^{70} promoter resulted in the successful expression of active nitrogenase, but no transcriptional regulation by either oxygen or fixed nitrogen was detectable in the recombinant *E. coli* [21]. The *P. stutzeri* A1501 NFI represents the largest nitrogen fixation assembly characterized in any diazotrophic species to date [1, 15, 21, 26]. In contrast to *Paenibacillus* sp. WLY78, a total of 11 operons in the *P. stutzeri* A1501 NFI were activated in a NifA-σ^{54}-dependent manner under nitrogen-fixing conditions [25]. Expression of the island is stringently regulated in response to oxygen and fixed nitrogen [9]. In this work, a series of recombinant nitrogen-fixing *E. coli* strains were obtained, and the regulatory mechanisms were investigated. It was observed that nitrogenase activity in the recombinant *E. coli* strain was dependent on external ammonia concentration, oxygen tension, and temperature, indicating that the NFI was also highly regulated in the new host, in a manner similar to its regulation in the parent strain. We provided further evidence of a regulatory coupling between the *E. coli* general nitrogen regulatory system and the heterologous *P. stutzeri nif* island in the recombinant *E. coli* strain.

This work will shed light on the structure and functional integrity of the *nif* island and increase understanding of how the NFI adapts to new cell circumstances in the natural environment. It will also be useful for the construction of novel and more robust nitrogen-fixing organisms through biosynthetic engineering.

Acknowledgments

We would like to thank Prof. Ray Dixon and Claudine Elmerich for many helpful discussions, and Dr. Qi Cheng and Ahmed Gomma for critical reading of the manuscript. This work was supported by grants from the National Basic Research Program of China (No. 2015CB755700), the National Nature Science Foundation of China (No. 31230004, 31470174 and 31400076), the National High Technology Research and Development (863) Program of China (No. 2012AA02A703), and the Guangdong Innovative and Entrepreneurial Research Team Program (No. 2013S033).

References

1. Arnold W, Rump A, Klipp W, Priefer UB, Puhler A. 1988. Nucleotide sequence of a 24,206-base-pair DNA fragment carrying the entire nitrogen fixation gene cluster of *Klebsiella pneumoniae*. *J. Mol. Biol.* **203:** 715-738.
2. Baba T, Ara T, Hasegawa M, Takai Y, Okumura Y, Baba M, *et al.* 2006. Construction of *Escherichia coli* K-12 in-frame, single-gene knockout mutants: the Keio collection. *Mol. Syst. Biol.* **2:** 2006. 0008.
3. Brown DR, Barton G, Pan Z, Buck M, Wigneshweraraj S. 2014. Nitrogen stress response and stringent response are coupled in *Escherichia coli*. *Nat. Commun.* **5:** 4115.
4. Camacho EM, Casadesus J. 2005. Regulation of *traJ* transcription in the *Salmonella* virulence plasmid by strand-specific DNA adenine hemimethylation. *Mol. Microbiol.* **57:** 1700-1718.
5. Cannon FC, Dixon RA, Postgate JR. 1976. Derivation and

properties of F-prime factors in *Escherichia coli* carrying nitrogen fixation genes from *Klebsiella pneumoniae*. *J. Gen. Microbiol.* **93:** 111-125.
6. Cannon FC, Dixon RA, Postgate JR, Primrose SB. 1974. Chromosomal integration of *Klebsiella* nitrogen fixation genes in *Escherichia coli*. *J. Gen. Microbiol.* **80:** 227-239.
7. Cheng Q. 2008. Perspectives in biological nitrogen fixation research. *J. Integr. Plant Biol.* **50:** 786-798.
8. Cheng Q, Yang J, Day A, Dowson-Day M, Dixon R. 2005. Evolutionary implication of nitrogenase-like proteins in the plant kingdom and prospects for *nif* gene transfer in model eukaryotes, pp. 387-389. *In* Wang YP, Lin M, Tian ZX, Elmerich C, Newton WE (eds.). *Biological Nitrogen Fixation, Sustainable Agriculture and the Environment*. Springer, Netherlands.
9. Desnoues N, Lin M, Guo X, Ma L, Carreno-Lopez R, Elmerich C. 2003. Nitrogen fixation genetics and regulation in a *Pseudomonas stutzeri* strain associated with rice. *Microbiology* **149:** 2251-2262.
10. Dixon R, Kahn D. 2004. Genetic regulation of biological nitrogen fixation. *Nat. Rev. Microbiol.* **2:** 621-631.
11. Dixon RA, Postgate JR. 1972. Genetic transfer of nitrogen fixation from *Klebsiella pneumoniae* to *Escherichia coli*. *Nature* **237:** 102-103.
12. Gavini N, Tungtur S, Pulakat L. 2006. Peptidyl-prolyl *cis/trans* isomerase-independent functional NifH mutant of *Azotobacter vinelandii*. *J. Bacteriol.* **188:** 6020-6025.
13. He S, Chen M, Xie Z, Yan Y, Li H, Fan Y, *et al.* 2008. Involvement of GlnK, a PII protein, in control of nitrogen fixation and ammonia assimilation in *Pseudomonas stutzeri* A1501. *Arch. Microbiol.* **190:** 1-10.
14. Kneip C, Lockhart P, Voss C, Maier UG. 2007. Nitrogen fixation in eukaryotes – new models for symbiosis. *BMC Evol. Biol.* **7:** 55.
15. Krause A, Ramakumar A, Bartels D, Battistoni F, Bekel T, Boch J, *et al.* 2006. Complete genome of the mutualistic, N_2-fixing grass endophyte *Azoarcus* sp. strain BH72. *Nat. Biotechnol.* **24:** 1385-1391.
16. Osoegawa K, Zhu B, Shu CL, Ren T, Cao Q, Vessere GM, *et al.* 2004. BAC resources for the rat genome project. *Genome Res.* **14:** 780-785.
17. Schumacher J, Behrends V, Pan Z, Brown DR, Heydenreich F, Lewis MR, *et al.* 2013. Nitrogen and carbon status are integrated at the transcriptional level by the nitrogen regulator NtrC *in vivo*. *mBio* **4:** e00881-e00813.
18. Setten L, Soto G, Mozzicafreddo M, Fox AR, Lisi C, Cuccioloni M, *et al.* 2013. Engineering *Pseudomonas protegens* Pf-5 for nitrogen fixation and its application to improve plant growth under nitrogen-deficient conditions. *PLoS One* **8:** e63666.
19. Thomason LC, Costantino N, Court DL. 2007. *E. coli* genome manipulation by P1 transduction. *Curr. Protoc. Mol. Biol.* **Chapter 1:** Unit 1. 17.
20. Venieraki A, Dimou M, Vezyri E, Vamvakas A, Katinaki PA, Chatzipavlidis I, *et al.* 2014. The nitrogen-fixation island insertion site is conserved in diazotrophic *Pseudomonas stutzeri* and *Pseudomonas* sp. isolated from distal and close geographical regions. *PLoS One* **9:** e105837.
21. Wang L, Zhang L, Liu Z, Zhao D, Liu X, Zhang B, *et al.* 2013. A minimal nitrogen fixation gene cluster from *Paenibacillus* sp. WLY78 enables expression of active nitrogenase in *Escherichia coli*. *PLoS Genet.* **9:** e1003865.
22. Wang X, Yang JG, Chen L, Wang JL, Cheng Q, Dixon R, Wang YP. 2013. Using synthetic biology to distinguish and overcome regulatory and functional barriers related to nitrogen fixation. *PLoS One* **8:** e68677.
23. Windgassen M, Urban A, Jaeger KE. 2000. Rapid gene inactivation in *Pseudomonas aeruginosa*. *FEMS Microbiol. Lett.* **193:** 201-205.
24. Xie YG, Han FF, Luan C, Zhang HW, Feng J, Choi YJ, *et al.* 2013. High-yield soluble expression and simple purification of the antimicrobial peptide OG2 using the intein system in *Escherichia coli*. *Biomed. Res. Int.* **2013:** 754319.
25. Yan Y, Ping S, Peng J, Han Y, Li L, Yang J, *et al.* 2010. Global transcriptional analysis of nitrogen fixation and ammonium repression in root-associated *Pseudomonas stutzeri* A1501. *BMC Genomics* **11:** 11.
26. Yan Y, Yang J, Dou Y, Chen M, Ping S, Peng J, *et al.* 2008. Nitrogen fixation island and rhizosphere competence traits in the genome of root-associated *Pseudomonas stutzeri* A1501. *Proc. Natl. Acad. Sci. USA* **105:** 7564-7569.
27. Yang J, Xie X, Wang X, Dixon R, Wang YP. 2014. Reconstruction and minimal gene requirements for the alternative iron-only nitrogenase in *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* **111:** E3718-E3725.
28. Ying BW, Fourmy D, Yoshizawa S. 2007. Substitution of the use of radioactivity by fluorescence for biochemical studies of RNA. *RNA* **13:** 2042-2050.