Synthesis of Nitrogenase by Paenibacillus Sabinae T27 in Presence of High Levels of Ammonia During Anaerobic Fermentation

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Research

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Abstract

Background

Biological nitrogen fixation catalyzed by nitrogenase is a high energy-intensive process, and thus nitrogenase synthesis and activity are inhibited by ammonium (NH_4^+). Microorganism fix nitrogen at high ammonium (30-300 mM) concentration has not been reported before.

Results

Paenibacillus sabinae T27, a Gram-positive, spore-forming diazotroph (N_2-fixing microorganism, showed nitrogenase activities not only in low (0-4 mM) concentration of NH_4^+, but also in high (30-300 mM) concentration of NH_4^+, no matter whether the cells of this bacterium were grown in flask or in fermentor on scale cultivation. qRT-PCR and western blotting analysis supported that Fe protein and MoFe protein were synthesized under both low (0-4 mM) and high (30-300 mM) concentration of NH_4^+. Liquid chromatography-mass spectrometry（LC-MS）analysis revealed that MoFe protein purified form cultures grown in nitrogen-limited condition or nitrogen-excess condition 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 -excess conditions were active.

Conclusions

Our results indicate that N_2 fixation occurs in presence of high (30-300 mM) concentration of NH_4^+ in *P. sabinae* T27. Nitrogen fixation under both low and high concentration of NH_4^+ was catalyzed by the same nitrogenases and the Fe protein was encoded by both *nifH* and *nifH2.* Our study will provide a clue for studying the mechanisms on nitrogen fixation in presence of the high concentration of NH_4^+.

Background

Biological nitrogen fixation, conversion of atmospheric N_2 into biologically usable NH_3, is one of the most important process 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 $\alpha_2\beta_2$ 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 [1-4]. 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 (NH_4^+) and O_2. The *nif* (nitrogen

fixation) genes are repressed by NH_4^+, the immediate product of the nitrogenase reaction, and nitrogenase proteins are inactivated and destroyed by oxygen (O_2) [5,6]. Regulation of nitrogen fixation by NH_4^+ 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 σ^{54}. NifA is the master regulator of nitrogen fixation and its expression and activity are regulated in cascades in response to NH_4^+ and O_2 [7,8]. Besides regulation at the gene expression level, nitrogenase activity is also regulated at post-translational level in some diazotrophs in response to NH_4^+, which is termed ammonia switch-off [9]. The nitrogenase activities in *Herbaspirillum seropedicae* [10], *Acetobacter diazotrophicus* [11] and *Pseudomonas stutzeri* [12] were inhibited to various degrees by NH_4^+. 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 concentration of NH_4^+. The ADP–ribose moiety on the covalent modified Fe protein of nitrogenase is reversed by DraG (dinitrogenase reductase activating glycohydrolase) under limited nitrogen conditions [13-15].

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 the bacterial fertilizers in agriculture [16-19]. *Paenibacilus 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 NH_4^+ and O_2 [20]. This bacterium exhibited the highest nitrogenase activity in the absence of NH_4^+ and no activity in the presence of more than 5 mM NH_4^+ 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 *glnRA* operon, represses *nif* expression under excess nitrogen [21].

The diazotrophic *Paenibacillus sabinae* T27 isolated from the rhizosphere of plant *Sabina squamata* is a novel species identified by our laboratory [22]. *P. sabinae* T27 exhibited much high nitrogenase activity and had more copies of *nifH*/*nifD*/*nifK* and *nif*-like genes [23,24]. In this study we find that *P. sabinae* T27 showed nitrogenase activity in presence of high concentration of NH_4^+. The same nitrogenases catalyze nitrogen fixation under both nitrogen-limited and nitrogen-excess conditions. Of the three copies of *nifH* (*nifH, nifH2* and *nifH3*), *nifH* and *nifH2* are involved in synthesis of Fe protein of nitrogenase, whereas MoFe protein of nitrogenase was encoded by *nifDK*.

Results

Paenibacillus sabinae T27 performs nitrogen fixation in presence of high concentration of ammonium

The Diazotrophs generally performed nitrogen fixation in low ammonia environments. To determine effect of ammonium on nitrogenase synthesis, the nitrogenase activity was measured by acetylene reduction when *P. sabinae* T27 were grown in flask containing different ammonium concentrations (0-400 mM) in absence of oxygen. We found that *P. sabinae* T27 exhibited high nitrogenase activities at low concentration of ammonium (0-3 mM) and high concentration of ammonium (30-300 mM), while very little activities were observed in the presence of 4-20 mM ammonium (Fig. 1). In contrast, nitrogenase activity was totally inhibited in *P. polymyxa* WLY78 when 2 mM NH_4Cl was used [21,25]. Also, nitrogenase activity was totally inhibited in *Azospirillum lipoferum* and *Azospirillum brasilense* when 1 mM NH_4Cl was used [26].

Dynamic analysis of nitrogenase activities of P. sabinae T27 in presence of the different concentrations of NH_4^+ 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 NH_4^+ and little activities in the presence of 4-20 mM NH_4^+, the bacterial cells were anaerobically cultivated in 7.5 L fermentor containing 20% (w/v) glucose and 2 mM glutamate supplemented with 0 mM, 10 mM and 100 mM NH_4Cl as the initial nitrogen source, respectively. During each fermentation, the growth rates (OD value) and nitrogenase activities of bacterial cells, and the concentration of NH_4^+ and glucose in medium were measured at 2 h intervals.

As shown in Fig. 2a, when P. sabinae T27 were grown in the medium containing 0 mM NH_4Cl, the cell densities increased and glucose concentration decreased gradually during the fermentation process. Notably, nitrogenase activity was rapidly detected at 2 h and then it increased as bacterial cells grew. Nitrogenase activity reached maximum at 14 h and then decreased.

When P. sabinae T27 cells were grown in the medium containing 10 mM NH_4Cl, the cell densities of P. sabinae T27 were much higher than those cultivated in medium containing 0 mM NH_4Cl. After 8 h of cultivation, the cell concentrations of the P. sabinae T27 increased gradually from OD_{600} 0.5 to 3, meanwhile the concentration of glucose was decreased. Then glucose was added in order to keep growth of bacterial cells at 10 h and 16 h, respectively. Meanwhile, NH_4^+ concentration in the medium decreased as culture titer increased and was almost completely depleted after 12 h of fermentation. However, no nitrogenase activity could be detected until after 12 h of cultivation at that time NH_4^+ in the 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 NH_4Cl, 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 NH_4Cl. Then, P. sabinae T27 maintained nitrogenase activities throughout fermentation and the

activities of reached a peak of 1169 nmol C_2H_4 OD_{600}^{-1} mL^{-1} h^{-1} at 16 h. Although NH_4^+ concentration is also decreased, it was constantly higher than 60 mM during the fermentation process (Figure 2C).

Taken together, fermentation experiments demonstrated that P. sabinae T27 on scale cultivation exhibited a little activity in the presence of 10 mM NH_4^+, but high nitrogenase activities in the absence of NH_4^+ and presence of 50-100 mM NH_4^+. The results were in agreement with the data obtained by cultivating P. sabinae T27 in Erlenmeyer flask containing 0 mM, 10 mM and 100 mM NH_4^+, respectively.

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

In order to investigate the effect of NH_4^+ 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 with the cell samples taken when nitrogenase activity was rapidly increased shown in Fig. 2b, c with arrow. qRT-PCR analysis demonstrated that the transcript levels of the nifBHDKENXorf1hesAnifV genes within the main nif cluster was highly induced under 0 and 100 mM NH_4^+ conditions in comparison to those under 10 mM NH_4^+ condition (Fig. 3), suggesting that nifBHDKENXorf1hesAnifV genes were involved in nitrogen fixation in presence of high NH_4^+. In contrast to nifBHDKENXorf1hesAnifV, nifHDK-like and multiple nifHBNE genes, with the exception of nifH2B2, were not significantly differently expressed under 0, 10 and 100 mM NH_4^+ conditions, suggesting that these genes did not function in nitrogen fixation under nitrogen-excess condition.

Nif protein expression in *P. sabinae* T27 during the fermentation processes

Western blot analysis with the extracts from fermentation also demonstrated that the Fe protein (NifH) and the α subunit (NifD) of the MoFe protein of nitrogenase were detected at 0 and 100 mM NH_4^+, respectively, consistent with nitrogenase activity and transcript levels under 0, 10 and 100 mM NH_4^+ conditions (Fig. 4). The data suggest that nitrogenase was synthesized both in the absence of ammonium and in the presence of high concentration of ammonium.

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

The cells of P. sabinae T27 after 16-20 h of fermentation at 0 and 100 mM NH_4^+ were anaerobically collected, respectively. Then, MoFe and Fe proteins were purified from the cell-free extracts by sequential anion exchange chromatography and preparative polyacrylamide gel electrophoresis (PAGE), respectively. The homogeneities of the purified MoFe and Fe proteins after preparative PAGE were at least 90% purity, as assessed by SDS-PAGE (Fig. 5). The MoFe protein component showed enrichment of two band (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 NH_4^+ had the same sizes as those purified from the bacterial cultures grown at 100 mM NH_4^+ (Fig. 5).

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 limitation and nitrogen excess condition (Additional file 1: Figure S1). 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.

The specific activities of the purified MoFe protein and Fe protein were analyzed by using the acetylene reduction assay after mixing MoFe protein and Fe protein. Nitrogenase activity was observed when the purified MoFe protein and Fe protein were mixed. On the contrary, the purified MoFe protein or Fe proteins along did not show activity. The data suggest that both Fe and MoFe proteins were pure for further assay. Like most of other nitrogenase components, both Fe and MoFe proteins were extremely sensitive to oxygen. The Fe protein levels in the extracts, fractions, or purified preparations were normally established by performing the assays in presence of an excess of MoFe protein, and vice versa. The MoFe and Fe protein activities varied among preparations, and maximum activity of MoFe and Fe proteins (805 and 667 nmol $C_2H_4 \cdot mg^{-1} \cdot min^{-1}$, respectively) was obtained under nitrogen limitation and (792 and 638 nmol $C_2H_4 \cdot mg^{-1} \cdot min^{-1}$, respectively) under nitrogen excess (Table 1).

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 NH_4^+ was correspondingly mixed with MoFe protein or Fe protein from 0 mM NH_4^+, the similar activities (690 nmol $C_2H_4 \cdot mg^{-1} \cdot min^{-1}$ and 671 nmol $C_2H_4 \cdot mg^{-1} \cdot min^{-1}$) were found. The data suggest that Fe protein and MoFe protein purified from 100 mM NH_4^+ had the specific activities as those purified from 0 mM NH_4^+ did.

Furthermore, in vitro nitrogenase activities were determined by mixing Fe protein or MoFe protein components of P. sabinae 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. sabinae T27 was purified from 0 mM NH_4^+ or from 100 mM NH_4^+, they could exhibit nitrogenase activities when mixed with the corresponding MoFe protein or Fe protein from A. vinelandii and K. oxytoca.

Discussion

Biological nitrogen fixation is a high energy-intensive process, and thus nitrogenase synthesis and activity are tightly regulated by ammonia (NH_4^+). Almost all of nitrogen-fixing organisms carry out

nitrogen fixation at low level of 0-2 mM NH_4^+ [5, 12, 15, 26, 27]. In this study, we reveal that *P. sabinae* T27 performed nitrogen fixation not only in low (0-3 mM) concentration of NH_4^+, but also in high (30-300 mM) concentration of NH_4^+. This is the first time to report that nitrogen fixation was carried out under the condition of high concentration of ammonium. This property has extended the currently known knowledge of nitrogen fixation.

Initially, we observed that *P. sabinae* T27 grown in flask containing low (0-3 mM) concentration of NH_4^+ or high (30-300 mM) concentration of NH_4^+ showed high nitrogenase activities, but it showed very low nitrogenase activity in 4-20 mM NH_4^+. Then, we cultivated *P. sabinae* T27 in fermentor and confirmed that the phenomenon of nitrogen fixation in high (30-300 mM) concentration of NH_4^+ was still observed. Given the multiplicity of *nif* and *nif*-like genes in *P. sabinae* T27, the discovery of nitrogen fixation under high ammonium concentration in the *Paenibacillus* raises questions concerning the similarity of nitrogenases under both nitrogen-limited and nitrogen-excess conditions. To answer this question, we purified both Fe protein and MoFe protein of nitrogenase from *P. sabinae* T27 grown in low (0 mM) NH_4^+ and in high (100 mM) NH_4^+, respectively. The purified MoFe proteins under both conditions showed the same size of about 55 kDa and the purified Fe protein with molecular size of about 35 kDa, similar to many other diazotrophs.

In vitro nitrogenase assay showed that the purified Fe protein and MoFe protein from *P. sabinae* T27 grown in both conditions were active. Then we analyzed the compositions of Fe protein and MoFe protein in amino acids by LC-MS and found that MoFe protein was encoded by *nifDK* and Fe protein was encoded by *nifH* 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 *Paenibacillus* genus [28]. However, the current qRT-PCR data were a little different from the previous reports that three *nifH* genes (*nifH*, *nifH2*, *nifH3*) of *P. sabinae* T27 were expressed under nitrogen-limited condition [24]. The study revealed the that *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.

Recently, we have revealed that GlnR activates *nif* gene transcription under nitrogen-limitation, whereas GlnR, together with glutaminase encoded by *glnA* within the *glnRglnA* 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 [25]. Increasing the copy numbers of GlnR or disrupting *glnA* gene of *P. polymyxa* WLY78 resulted to nitrogen fixation in the presence of excess ammonia [21]. The ability to synthesize nitrogenase in the presence of excess ammonia were also found in several different

diazotrophs whose mutants constructed by deletion or disruption of the transcriptional regulation genes (e.g. *glnB*, *glnK*, *nifL*) [29-34]. Whether GlnR and glutaminase encoded by *glnA* were involved regulation of *P. sabinae* T27 under both low and high concentrations of NH_4^+ needs to be investigated in the near future.

Conclusions

P. sabinae T27 exhibited nitrogen fixation in both low (0-3 mM) and high (30-300 mM) concentration 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^+. MoFe protein of nitrogenase was encoded by *nifDK* and Fe protein of nitrogenase was encoded by *nifH* and *nifH2*. The nitrogenases purified from *P. sabinae* T27 grown in both nitrogen-limited and -excess conditions were active and also showed activities in the cross-reactions with the nitrogenase components of *K. oxytoca* and *A. vinelandii.* The nitrogenases obtained in both nitrogen-limited and -excess conditions had the same molecular sizes and the same amino acids compositions.

Material And Methods

Medium and culture conditions

P. sabinae T27 was grown overnight in LD medium (per liter contains: 2.5 g NaCl, 5 g yeast and 10 g tryptone) at 30°C with shaking. Then, the cultures were transferred to a 100 mL amounts in 250 mL bottles or 500 mL in 1 L bottles containing 10 mmol/L NH_4Cl supplemented in nitrogen-limited medium. Nitrogen-limited medium contained (per liter) 10.4 g Na_2HPO_4, 3.4 g KH_2PO_4, 26 mg $CaCl_2{\cdot}2H_2O$, 30 mg $MgSO_4$, 0.3 mg $MnSO_4$, 36 mg Ferric citrate, 7.6 mg $Na_2MoO_4{\cdot}2H_2O$, 10 mg p-aminobenzoic acid, 5 μg biotin, 2 mM glutamate and 4 g glucose as carbon source. Inoculum size was 2% (vol/vol) for flask cultures and 10% (vol/vol) for fermentor cultures.

Fermentation and collection of the bacteria lcells

The initial protocol utilized to efficiently cultivate *P. sabinae* T27 on large scale for expression of nitrogenase component proteins was based on the synthesis of protocols developed for *P. polymyxa* [35] and *K. oxytoca* [36] with modifications. *P. sabinae* T27 were cultured in a 7.5 L fermentor with a working volume of 5 L. During fermentation process, temperature and pH were controlled at 30°C and 6.8, respectively. During the fermentation pH was automatically controlled by addition of KOH. Cultures were continuously stirred and bubbled with N_2 gas. Cell growth was monitored by measuring OD_{600} and nitrogenase activity was assayed by using C_2H_2 reduction method at 2 h intervals. The bacterial cultures were harvested under a nitrogen atmosphere by centrifugation at 3,000 × g for 15 min. The cell pellet was stored in liquid nitrogen for further use.

Nitrogenase assays in vivo

Acetylene reduction assays were performed to measure nitrogenase activity as described previously by Wang [25]. To detect whole cell nitrogenase activity in the fermentation broth, 1 ml of N_2-fixing cultures were transferred to 10-ml vials that were evacuated and flushed with argon. Then, C_2H_2 (10% of the headspace volume) was injected into the vials. After incubating the cultures for a 30 min, 100 μL of gas was withdrawn and injected into a TP-2060 gas chromatograph to quantify ethylene (C_2H_4) production. The nitrogenase activity was expressed in nmol $C_2H_4 \cdot OD_{600}^{-1} \cdot mL^{-1} \cdot h^{-1}$.

Measurement of glucose and NH_4^+ concentrations in the culture broth

For measuring glucose and NH_4^+ concentrations, 1 mL of culture was taken from fermentor 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) [37]. NH_4^+ concentration was measured by using the indophenol method [38].

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 Additional file 2: Table S1. The relative expression level was calculated using $2^{-\Delta\Delta Ct}$ method [39]. 16S rRNA was set as internal control and the expression levels of genes under 10 mM NH_4^+ conditions were arbitrarily set to 1.0. Each experiment was performed in triplicate.

Western blot assays for NifH and NifD 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 protein were detected using polyclonal anti-NifH and anti-NifD respectively. Binding of antibodies was visualized using chemiluminescence detected by ECL.

Purification of Fe protein and MoFe protein of nitrogenase

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 N_2 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 a steadily blowing N_2. The broken cell preparation was enzymolysised by DNase (5 mg)-RNase (10 mg) at 30°Cfor 30 min and was terminated at 55°C for 5 min and then cooled to room temperature and finally was centrifuged at 10,000 x 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 $MgCl_2$. 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 $MgCl_2$ 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 25 mM Tris-HCl (pH 7.4)-5 mM $MgCl_2$ and adsorbed on line to a DEAE-52 cellulose column (1.5 by 10 cm). Finally, pure MoFe protein was eluted from the DEAE-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 reductant as described by Guo et al., 2014. The reaction mixture (1 mL) contained 40 mmol of creatine phosphate (CP), 0.125mg (>150 U/mg) of creatine phosphokinase (CPK), 10 mmol of $MgCl_2$, 40 mmol of MOPS-KOH buffer (pH 7.4), 5 mmol of ATP, and 3.5 mg/mL of $Na_2S_2O_4$. Reactions were conducted in 10 mL vials with crude extract or purified

enzyme for 30 min at 30°C in a 90 % Ar/10 % C_2H_2 atmosphere. A 100 µl of gas sample was withdrawn and injected into a TP-2060 gas chromatograph to quantify ethylene (C_2H_4) production. The nitrogenase activity was expressed in nmol $C_2H_4 \cdot mg^{-1} \cdot min^{-1}$. 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* was kindly provided by Dr. Wei Jiang, China Agricultural University.

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

The purified of 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.

Declarations

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Author Contributions

QL performed all experiments, and drafted the manuscript. XJH, PXL, HWZ and MYW assisted in the fermentations. SFC conceived the study, guided its coordination and wrote the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

All data generated or analysed during this study are included in this published article and are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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Tables

Table 1 Purification of MoFe protein and Fe protein under nitrogen-limited and nitrogen-excess conditions.

Fraction	Specific activity (nmol $C_2H_4 \cdot mg^{-1} \cdot min^{-1}$)	
	nitrogen limitation	excess nitrogen
MoFe protein		
Cell-free extract	27	22
Effluent from 1st DEAE-52	116	106
Effluent from 2nd DEAE-52	278	284
MoFe protein fraction after preparative PAGE	805	792
Fe protein		
Cell-free extract	27	22
Effluent from 1st DEAE-52	80	89
Effluent from 2nd DEAE-52	209	196
Fe protein fraction after preparative PAGE	667	635

Table 2 Nitrogenase activities of the purified nitrogenase components from the intraspecies or interspecies. Expressed as nanomoles of C_2H_4 formed per min per milligram of protein.

	P. sabinae T27 Fe protein (nitrogen excess)	*P. sabinae* T27 Fe protein (nitrogen limitation)	*K. oxytoca* Fe protein	*A. vinelandii* 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

Figures

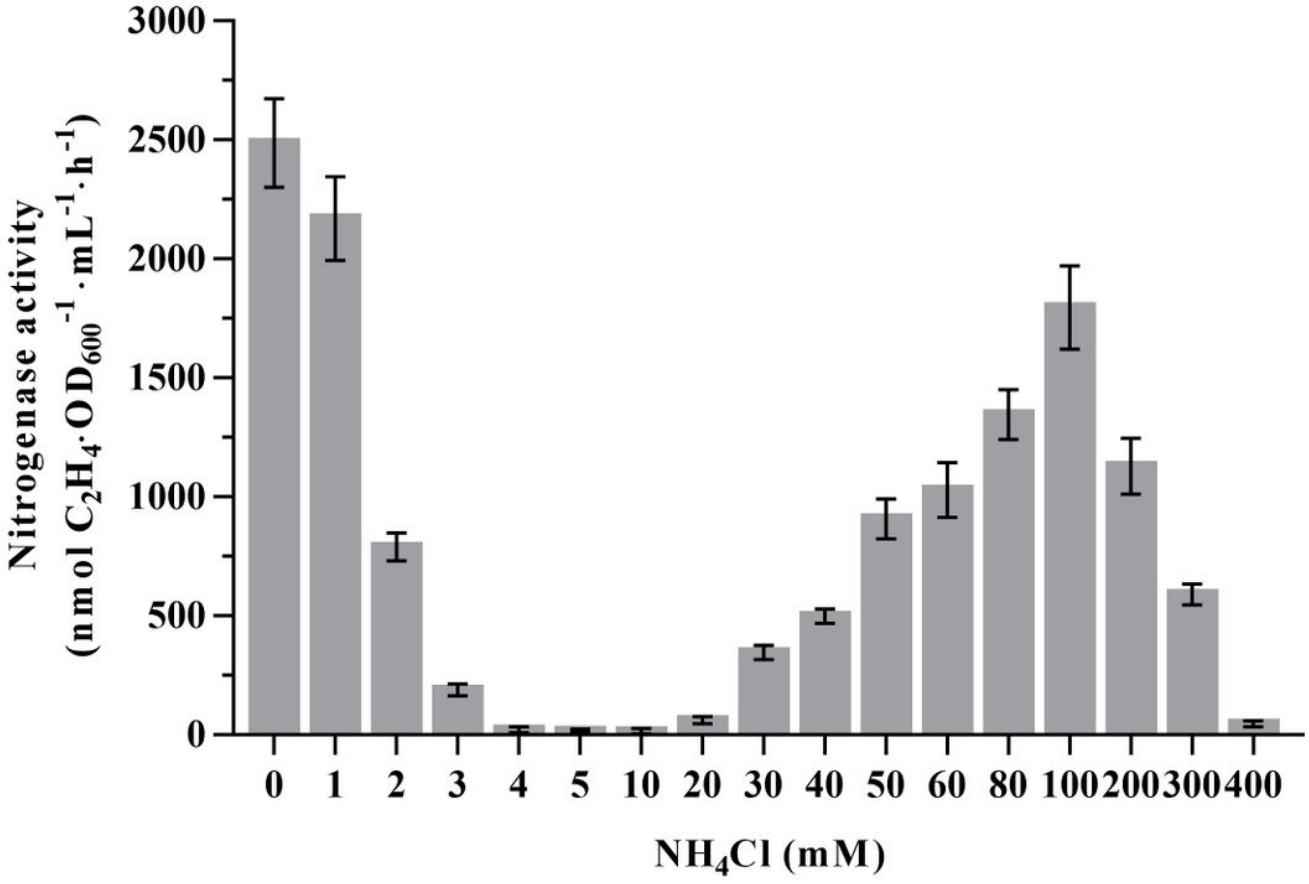

Figure 1

The nitrogenase activity of P. sabinae T27 grown anaerobically in medium containing different concentration of NH4Cl.

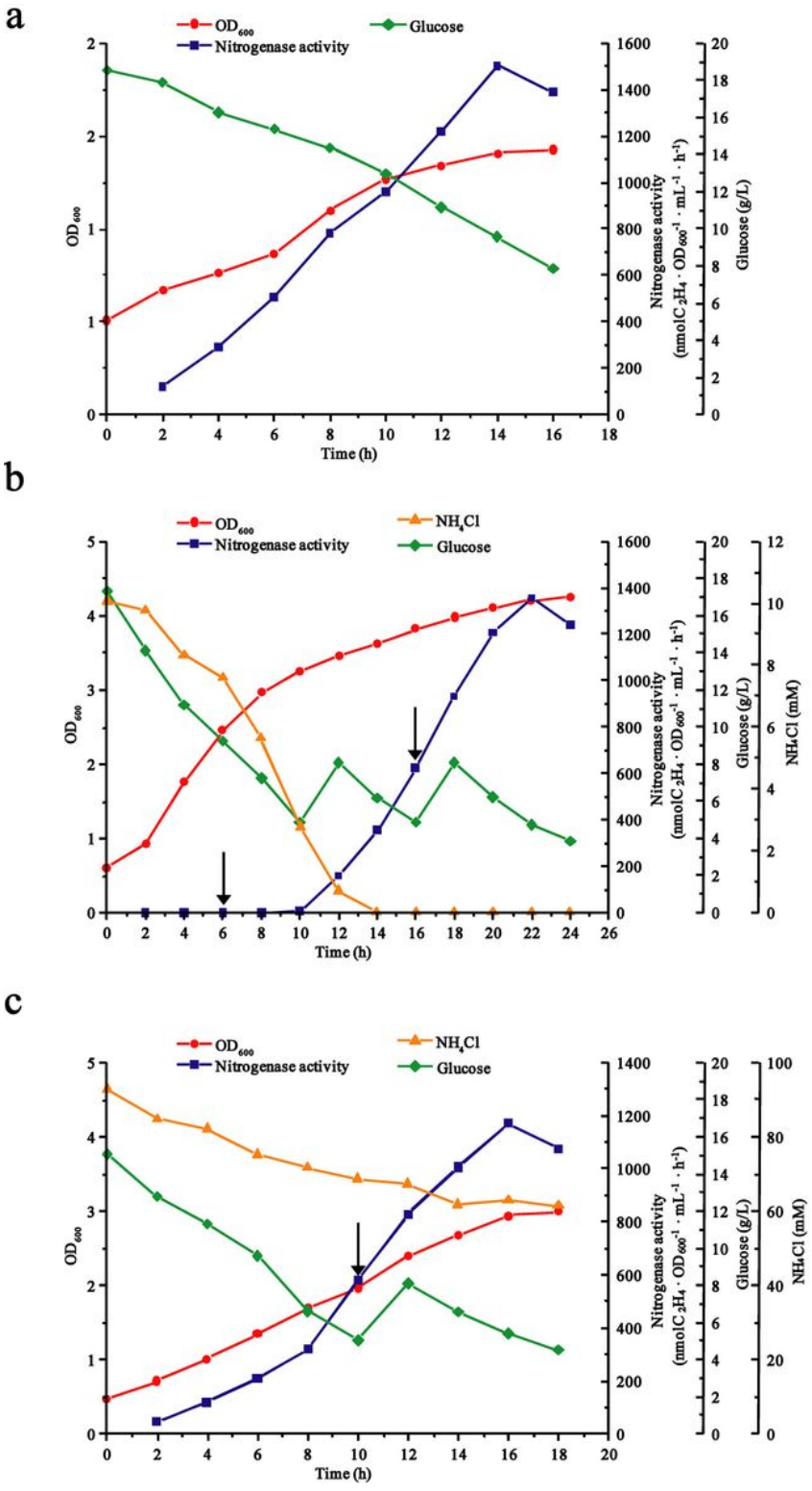

Figure 2

The fermentation of P. sabinae T27 in different concentration of NH4Cl. 0 mM (a), 10 mM (b) and 100 mM (c). Arrow indicates the time points where RNA samples were harvested for qRT-PCR. Glucose was added to the medium when its concentration was lower than 5g/L.

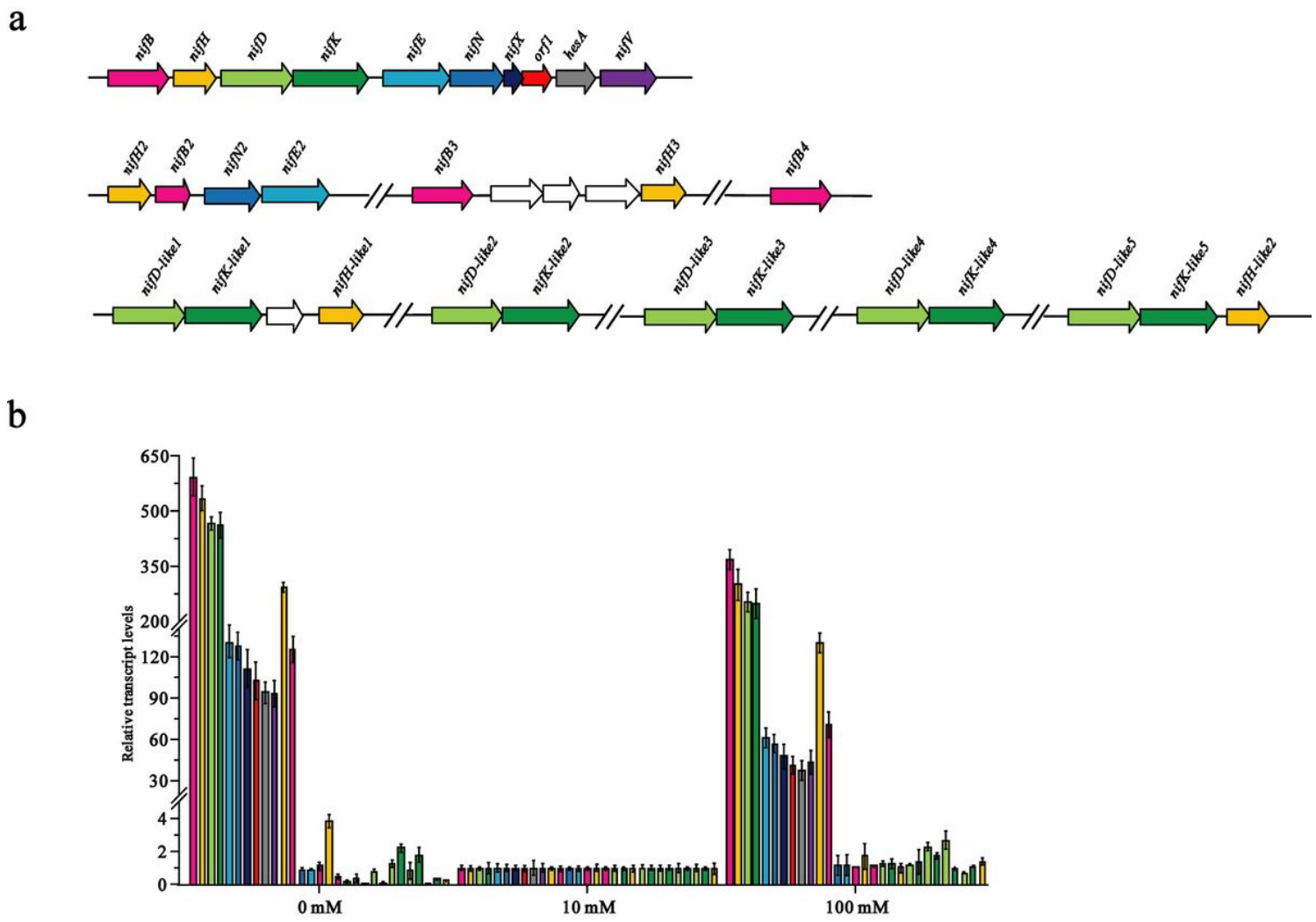

Figure 3

The nif and nifHDK-like transcription of P. sabinae T27 grown anaerobically under 0, 10 and 100 mM NH4+ conditions, respectively. (a) The organization of nif and nifHDK-like genes of P. sabinae T27. (b) qRT-PCR analysis of transcripts of nif and nifHDK-like genes of P. sabinae T27 grown anaerobically under 0, 10 and 100 mM NH4+ conditions, respectively. The relative expression level was calculated using2-ΔΔCt method. The transcription levels of genes under 10 mM NH4+ condition was arbitrarily set to 1.0.

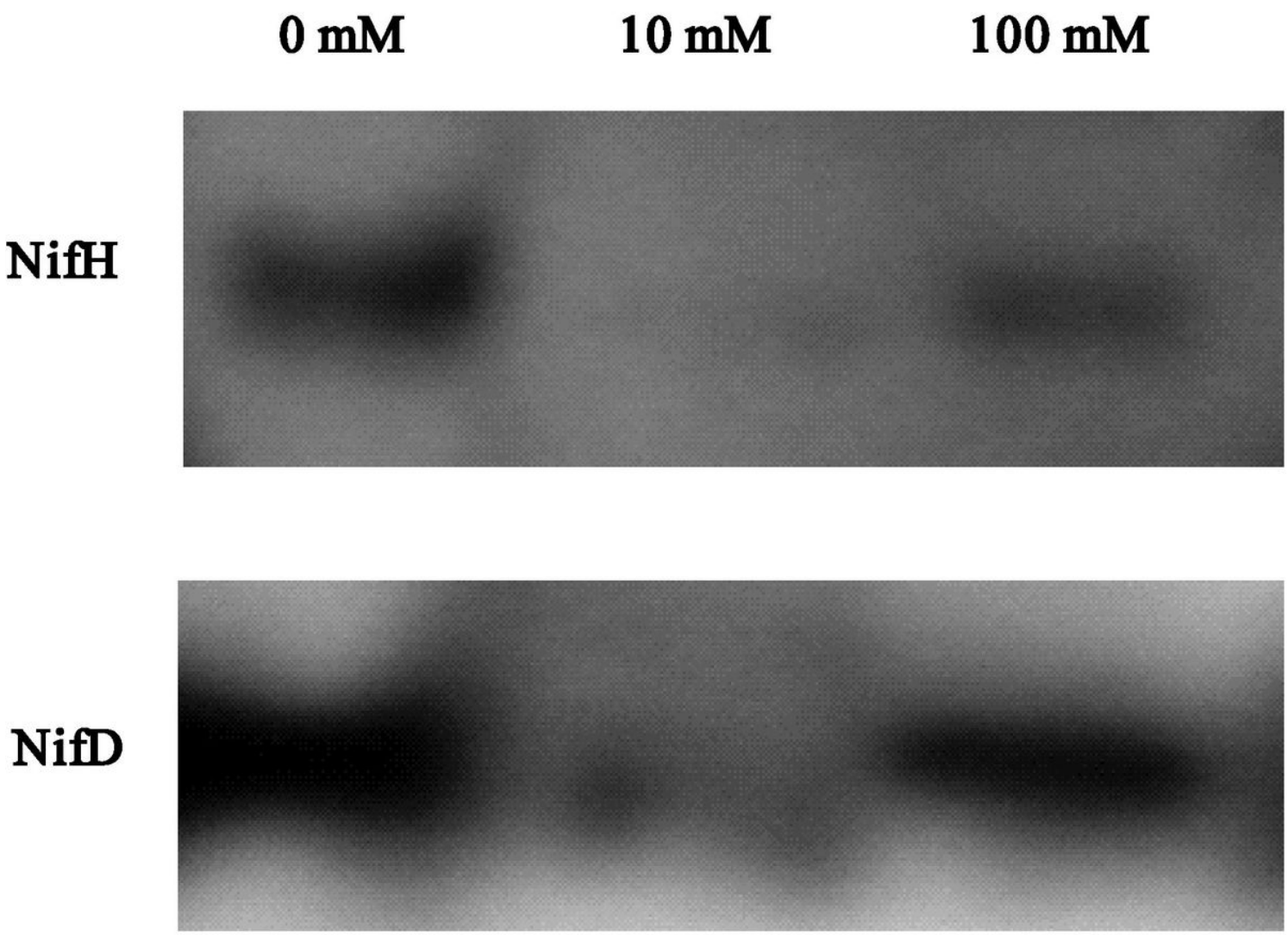

Figure 4

Western blot analysis of NifH and NifD proteins of P. sabinae T27 during fermentation with 0, 10 and 100 mM NH4+, respectively.

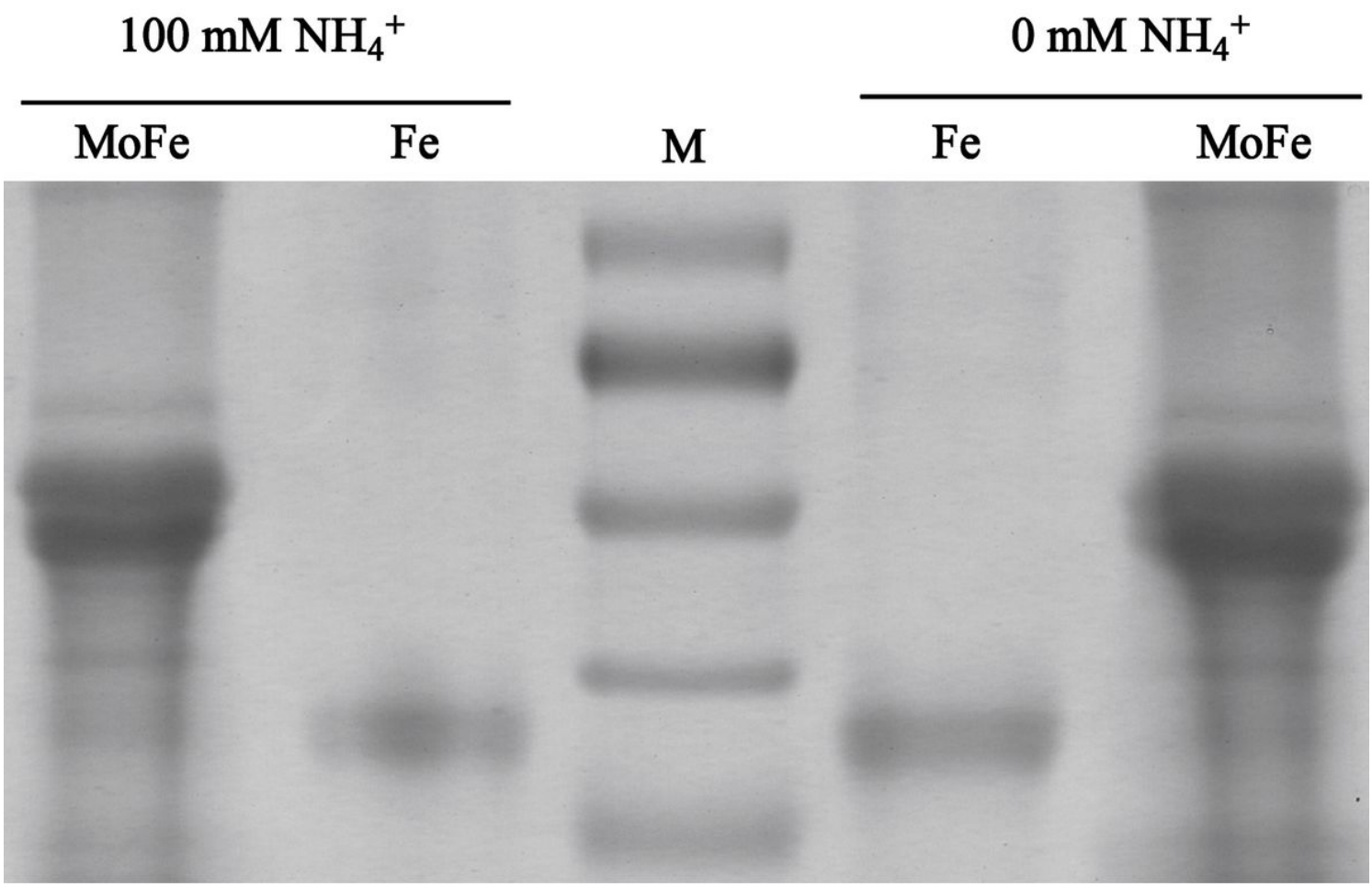

Figure 5

SDS-PAGE analysis of purified Fe and MoFe proteins under nitrogen-limited and nitrogen-excess conditions.

Supplementary Files

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