Synthesis of nitrogenase by *Paenibacillus sabinae* T27 in presence of high levels of ammonia during anaerobic fermentation

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Abstract

Biological nitrogen fixation is usually inhibited by fixed nitrogen. *Paenibacillus sabinae* T27, a Gram-positive, spore-forming diazotroph, possesses high nitrogenase activity and has 3 copies of *nifH* (*nifH*, *nifH2*, *nifH3*), a copy of *nifDK*, and multiple *nifHDK*-like genes. In this study, we found that *P. sabinae* T27 showed nitrogenase activities not only in low (0–3 mM) concentrations of *NH₄⁺* but also in high (30–300 mM) concentrations of *NH₄⁺*, no matter whether this bacterium was grown in a flask or in a fermenter on scale cultivation. qRT-PCR and western blotting analyses supported that Fe protein and MoFe protein were synthesized under both low (0–3 mM) and high (30–300 mM) concentrations of *NH₄⁺*. Liquid chromatography-mass spectrometry (LC-MS) analysis revealed that MoFe protein was encoded by *nifDK* and Fe protein was encoded by both *nifH* and *nifH2*. The cross-reaction suggested the purified Fe and MoFe components from *P. sabinae* T27 grown in both nitrogen-limited and nitrogen-excess conditions were active. This is the first time to report that diazotrophs show nitrogenase activity in presence of high (30–300 mM) concentrations of *NH₄⁺*. Our study will provide a clue for studying the mechanisms of nitrogen fixation in presence of the high concentration of *NH₄⁺*.

Key Points

- *P. sabinae* T27 can synthesize active nitrogenase in presence of high levels of ammonia.
- Fe and MoFe proteins of nitrogenase purified in absence of ammonia are the same as those purified from the high concentration of ammonia.
- Fe protein is encoded by *nifH* and *nifH2*, and MoFe protein is encoded by *nifDK*.

Keywords *Paenibacillus sabinae* · Nitrogen fixation · Nitrogenase · High concentration of ammonium

Introduction

Biological nitrogen fixation, conversion of atmospheric *N₂* into biologically usable *NH₃*, is one of the most important processes in the global nitrogen cycle. Most biological nitrogen fixation is catalyzed by molybdenum-dependent nitrogenase that 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 (Buren et al. 2020; Hoffman et al. 2014; Hu and Ribbe 2011; Sickerman et al. 2017). Nitrogenase proteins are hard to characterize because of their complexity and extreme oxygen sensitivity.

Biological nitrogen fixation catalyzed by nitrogenase is a high energy-intensive process, and thus nitrogenase synthesis and activity are tightly regulated by ammonia (*NH₄⁺*) and *O₂*. The *nif* (nitrogen fixation) genes are repressed by *NH₄⁺*, the immediate product of the nitrogenase reaction, and
nitrogenase proteins are inactivated and destroyed by oxygen (O₂) (Dixon and Kahn 2004; Klugkist and Haaker 1984). Regulation of nitrogen fixation by NH₄⁺ in the Gram-negative diazotrophs (e.g., Klebsiella oxytoca, Azotobacter vinelandii) is well characterized. In these bacteria, transcription of nif genes is activated by NifA, together with the RNA polymerase σ₅⁴. NifA is the master regulator of nitrogen fixation, and its expression and activity are regulated in cascades in response to NH₄⁺ and O₂ (Bush and Dixon 2012; Pozza-Carrion et al. 2014). Besides regulation at the gene expression level, nitrogenase activity is also regulated at post-translational level in some diazotrophs in response to NH₄⁺, which is termed ammonia switch-off (Zumft and Castillo 1978). The nitrogenase activities in Herbaspirillum seropedicae (Fu and Burris 1989), Acetobacter diazotrophicus (Reis and Dobereiner 1998), and Pseudomonas stutzeri (Desnoues et al. 2003) were inhibited to various degrees by NH₄⁺. The mechanisms of ammonia switch-off are clarified in Rhodospirillum rubrum, Azospirillum brasilense, and Rhodobacter capsulatus where nitrogenase is inactivated by DraT (dinitrogenase reductase ADP-ribosyltransferase) that catalyzes ADP ribosylation of the Fe protein of nitrogenase in response to high concentrations of NH₄⁺. The ADP–ribose moiety on the covalent modified Fe protein of nitrogenase is reversed by DraG (dinitrogenase reductase activating glycohydrolase) under limited nitrogen conditions (Huerdo et al. 2012; Mouré et al. 2019; Steenhoudt and Vanderleyden 2000).

Paenibacillus is a large genus of Gram-positive, facultative anaerobic, endospore-forming bacteria, and some diazotrophic Paenibacillus species and strains have been extensively used as bacterial fertilizers in agriculture (Grady et al. 2016; Khan et al. 2020; Li et al. 2019; Shi et al. 2016a). Paenibacillus polymyxa WLY78 is an extensively studied diazotroph that carries a minimal and compact nif cluster comprising nine genes (nifB, nifH, nifD, nifK, nifE, nifN, nifX, hesA, nifV) encoding Mo-nitrogenase. The nif gene transcription of P. polymyxa WLY78 was strongly regulated by NH₄⁺ and O₂ (Shi et al. 2016b). This bacterium exhibited the highest nitrogenase activity in the absence of NH₄⁺ and no activity in the presence of more than 5 mM NH₄⁺ under anaerobic conditions. Recent studies have revealed that GlnR (a central regulator of nitrogen metabolism) activates nif gene transcription under nitrogen limitation, whereas GlnR, together with glutamine synthetase (GS) encoded by glnA within the glnRA operon, represses nif expression under excess nitrogen (Wang et al. 2018).

The diazotrophic Paenibacillus sabinae T27 (=CCBAU 10202ᵀ=DSM 17841ᵀ) isolated from the rhizosphere of plant Sabina squamata is a novel species identified by our laboratory (Ma et al. 2007). P. sabinae T27 exhibited much high nitrogenase activity and had more copies of nifH/nifD/nifK and nif-like genes (Hong et al. 2012; Li et al. 2014). In this study, we find that P. sabinae T27 showed nitrogenase activity in presence of high concentrations of NH₄⁺. The nitrogen fixation is catalyzed by the same nitrogenases under both nitrogen-limited and nitrogen-excess conditions. Of the three copies of nifH (nifH₁, nifH₂, and nifH₃), nifH₁ and nifH₂ are involved in the synthesis of Fe protein of nitrogenase, whereas MoFe protein of nitrogenase was encoded by nifDK.

**Materials and methods**

**Strains and media**

*Paenibacillus sabinae* T27 used here was isolated from the rhizosphere of plant Sabina squamata by our laboratory (Ma et al. 2007). This strain was routinely grown in LD medium (per liter contains 2.5 g NaCl, 5 g yeast, and 10 g tryptone) at 30°C for 14 h with shaking at 200 rpm (Wang et al. 2013). For assays of nitrogenase activity, *P. sabinae* T27 was grown in nitrogen-limited medium supplemented with 0–400 mM NH₄Cl under anaerobic condition. Nitrogen-limited medium contained (per liter) 10.4 g Na₂HPO₄, 3.4 g KH₂PO₄, 26 mg CaCl₂·2H₂O, 30 mg MgSO₄, 0.3 mg MnSO₄, 36 mg ferrie citrate, 7.6 mg Na₂MoO₄·2H₂O, 10 mg p-aminobenzoic acid, 5 μg biotin, 2 mM glutamate, and 4 g glucose as carbon source.

**Measurement of nitrogenase activity**

Acetylene reduction assays were performed to measure nitrogenase activity as described previously by Wang et al. (2013). For doing this, *P. sabinae* T27 was initially grown in 5 mL liquid LD medium overnight and then was transferred to a 250-mL shaking flask containing 100 mL nitrogen-limited medium (supplemented 10 mM NH₄Cl as nitrogen source) and grown for 14 h at 30°C with shaking at 200 rpm. The cultures were collected by centrifugation, washed three times with sterilized water, and then resuspended in nitrogen-limited medium containing different concentrations of NH₄Cl and no nitrogen-limited medium containing 0–400 mM NH₄Cl under anaerobic condition to a final OD₆₀₀ of 0.4. Then, 4 mL of the culture was transferred to a 25-mL test tube and the test tube was sealed with a rubber stopper. The headspace in the tube was then evacuated and replaced with argon gas. After incubating the cultures for 4 h at 30°C, 2.1 mL C₂H₂ (10% of the headspace volume) was injected into the test tubes. After incubating the cultures for a further 30 min, 100 μL of gas was withdrawn and injected into a TP-2060 gas chromatograph to quantify C₂H₂ production. The nitrogenase activity was expressed as nmol C₂H₂/mg protein/h. The experiments were repeated three or more times.

For measuring the effect of glucose on nitrogenase activity, nitrogen-limited medium containing different concentrations (0, 10, and 100 mM) of NH₄Cl was supplemented with 2, 4, 8,
16, 20, 25, and 30 g/L, respectively, and the cultures were also grown under anaerobic conditions.

**Fermentation and collection of the bacterial cells**

*P. sabinae* T27 were cultured in a 7.5-L fermenter (Baoxing Bio-engineering Equipment Co., Shanghai, China) with a working volume of 5 L after 10% (vol/vol) inoculation. A single bacterial colony on LD plate was cultured in a 50-mL flask filled with 20 mL liquid LD for 14 h at 30 °C with shaking at 200 rpm. Then, the 20-mL liquid culture was transferred into a 1000-mL flask filled with 500 mL nitrogen-limited medium, supplemented with 10 mM NH₄Cl as nitrogen source and 4 g/L glucose as carbon source and cultured under the same conditions for 14 h. Finally, the 500-mL culture was transferred into a 7.5-L fermenter filled with 4.5 L nitrogen-limited medium containing 20 g/L glucose and 0 mM, 10 mM, and 100 mM NH₄Cl as the initial nitrogen source, respectively. During fermentation process, temperature was maintained at 30°C, pH was controlled at 6.8 by addition of 5 M KOH, and cultures were continuously stirred at 200 rpm and bubbled with N₂ gas at a flux of 0.8 mL/min. Culture samples were taken to determine OD₆₀₀ and nitrogenase activity, concentrations of glucose, and NH₄⁺ at 2-h intervals. Cells were harvested under N₂ flow by centrifugation at 3000×g for 15 min when maximal activity was attained. The cell paste was frozen immediately and stored in liquid nitrogen. All the experiments were repeated five or more times.

**Cell density, nitrogenase activity, and glucose and NH₄⁺ concentrations in the fermentation broth**

Cell density of *P. sabinae* T27 cells was estimated from OD₆₀₀ value. Nitrogenase activity was assayed by the method of Guo et al. (2014). In total, 1 mL of fermenter culture was taken and transferred to 10-mL sealed vials for 30 min in a 10% C₂H₂. In total, 100 μL of gas was analyzed by gas chromatography. For measuring glucose and NH₄⁺ concentrations, 1 mL of culture broth was taken from the fermenter in three duplicates and centrifuged for 10 min at 12,000 rpm to obtain cell-free supernatant. Afterwards, 500 μL of the supernatants was stored at −20 °C for further use. Glucose concentration was measured by using the 3,5-dinitrosalicylic acid (DNS) (Gusakov et al. 2011). NH₄⁺ concentration was measured by using the indophenol method (Chaney and Marbach 1962).

**RNA preparation and qRT-PCR analysis**

Total RNA was prepared from fermentation samples at the rapid increase phase of nitrogenase activity using RNAiso Plus (Takara, Japan) according to the manufacturer’s protocol. Remove of genome DNA and synthesis of cDNA were performed using PrimeScript™ RT reagent Kit with gDNA Eraser (Takara, Japan). qRT-PCR was performed on Applied Biosystems 7500 Real-Time System (Life Technologies) and detected by the SYBR Green detection system with the following program: 95 °C for 15 min, 1 cycle; 95 °C for 10 s and 60 °C for 30 s, 40 cycles. Primers used for qRT-PCR are listed in Table S1. The relative expression level was calculated using the 2−ΔΔCt method (Livak and Schmittgen 2001). 16S rRNA was set as internal control and the expression levels of genes under 10 mM NH₄⁺ conditions were arbitrarily set to 1.0. Each experiment was performed in triplicate.

**Western blot assays for NifH, NifD, and NifK expression**

The cell pellets collected from 1-mL fermentation culture were dissolved in 100 μL of sodium dodecyl sulfate (SDS) gel-loading buffer and boiled for 5 min, and then, 20 μL was loaded onto the stacking gel. Proteins were separated by SDS-PAGE and transferred to a polyvinylidene fluoride (PVDF) membrane, and the Fe and MoFe proteins were detected using monoclonal anti-NifH, anti-NifD, and anti-NifK, respectively. Binding of antibodies was visualized using chemiluminescence detected by ECL.

**Purification of Fe protein and MoFe protein of nitrogenase**

Nitrogenase purification from *P. sabinae* T27 was anaerobically performed as described previously (Emerich and Burris 1978; Guo et al. 2014) with modifications. Purification of Fe protein and MoFe protein of nitrogenase was anaerobically performed. All buffers used were deoxygenated on a gassing manifold by repeated evacuation and flushing with high-purity gases by passage through a heated copper catalyst. Solutions finally were sparged with prepurified N₂ before the addition of dithionite. Approximately 80 g of frozen cell paste was thawed in 200 mL of 50 mM Tris-HCl buffer (pH 8.0) containing 2 mM dithionite. The cells were autolyzed at room temperature for 30 min with lysozyme (200 mg), and then they were sonicated for 30 min in ice bath under continuous stirring and steadily blowing N₂. The broken cell preparation was enzymolysised by DNase (5 mg)-RNase (10 mg) at 30°C for 30 min, and then enzyme lysis was terminated at 55°C for 5 min and the enzymolysised preparation was cooled to room temperature. Finally, the enzymolysised preparation was centrifuged at 10,000×g for 1 h to remove particulates. The dark brown supernatant (crude extract) was introduced onto a fully reduced DEAE-52 cellulose column (3.5 by 20 cm) preequilibrated anaerobically at room temperature with 25 mM Tris-HCl buffer (pH 7.4). The MoFe protein fraction was eluted as a dark brown band with column buffer.
containing 230 mM NaCl, and the Fe protein fraction was eluted as a yellow band with the same buffer containing 90 mM MgCl₂. The flow rate was approximately 150 mL/h.

For further purification, the MoFe protein fraction and Fe fraction were diluted with two volumes of column buffer to lower the NaCl concentration, respectively. Each fraction was then applied to a second DEAE-52 cellulose column (1.5 by 10 cm) which was preequilibrated with column buffer. The MoFe protein fraction was eluted with 230 mM NaCl in 25 mM Tris-HCl (pH 7.4) buffer and the Fe protein fraction was eluted with 90 mM MgCl₂ in 25 mM Tris-HCl (pH 7.4). The MoFe protein or Fe protein was finally purified by using preparative polyacrylamide gel electrophoresis (PAGE) which consists of 7% separating gel (4 cm), 4% stacking gel (1 cm), and 13% lower gel (3 cm). For doing preparative (PAGE), each protein faction collected from a second DEAE-52 cellulose column was diluted with 40% (wt/vol) sucrose added and then used as loading samples. The electrophoresis unit consisted of a water-jacketed column (13 cm long) and electrophoresis equipment. The lower electrode reservoir was prepared aerobically and contained 18.5 mM Tris adjusting with glycine to pH 8.5. The upper electrode reservoir was anaerobic and contained 10 mM Tris adjusting with glycine to pH 8.3. The gel was prerun with 300 mM Tris-HCl buffer (pH 8.9) for 2.5 h at 60 V. About 100 mg (10 mL) of concentrated MoFe fraction was applied to the stacking gel, and electrophoresis was continued at the same voltage for 10 h. The voltage then was raised to 200 V. The MoFe protein, visible as a dark brown band, was eluted with 50 mM Tris-HCl (pH 8.0) and then adsorbed on line to a DEAE-52 cellulose column (1.5 by 10 cm). Finally, pure MoFe protein was eluted from the DEAE-52 cellulose column with 230 mM NaCl in column buffer. The Fe protein appeared as a yellow band and was eluted much earlier than the MoFe protein. All purified proteins were frozen and stored in liquid nitrogen.

The adequacy of the anaerobic technique applied during purification was checked routinely by injecting a small quantity of buffer into a solution of methyl viologen.

**In vitro nitrogenase activity assay**

The nitrogenase activity in the mixtures of the purified Fe protein and MoFe protein in vitro was determined by acetylene reduction with sodium dithionite as a reductant as described by Guo et al. (2014). The reaction mixture (1 mL) contained 40 mM of creatine phosphate (CP), 0.125 mg (>150 U/mg) of creatine phosphokinase (CPK), 10 mM of MgCl₂, 40 mM of MOPS-KOH buffer (pH 7.4), 5 mmol of ATP, and 3.5 mg/mL of Na₂S₂O₄. Reactions were conducted in 10-mL vials with crude extract or purified enzyme for 30 min at 30°C in a 90% Ar/10% C₂H₂ atmosphere. A 100 μL of gas sample was withdrawn and injected into a TP-2060 gas chromatograph to quantify ethylene (C₂H₄) production.

The nitrogenase activity was expressed in nmol C₂H₄·mg⁻¹·min⁻¹. To determine the specific activities of MoFe or Fe proteins, we performed assays with saturating amounts of the complementary nitrogenase protein. The purified Fe protein and MoFe protein of *Azotobacter vinelandii* and *Klebsiella oxytoca* were kindly provided by Dr. Wei Jiang, China Agricultural University.

**SDS-PAGE and liquid chromatography-mass spectrometry (LC-MS) analysis**

The purified MoFe and Fe protein samples were dissolved in sodium dodecyl sulfate (SDS) gel-loading buffer, boiled for 5 min, and then 5 μL was loaded onto the stacking gel, respectively. After electrophoresis, each protein in the separating gel was visualized by staining with Coomassie Brilliant Blue G. Selected gel bands containing visible stained protein were destained and digested. Then, LC-MS qualitative analysis was performed. The resulting data were searched for protein candidates with a database search using MASCOT software.

**Results**

***Paenibacillus sabinae*** T27 performs nitrogen fixation in presence of high concentrations of ammonium

Nitrogenase synthesis is normally repressed in the presence of ammonium. To determinate the effect of ammonium on nitrogenase synthesis of *P. sabinae* T27, the nitrogenase activity was measured by acetylene reduction when *P. sabinae* T27 were grown in test tubes filled with 4 mL medium containing 4 g/L glucose as carbon source and different ammonium concentrations of 0–400 mM) in absence of oxygen. We found that *P. sabinae* T27 exhibited high nitrogenase activities at both low concentrations of ammonium (0–3 mM) and high concentrations of ammonium (30–300 mM) when the cells were in the growth phase, while very little activities were observed in the presence of 4–20 mM ammonium (Fig. 1). Our result that *P. sabinae* T27 displayed the nitrogenase activity in presence of high concentrations of NH₄Cl is very different from previous studies on other diazotrophs. The nitrogenase activity of *P. polymyxa* WLY78 was totally inhibited by 2 mM NH₄Cl (Wang et al. 2013; Wang et al. 2018). Also, nitrogenase activities of *Azospirillum lipoferum* and *Azospirillum brasilense* were totally inhibited by 1 mM NH₄Cl (Hartmann et al. 1986).

**Dynamic analysis of nitrogenase activities of *P. sabinae* T27 in presence of the different concentrations of NH₄⁺ during fermentation process**

To investigate whether *P. sabinae* T27 on scale cultivation exhibited high nitrogenase activities at both low (0–3 mM)
and high (30–300 mM) concentrations of NH4+ and little activities in the presence of 4–20 mM NH4+, the bacterial cells were anaerobically cultivated in a 7.5-L fermenter with a working volume of 5 L after 10% (vol/vol) inoculation and initial NH4Cl concentration of approximately 0 mM, 10 mM, and 100 mM, respectively. Generally, the growth rate of the bacterial cells is much higher in the fermenter than in test tube, since the temperature and pH of culture in the fermenter is well adjusted. We deduced that 4 g/L glucose as solo carbon source used in test tube culture was not sufficient to support growth and N2 fixation in a 7.5-L fermenter. Thus, the optimal source used in test tube culture was not sufficient to support well adjusted. We deduced that 4 g/L glucose as solo carbon source, since the temperature and pH of culture in the fermenter is much higher in the fermenter than in test tube, and 100 mM, respectively. Generally, the growth rate of the bacterial cells was used as solo carbon source, the high growth rate of the bacterial cells is much higher in the fermenter than in test tube, and the concentration of NH4+ in medium was nearly depleted, consistent with the nitrogenase activities observed in the cultivation on flask scale (Fig. 2b).

When P. sabinae T27 cells were grown in the medium containing 100 mM NH4Cl, the nitrogenase activities increased as the bacterial cells grew. Importantly, nitrogenase activity was rapidly detected after 2 h of cultivation, just as it was observed when P. sabinae T27 cells were grown in the medium containing 0 mM NH4Cl. Then, nitrogenase activities were maintained throughout fermentation and the activities reached a peak of 3569 nmol C2H4/mg protein/h at 16 h. Although NH4+ concentration decreased as cultivation time increased, concentration of NH4Cl was constantly higher than 60 mM during the fermentation process (Fig. 2c).

Taken together, fermentation experiments demonstrated that P. sabinae T27 on scale cultivation exhibited a little activity in the presence of 10 mM NH4+, but high nitrogenase activities in the absence of NH4+ and presence of 50–100 mM NH4+. The results were in agreement with the data obtained by cultivating P. sabinae T27 in test tubes containing 0 mM, 10 mM, and 100 mM NH4+, respectively.

**Transcriptional analysis of the nif and nif-like genes of P. sabinae T27 during the fermentation processes**

In order to investigate the effect of NH4+ concentration on the transcription, the transcript levels of the nif and nif-like genes of P. sabinae T27 under fermentation conditions were analyzed by qRT-PCR. The cell samples for qRT-PCR were taken when nitrogenase activity was rapidly increased (Fig. 2). qRT-PCR analysis demonstrated that the transcript levels of the nifBHDKENXorf1hesAnifV genes within the main nif cluster were highly induced under 0 and 100 mM NH4+ conditions in comparison to those under 10 mM NH4+ condition (Fig. 3b), suggesting that nifBHDKENXorf1hesAnifV genes were involved in nitrogen fixation in presence of the high concentration of NH4+. In addition to nifBHDKENXorf1hesAnifV, the nifH2B2 genes were highly expressed under 0 and 100 mM NH4+ conditions in comparison to those under 10 mM NH4+ condition. However, the nifHDK-like and multiple nifHBNE genes were not significantly differently expressed under 0, 10, and 100 mM NH4+ conditions, suggesting that these genes did not function in nitrogen fixation under nitrogen-excess condition.
Nif protein synthesis of *P. sabinae* T27 during the fermentation processes

Western blot analysis with the extracts from fermentation also demonstrated that the Fe protein (NifH) and the MoFe protein (NifD and NifK) of the nitrogenase were detected at 0 and 100 mM NH₄⁺, respectively (Fig. 3c). The data are consistent with nitrogenase activity and transcript levels under 0 and 100 mM NH₄⁺ conditions. The data suggest that nitrogenase was synthesized both in the absence of ammonium and in the presence of high concentrations of ammonium.

The specific activities of the purified nitrogenase components in presence of high concentration of ammonium

The cells of *P. sabinae* T27 after 16–20 h of fermentation at 0 and 100 mM NH₄⁺ were anaerobically collected, respectively. As shown in Table 1, MoFe and Fe proteins were purified from the cell-free extracts by sequential anion exchange chromatography and preparative polyacrylamide gel electrophoresis (PAGE), respectively. The specific activity of the MoFe protein in the extracts, fractions, or purified preparations was normally established by performing the assays in presence of an excess of Fe protein, and vice versa. None of the final preparations of MoFe protein or Fe protein had acetylene-reducing activity alone, but they exhibited activity after complementation with each other. The specific activity assays performed with purified complementary component from *A. vinelandii* also confirmed the observations. When nitrogenase was recombined by adding increasing amounts of Fe or MoFe protein to a fixed amount of MoFe or Fe protein, the specific activity of MoFe or Fe protein was increased but did not rise further with excess complementary protein. The results are shown in Fig. S2. The optimal combination of the Fe and MoFe proteins for maximum nitrogenase activity was at a molar ratio of approximately 1 MoFe...
protein to 13 Fe protein and 1 Fe protein to 2.5 MoFe protein, respectively. The MoFe and Fe protein activities varied among preparations, and maximum activity of MoFe and Fe proteins (805 and 667 nmol C₂H₄·mg⁻¹·min⁻¹, respectively) was obtained under nitrogen limitation and (792 and 635 nmol C₂H₄·mg⁻¹·min⁻¹, respectively) under nitrogen excess (Table 1).

As shown in Fig. 4, the homogeneities of the purified MoFe and Fe proteins after preparative PAGE were at least 90% purity, as assessed by SDS-PAGE (Fig. 4). The MoFe protein component showed enrichment of two bands (corresponding to α and β subunits) with an apparent molecular size near 55 kDa and the Fe protein component showed enrichment of a band with an apparent molecular size of about 35 kDa. The MoFe protein and the Fe protein purified from the bacterial cultures grown at 0 mM NH₄⁺ had the same sizes as those purified from the bacterial cultures grown at 100 mM NH₄⁺.

Furthermore, the bands corresponding to MoFe protein and Fe protein under nitrogen-limited and nitrogen-excess conditions were excised, digested, and analyzed in a qualitative fashion by LC-MS to discern the identity of the bands. The composition of MoFe protein was identified as portions of NifD and NifK, and Fe protein components were recognized as portions of NifH and NifH₂ under both nitrogen-limited and nitrogen-excess conditions (Fig. S3). These data suggest that the nitrogenases of P. sabinae T27 grown under both conditions were similar in composition and that the MoFe encoded by the same nifDK and Fe proteins encoded by the same nifHnifH2 are responsible for nitrogenase activities.

**In vitro nitrogenase activities of the purified nitrogenase components from the intraspecies or interspecies**

In vitro nitrogenase activities in the mixtures of Fe and MoFe protein components from the intraspecies or interspecies were determined. As shown in Table 2, when Fe protein or MoFe protein from 100 mM NH₄⁺ was correspondingly mixed with...
MoFe protein or Fe protein from 0 mM NH₄⁺, the similar activities (690 nmol C₂H₄·mg⁻¹·min⁻¹ and 671 nmol C₂H₄·mg⁻¹·min⁻¹) were found. The data suggest that Fe protein and MoFe protein purified from 100 mM NH₄⁺ had the specific activities as those purified from 0 mM NH₄⁺.

Furthermore, in vitro nitrogenase activities were determined by mixing Fe protein or MoFe protein components of P. sabiniae T27 with the corresponding MoFe protein or Fe protein of Azotobacter vinelandii and Klebsiella oxytoca cultivated under nitrogen-fixing conditions. No matter Fe protein or MoFe protein of P. sabiniae T27 was purified from 0 mM NH₄⁺ or from 100 mM NH₄⁺, they could exhibit nitrogenase activities when mixed with the corresponding MoFe protein or Fe protein from A. vinelandii and K. oxytoca.

### Table 1

| Fraction                  | Nitrogen limitation | Excess nitrogen |
|--------------------------|--------------------|----------------|
|                          | Specific activity (nmol C₂H₄/mg protein/min) | Specific activity (nmol C₂H₄/mg protein/min) |
| -a + P. sabiniae b + A. vinelandii c |                         |                 |
| MoFe protein             |                     |                 |
| Cell-free extraction     | 27                  | 22             |
| First DEAE-52            | 56                  | 50             |
| Second DEAE-52           | 18                  | 14             |
| Preparative PAGE         | 0                   | 0              |
| Fe protein               |                     |                 |
| Cell-free extraction     | 27                  | 22             |
| First DEAE-52            | 40                  | 36             |
| Second DEAE-52           | 14                  | 15             |
| Preparative PAGE         | 0                   | 0              |

*a Specific activity of a component was obtained in absence of the complementary component

*b Specific activity of a component was obtained in presence of saturating amounts of the complementary component from P. sabiniae T27

*c Specific activity was complemented with the purified Fe protein or MoFe protein of Azotobacter vinelandii

### Discussion

Biological nitrogen fixation is a high-energy-intensive process, and thus nitrogenase synthesis and activity are tightly regulated in response to ammonium concentration. Almost all nitrogen-fixing organisms carry out nitrogen fixation at low levels of 0–2 mM NH₄⁺ (Desnoues et al. 2003; Hartmann et al. 1986; Inomura et al. 2018; Klugkist and Haaker 1984; Steenhoudt and Vanderleyden 2000). In this study, we reveal that P. sabiniae T27 had nitrogenase activity not only in low (0–3 mM) concentrations of NH₄⁺ but also in high (30–300 mM) concentrations of NH₄⁺. The transcription levels of the nifBHDKENXorf1hesAnifV within the complete nif gene cluster of P. sabiniae T27 were also significantly upregulated in the presence of high ammonium concentration. This is the first time to report that nitrogen fixation was performed in presence of high concentration of ammonium. This property has extended the currently known knowledge of nitrogen fixation.

Initially, we observed reasonably high nitrogenase activity when P. sabiniae T27 was grown in 25-mL test tubes filled with 4 mL nitrogen-limited medium containing 4% glucose as carbon source under low (0–3 mM) concentrations of NH₄⁺ or high (30–300 mM) concentrations of NH₄⁺. However, only a little nitrogenase activity was not observed when P. sabiniae T27 was grown in the same medium containing 4–20 mM NH₄⁺. To confirm that the novel phenomenon of nitrogen fixation in presence of the high concentration of ammonium was still present in scale cultivation, the bacterial cells were anaerobically cultivated in a 7.5-L fermenter filled with 5 L nitrogen-limited medium containing approximately 0 mM, 10

![Fig. 4 SDS-PAGE analysis of purified Fe and MoFe proteins under nitrogen-limited and nitrogen-excess conditions. M, molecular weight markers (kDa), from top: 95, 72, 55, 43, and 43](image)
mM, and 100 mM NH₄Cl as the initial nitrogen source, respectively. We found that the 4 g/L glucose used in test tube culture was not sufficient to support cell growth and drive nitrogen fixation for *P. sabinae* T27 on large-scale culture. Thus, we investigated the effects of different concentrations of glucose on growth and nitrogenase activity of *P. sabinae* T27 and found that 20 g/L glucose was suitable for high cell density and nitrogenase activity. Thus, the nitrogen-limited medium containing 20 g/L glucose as carbon source and different concentrations of ammonia was used to grow *P. sabinae* T27 on large scale. It was reported that *Glucanacetobacter diazotrophicus* grew and fixed N₂ in medium containing up to 30% sucrose (Fisher and Newton 2005), and the higher sucrose levels even protected nitrogenase against inhibition by ammonium (Reis and Dobereiner 1998). But *G. diazotrophicus* produced a higher amount of nitrogenase in medium containing 10% (w/v) glucose than in the same medium containing sucrose (Owens and Tezcan 2018). Initially, *P. sabinae* T27 was grown in the medium containing 40 g/L sucrose which was usually used to cultivate *K. oxytoca* on large scale for purifying nitrogenase complex (Tumer and Gibson 1980), but *P. sabinae* T27 displayed low nitrogenase activity. Our results combined other reports suggested that different N₂-fixing microorganisms may require different recipes to achieve balance of the cell growth and nitrogenase expression.

|   | *P. sabinae* T27 | *P. sabinae* T27 | *K. oxytoca* | *A. vinelandii* |
|---|-----------------|-----------------|-------------|----------------|
|   | Fe protein (nitrogen excess) | Fe protein (nitrogen limitation) | Fe protein | Fe protein |
| *P. sabinae* T27 MoFe protein (excess nitrogen) | 720±33 | 671±40 | 493±35 | 437±46 |
| *P. sabinae* T27 MoFe protein (nitrogen limitation) | 690±37 | 758±51 | 503±29 | 446±30 |
| *K. oxytoca* MoFe protein | 608±24 | 663±32 | 1809±97 | - |
| *A. vinelandii* MoFe protein | 519±28 | 546±44 | - | 1651±113 |

In vitro nitrogenase assay showed that the purified Fe protein and MoFe protein from *P. sabinae* T27 grown in both conditions were active. Heterologous complementation of the nitrogenase components of *P. sabinae* T27 with components of other species further demonstrated *P. sabinae* T27 could synthesize active nitrogenase in presence of high levels of ammonia and this also revealed a great deal of similarity among the various nitrogenase components. We analyzed the compositions of Fe protein and MoFe protein in amino acids by LC-MS and found that MoFe protein was encoded by *nifHDK* with the exception of *nifB2H2*. The purification protocol will provide help for studying the properties of nitrogenase of *P. sabinae* T27. In vitro nitrogenase assay showed that the purified Fe protein and MoFe protein from *P. sabinae* T27 grown in both conditions were active. Heterologous complementation of the nitrogenase components of *P. sabinae* T27 with components of other species further demonstrated *P. sabinae* T27 could synthesize active nitrogenase in presence of high levels of ammonia and this also revealed a great deal of similarity among the various nitrogenase components. We analyzed the compositions of Fe protein and MoFe protein in amino acids by LC-MS and found that MoFe protein was encoded by *nifHDK* and Fe protein was encoded by *nifH1* and *nifH2*, no matter that *P. sabinae* T27 was grown in nitrogen-limited or nitrogen-excess conditions. qRT-PCR also revealed that *nifDK* and *nifH/nifH2* were significantly transcribed in both conditions, consistent with the amino acid compositions of nitrogenase components observed by LC-MS analysis. Our results were in agreement with the reports that the amino acid residues of both *NifH1* and *NifH2* were present in Fe protein in *Clostridium pasteurianum*, a member of Firmicutes which includes the *Paenibacillus* genus (Kasap and Chen 2005). However, the current qRT-PCR data were a little different from the previous reports that three *nifH* genes (*nifH1*, *nifH2*, *nifH3*) of *P. sabinae* T27 were expressed under nitrogen-limited condition (Li et al. 2014). The study revealed that the *nifHDK*-like and other multiple *nif* genes, with the exception of *nifB2H2*, were also not
significantly differently expressed under nitrogen-excess condition, indicating that these genes were not related to nitrogenase activity of *P. sabinae* T27 under high concentrations of ammonium.

It is well known that biological nitrogen fixation is highly regulated at the transcriptional level by a complicated regulatory network that responds to multiple environmental cues (Dixon and Kahn 2004). In this study, qRT-PCR also confirmed the novel findings. But why nitrogenase of *P. sabinae* T27 is expressed in presence of 30–300 mM NH$_4^+$ but not expressed in presence of 4–20 mM NH$_4^+$? Our previous study had revealed that GlnR activates *nif* gene transcription under nitrogen-limited condition, whereas GlnR, together with glutaminase encoded by *glnA* within the *glnR*-*glnA* operon, represses *nif* gene transcription under nitrogen-excess condition in *P. polymyxa* WLY78 which fixes nitrogen only in nitrogen-limited (0–1 mM NH$_4^+$) condition (Wang et al. 2013). Increasing the copy numbers of GlnR or disrupting *glnA* gene of *P. polymyxa* WLY78 resulted in nitrogen fixation in the presence of excess ammonia (100 mM NH$_4^+$) (Wang et al. 2018). Whether GlnR and glutaminase encoded by *glnA* were involved in regulation of *P. sabinae* T27 under high concentrations of NH$_4^+$ is being investigated by our lab. Recently, we have performed genome-wide transcription profiling analysis of *P. sabinae* T27 grown in medium containing 0, 10, and 100 mM NH$_4^+$ conditions. We found that *ald* gene which encodes alanine dehydrogenase (Ald) is significantly upregulated in presence of 100 mM NH$_4^+$ but not in presence of 10 mM NH$_4^+$ (results unpublished). Alanine dehydrogenases are found in some bacterial species which convert ammonium and pyruvate into alanine under high ammonium condition (Pernil et al. 2010; Wang et al. 2014). It is likely that the 4–20 mM NH$_4^+$ may not be enough to activate *ald* expression in *P. sabinae* T27 and the role of *ald* involved in nitrogen fixation is currently under investigation. The novel mechanism involved by *P. sabinae* T27 might be clarified in the near future.

In summary, we revealed that *P. sabinae* T27 exhibited nitrogen fixation in both low (0–3 mM) and high (30–300 mM) concentrations of NH$_4^+$. As we know, this is the first time to report that active nitrogenase can be synthesized in presence of such high (30–300 mM) levels of NH$_4^+$. The nitrogenases purified from *P. sabinae* T27 grown in both nitrogen-limited and nitrogen-excess conditions had the same molecular sizes and the same amino acid compositions. MoFe protein of nitrogenase was encoded by *nifDK* and Fe protein of nitrogenase was encoded by *nifH* and *nifH2*.

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**Author contribution** Q.L. performed all experiments and drafted the manuscript. X.J.H., P.X.L., H.W.Z., and M.Y.W. assisted in the fermentations. S.F.C. conceived the study, guided its coordination, and wrote the manuscript. All authors read and approved the final manuscript.

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**Data availability** All data generated or analyzed during this study are included in this published article and its supplementary information files.

**Declarations**

**Ethical statement** This article does not contain any studies with human participants or animals performed by the authors.

**Conflict of interest** The authors declare no competing interests.

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