Bioelectrochemical Fixation of Nitrogen to Extracellular Ammonium by Pseudomonas stutzeri

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ABSTRACT Diazotrophs can produce bioavailable nitrogen from inert N2 gas by bioelectrochemical nitrogen fixation (e-BNF), which is emerging as an energy-saving and highly selective strategy for agriculture and industry. However, current e-BNF technology is impeded by requirements for NH4+ assimilation inhibitors to facilitate intracellular ammonia secretion and precious metal catalysts to generate H2 as the energy-carrying intermediate. Here, we initially demonstrate inhibitor- and catalystless extracellular NH4+ production by the diazotroph Pseudomonas stutzeri A1501 using an electrode as the sole electron donor. Multiple lines of evidence revealed that P. stutzeri produced 2.32 ± 0.25 mg/liter extracellular NH4+ at a poised potential of −0.3 V (versus standard hydrogen electrode [SHE]) without the addition of inhibitors or expensive catalysts. The electron uptake mechanism was attributed to the endogenous electron shuttle phenazine-1-carboxylic acid, which was excreted by P. stutzeri and mediated electron transfer from electrodes into cells to directly drive N2 fixation. The faradaic efficiency was 20% ± 3%, which was 2 to 4 times that of previous e-BNF attempts using the H2-mediated pathway. This study reports a diazotroph capable of producing secretable NH4+ via extracellular electron uptake, which has important implications for optimizing the performance of e-BNF systems and exploring the novel nitrogen-fixing mode of syntrophic microbial communities in the natural environment.

IMPORTANCE Ammonia greatly affects global ecology, agriculture, and the food industry. Diazotrophs with an enhanced capacity of extracellular NH4+ excretion have been proven to be more beneficial to the growth of microalgae and plants, whereas most previously reported diazotrophs produce intracellular organic nitrogen in the absence of chemical suppression and genetic manipulation. Here, we demonstrate that Pseudomonas stutzeri A1501 is capable of extracellular NH4+ production without chemical suppression or genetic manipulation when the extracellular electrode is used as the sole electron donor. We also reveal the electron uptake pathway from the extracellular electron-donating partner to P. stutzeri A1501 via redox electron shuttle phenazines. Since both P. stutzeri A1501 and potential electron-donating partners (such as electroactive microbes and natural semiconductor minerals) are abundant in diverse soils and sediments, P. stutzeri A1501 has broader implications on the improvement of nitrogen fertilization in the natural environment.

KEYWORDS bioelectrochemical system, electron uptake mechanism, nitrogen fixation, phenazine, Pseudomonas stutzeri

Ammonia, which is fundamental to the synthesis of amino acids and nucleotides, greatly affects global ecology, agriculture, and the food industry (1, 2).
conventional Haber-Bosch process, allowing enormous NH₃ production from N₂, is widely applied, but this technology is extremely energy intensive (14 kWh/kg NH₃-N), since it requires high temperature and high pressure (3). Alternatives performed under ambient conditions, including photocatalysis, electrocatalysis, transition metal catalysis, and plasma induction, are relatively energy saving (4, 5). Nevertheless, these approaches suffer from the use of expensive catalysts, poor selectivity, and low conversion efficiency (6). Electro- and photocatalysis with nitrogenases can overcome the limitations of selectivity and efficiency (7, 8), whereas this system faces the challenges of oxygen-induced irreversible damage and electron shuttling, such as the required methylviologen addition (9).

Recently, there has been increasing attention paid to bioelectrochemical nitrogen fixation (e-BNF) using whole-cell diazotrophs in bioelectrochemical systems that fix N₂ to bioavailable nitrogen in the cathode compartment (10). Ortiz-Medina et al. (9) and Rago et al. (10) fixed N₂ using microbial electrolysis cells with a mixed microbial community. Liu et al. (11) performed the efficient synthesis of solid N biomass and NH₃ from N₂ and H₂O by coupling hydrogen generation from electrocatalytic water splitting to the H₂-oxidizing autotrophic microorganism Xanthobacter autotrophicus, and Soundararajan et al. (12) demonstrated that the phototrophic bacterium Rhodopseudomonas palustris in the cathodic compartment of an electrochemical cell can also capture both CO₂ and N₂ using H₂ and photons as the energy sources. These studies enable sustainable nitrogen fixation along with the continuous growth of microorganisms.

However, current e-BNF studies have several limitations. (i) From the perspective of nitrogen fixation products, diazotrophs with an enhanced capacity of extracellular NH₄⁺ excretion have been proven to be more beneficial to the growth of microalgae and plants (13–16). However, in the e-BNF system, extracellular NH₄⁺ is obtained only when the NH₄⁺ assimilation inhibitors (e.g., methionine sulfoximine and phosphinothricin) are added (9, 11), which is not a sustainable approach in real-life applications. (ii) From the perspective of electron uptake, most diazotrophs use H₂ as an energy-carrying intermediate in the e-BNF system (11, 12). High applied voltages, even up to 3 V, or expensive metallic catalysts are typically required to overcome sluggishness in H₂ generation in the e-BNF system. Moreover, the low solubility and high explosiveness of H₂ limit the usefulness of the H₂-mediated route; thus, extracellular electron uptake (i.e., direct electron transfer via cytochrome or indirect electron transfer via soluble endogenous electron shuttle from electrodes to microorganisms) is expected to be a better choice for applications (17). To date, diazotrophs capable of extracellular NH₄⁺ production through extracellular electron uptake from electrodes have not been reported.

_Pseudomonas stutzeri_ A1501, originally isolated from the rice rhizosphere, exhibits excellent nitrogen fixation performance and adapts to a range of environmental stresses and nutrient availabilities (18). Its complete genome has been sequenced and annotated (19). Similar sequences of PhzA-G that are identified as phenazine biosynthesis proteins (20–22) have been found in _P. stutzeri_. Phenazines are a representative type of soluble electron shuttle involved in indirect extracellular electron transfer (23–25), indicating that _P. stutzeri_ performs extracellular electron uptake. So far, the e-BNF ability of _P. stutzeri_ has not been recognized.

We employ _P. stutzeri_ to set up an inhibitor- and H₂-less e-BNF system for extracellular NH₄⁺ production. The nitrogenase activity, nitrogen-fixing gene transcription, and ^1⁵N isotope labeling were measured to investigate the bioelectrochemical nitrogen-to-extracellular NH₄⁺ fixation, while electrochemical measurements and liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis were carried out to explore the electron uptake mechanism of _P. stutzeri_ in the e-BNF system.

**RESULTS**

**Bioelectrochemical nitrogen to extracellular NH₄⁺ fixation by _P. stutzeri_.** The bioelectrochemical extracellular NH₄⁺ production of _P. stutzeri_ rose rapidly from day 3 and the final NH₄⁺ accumulation reached 2.32 ± 0.25 mg/liter during the 13-day
experiment, while the NH$_4^+$ production of the open circuit control was only 0.45 ± 0.03 mg/liter and the dead cell and no cell controls yielded less than 0.20 ± 0.01 mg/liter (Fig. 1A). The biological ATP-dependent reduction of N$_2$ to NH$_3$ was carried out by the nitrogenase enzyme complex. The maximum nitrogenase activity of P. stutzeri in the e-BNF group appeared on day 6, which was approximately 12 times higher
than that in the open circuit control (Fig. 1B). Therefore, it can be assumed that most of the NH$_4^+$ production in the open circuit control comes from the organic nitrogen ammoniation but not nitrogen fixation. The nitrogenase complex consists of the MoFe protein encoded by *nifD* and *nifK* and the Fe protein encoded by *nifH* (26). *nifA* encodes a positive regulator of the nitrogen fixation operon (27). As shown in Fig. 1C, all four key nitrogen fixation genes of *e*-BNF had more than 3-fold greater transcript abundances than those in the open circuit control and the no N$_2$ control.

Total nitrogen detection and the $^{15}$N isotope labeling experiment were used to confirm that NH$_4^+$ formation occurred through N$_2$ fixation rather than organic nitrogen ammoniation. The increase in the total nitrogen was nearly 3 mg/liter in the *e*-BNF group, while the total nitrogen increase of the open circuit control was only 0.55 ± 0.28 mg/liter and those of the no N$_2$, dead cell, and no cell controls were below 0.10 ± 0.01 mg/liter (Fig. 1D). The peak height values of $^{15}$N/$^{14}$N and $^{15}$N/$^{14}$N (Fig. 1E and F) were used to calculate the $^{15}$N/$^{14}$N atom ratio by the built-in software of the isotope mass spectrometer, and a higher peak height ratio of $^{15}$N/$^{14}$N in the chromatogram led to a higher $^{15}$N/$^{14}$N atom ratio, which means that more $^{15}$N is fixed. In our study, the $^{15}$N/$^{14}$N ratio in *P. stutzeri* of the *e*-BNF group is higher than those without the *e*-BNF system (12.517% versus 4.103%), demonstrating that a bioelectrochemical N$_2$ fixation process indeed occurred in the *e*-BNF system.

Pronounced electron uptake was observed after an adaptation period of 2 days and then reached a maximum of approximately 33 mA/m$^2$ in the *e*-BNF group, whereas there were only negligible currents in all controls, indicating that *P. stutzeri* can utilize electrons from electrodes (Fig. 2A). The variation trend of electron uptake matched the extracellular NH$_4^+$ production pattern (Fig. 1A). The electrons recovered in NH$_4^+$ were positively related to the electron uptake from the electrode with a correlation coefficient (R) of 0.8479 (Fig. 2B). The faradaic efficiency for extracellular NH$_4^+$ was 15% ± 2% and that for total nitrogen reduction was 20% ± 3%, which were much higher than values in previous studies (see Table S2 in the supplemental material). The current was generated noticeably once N$_2$ was supplied and disappeared significantly when N$_2$ was gradually diluted by Ar (Fig. S1). The strong N$_2$ dependence of biocurrents indicated that bioelectrochemical nitrogen fixation was a main mechanism of electron uptake.

**Effects of inhibitor addition and genetic manipulation in the *e*-BNF of *P. stutzeri*.** An NH$_4^+$ assimilation inhibitor, phosphinothricin, which can block the biomass increases and allow all NH$_3$ produced to diffuse into the extracellular medium, was added to the group with electrodes and the group with lactate as the electron donor to measure their total NH$_4^+$ yields. As shown in Fig. 3A, in the absence of the NH$_4^+$ assimilation inhibitor, extracellular NH$_4^+$ production by *P. stutzeri* was found only in the culture in the *e*-BNF system and not in the culture with lactate as the electron donor. In the presence of the NH$_4^+$ assimilation inhibitor, the total NH$_4^+$ yield of *P. stutzeri* in the
The e-BNF system reached 3.02 ± 0.04 mg/liter, which was over 4 times greater than that of microbial nitrogen fixation with lactate and 30% higher than that of *P. stutzeri* without NH$_4^+$ assimilation inhibitor.

AmtB is the NH$_4^+$ carrier protein that transports NH$_4^+$ into the interior of cells for nitrogen assimilation metabolism when the intracellular NH$_4^+$ level is insufficient (28, 29). The transcript abundances of *amtB1* and *amtB2* genes, encoding the ammonium transporter of *Pseudomonas stutzeri* A1501, and the extracellular NH$_4^+$ production of the *amtB1-amtB2* double deletion mutant of *Pseudomonas stutzeri* in the e-BNF system.

**FIG 3** (A) Extracellular NH$_4^+$ production of *Pseudomonas stutzeri* A1501 using different electron donors with and without the addition of the NH$_4^+$ assimilation inhibitor phosphinothricin, (B) relative transcript abundance of the *amtB1* and *amtB2* genes that encode the ammonium transporter of *Pseudomonas stutzeri* A1501, and (C) extracellular NH$_4^+$ production of the *amtB1-amtB2* double deletion mutant of *Pseudomonas stutzeri* in the e-BNF system.
double deletion mutant strain. Figure 3C reveals that the bioelectrochemical extracellular NH$_4^+$ production of the mutant was 40% higher than that of the wild type.

**Roles of planktonic and electrode-attached *P. stutzeri* in electron uptake.** To identify the roles of planktonic and attached cells, we first established some e-BNF systems with working electrodes shielded by a dialysis membrane to physically isolate *P. stutzeri* and the electrodes. Electrical results showed that whether the electrodes were in the dialysis bag or not, there was significant electron uptake by *P. stutzeri* (Fig. 4A). Current uptakes of the e-BNF system with shielded electrodes had similar start-up rates and an only 12% decrease in current densities compared to those of electrodes without the dialysis membrane, demonstrating that physical contact between cells and electrodes was unnecessary for the electron uptake by *P. stutzeri*.

To determine the roles of planktonic and electrode-attached cells and their electron transfer mechanism, once a stable current uptake was obtained at day 6, different medium replacements were carried out in the e-BNF system without dialysis membranes (Fig. 4B). In the first experimental group, the entire original electrolyte was centrifuged for the collection of planktonic cells, and then all planktonic cells were returned with fresh medium to the chambers containing electrode-attached biofilms. Electron transfer began after 2 days of incubation in this group. In the second group, when only 50% of the original medium and 50% of the planktonic cells were replaced by fresh medium, electron uptake immediately started without any lag period. In the third group, we only replaced the biofilm-coated electrode with a sterile electrode, and the current density was immediately restored to 60% ± 4% of its original level.

In addition, the spatial structures of biofilms attached to the electrodes were observed. Figure S2 reveals that loose biofilms with a thickness of 18 μm were attached to the electrode in the e-BNF system, and the control groups also formed some loose and thinner biofilms with lower activity. The biomass increase of cells attached to electrodes was 1.39 ± 0.08 mg, while that of planktonic cells was 7.74 ± 0.64 mg, indicating that most (85%) of the *P. stutzeri* proteins were suspended in the electrolyte. Together, these results reveal that planktonic cells are responsible for the majority of electron uptake, and their metabolites should include a certain electron shuttle that mediates electron transfer from electrodes to planktonic and attached cells.

**Identification of the electron shuttle involved in *P. stutzeri*.** Cyclic voltammetry (CV) and differential pulse voltammetry (DPV) can provide evidence for the electron transfer mechanism of both electrode-attached and planktonic cells and preliminarily estimate the possible electron shuttle. As noted by Rabaey et al. (30), electron shuttles that are reversibly oxidized and reduced should have at least one peak on the upper curve and one peak on the lower curve in a CV plot. If one of the peaks disappears, this component would be regarded as permanently oxidized or reduced and lacking the basic requirements for an electron shuttle. The intercept of the x axis with a line connecting the upper and lower peaks indicates the formal potential of the redox mediator, and the peak intensity indicates its quantity.

To investigate the electron uptake pathway of the attached biofilm, CV detection was conducted in the e-BNF system with a *P. stutzeri*-colonized working electrode, and the suspended cells were filtered. The CV curve of the bioelectrochemical group had four obvious peaks: two oxidation peaks at −0.024 V and 0.089 V (versus saturated calomel electrode [SCE]) and two reduction peaks at −0.249 V and −0.069 V (versus SCE) (Fig. 4C). None of the CV curves of the controls had peaks as distinct as those of the e-BNF group. The four peaks disappeared after the original electrolyte was replaced by fresh medium in the bioelectrochemical group, demonstrating that the redox electron shuttle was a certain metabolite secreted by *P. stutzeri*.

To determine the inward electron transfer mechanism of the planktonic cells, the catholyte was tested by DPV in a three-electrode test vial (Fig. 4D). The fresh medium control did not contain any electroactive species. After e-BNF operation, both the catholyte effluent and the supernatant without cells had a peak centered on ca. −0.4 V (versus SCE), suggesting that some self-produced soluble electroactive species were produced (31). When additional phenazine-1-carboxylic acid was added to the catholyte
FIG 4  (A) Current uptake with a graphite plate working electrode shielded from *Pseudomonas stutzeri* A1501 by a dialysis membrane, (B) current uptake of e-BNF systems with different medium-replacement methods, (C) CV analyses under the turnover condition, (D) DPV analyses of the catholyte effluent, (E) LC-MS/MS of electron shuttles of *Pseudomonas stutzeri* A1501 in the e-BNF system, and (F) current uptake by *Pseudomonas stutzeri* A1501 in the e-BNF with different concentrations of phenazine-1-carboxylic acid.
effluent group, the peak height at $-0.4$ V increased. The additional phenazine-1-carboxylic acid could also bring out a peak at $-0.4$ V in the cells with fresh medium control. These results prove that the self-produced soluble electroactive species were probably phenazines. There was a peak shift between the CV and the DPV curves (Fig. 4C and D), since different working electrodes, counter electrodes, and structures of the text vials were used in the CV and the DPV measurements, which would lead to a 250-mV shift of the potential (Fig. S3), and different cell states (i.e., attached or planktonic) between these two measurements would also cause a peak shift.

LC-MS/MS analysis was used to identify the electroactive species excreted by *P. stutzeri* A1501. Matching the spectra of the electrolyte to the spectra in an existing database (mzCloud library) showed that the peaks of the precursor ion at $m/z$ 225.10983 and the other four fragment ions at $m/z$ 151.11209, $m/z$ 179.06078, $m/z$ 207.05478, and $m/z$ 227.04033 were all similar to the standard MS/MS peaks of phenazine-1-carboxylic acid ($m/z$ 225.06638, $m/z$ 151.04160, $m/z$ 179.06229, $m/z$ 207.05711, and $m/z$ 227.07121) (Fig. 4E). Thus, the redox electron shuttle was putatively identified as phenazine-1-carboxylic acid, which has been verified to act as the redox mediator for *Pseudomonas aeruginosa* (25). The phenazine synthesis pathway includes genes *phzA* to *phzG* (*phzA-G*) to get phenazine-1-carboxylic acid from chorismate (20–22). Thus, amino acid sequence alignment of proteins in *Pseudomonas stutzeri* A1501 and *PhzA-G* proteins in *Pseudomonas aeruginosa*, *Pseudomonas chlororaphis*, and *Pseudomonas fluorescens*, which could produce phenazine, were conducted. Several amino acid sequences in *Pseudomonas stutzeri* A1501 and *PhzA-G* had over 30% identity and over 60% coverage, implying that they were homologous (Table S1). Further mutant study of these homologous proteins is ongoing.

The concentration of phenazine-1-carboxylic acid excreted by *P. stutzeri* A1501 was measured using high-performance liquid chromatography (HPLC; Thermo U3000) by the same method as that in Bosire and Rosenbaum (32); however, the concentration was below the detection limit of 200 nM. Thus, we added different concentrations of phenazine-1-carboxylic acid to start up the e-BNF inoculated with *P. stutzeri*. As shown in Fig. 4F, 50 nM was enough to obtain a rapid startup of current uptake within 2 h. With the addition of 100 nM phenazine-1-carboxylic acid, the current density can reach 30 mA/m², which was close to the maximum current density of the e-BNF (Fig. 2A) within 8 h, suggesting that the excretive phenazine-1-carboxylic acid concentration of *P. stutzeri* was approximately 100 nM.

**Analysis of electron transfer pathway in the inner membrane of *P. stutzeri*.**

Electron transport blockers were used to reveal the electron transfer pathway in the inner membrane. As shown in Fig. 5, the current uptake was reduced to various degrees depending on the blocker used. Antimycin A (50 μM), quinacrine (200 μM), and dicyclohexylcarbodiimide (300 μM) had strong and unrecoverable inhibitory effects on inward electron transfer that inhibited 100%, 32%, and 100% of the current uptake, respectively. About 34% of the current uptake was inhibited by rotenone (500 μM) and then recovered to 97% of the initial current uptake within 17 h. About 49% of the current uptake was blocked by dicumarol (200 μM); however, it returned to 94% of the initial current uptake within 10 h. Lactate was used as the electron donor instead of the electrode to determine whether these dosages of blockers influenced the extracellular electron transfer or metabolic activity of *P. stutzeri*. As shown in Fig. 5, only quinacrine (200 μM) significantly suppressed 97% of the metabolic activity of *P. stutzeri*, while antimycin A (50 μM), dicumarol (200 μM), and dicyclohexylcarbodiimide (300 μM) had slight effects (i.e., 12%, 15%, and 1%, respectively) and rotenone (500 μM) showed no effect.

**DISCUSSION**

In this work, all the pieces of chemical, proteinic, and transcriptional evidence support each other to demonstrate that wild-type *P. stutzeri* can perform nitrogen fixation to extracellular NH₄⁺ in the e-BNF system without any special chemical or genetic treatment, while Table S2 in the supplemental material shows that in previous studies, bioelectrochemical nitrogen to extracellular NH₄⁺ fixation should be achieved through
FIG 5 Effects of electron transport blockers on the currents of inward extracellular electron transport from the electrodes to Pseudomonas stutzeri.
inhibitor addition to block the NH₄⁺ assimilation pathway and allow NH₃ to passively diffuse out into the extracellular medium (9, 10). Generally, diazotrophs reduce N₂ to NH₃ with the nitrogenase enzymes in the cytoplasm, and then the NH₃ is excreted into the extracellular environment via passive diffusion. Ammonia is rapidly protonated to NH₄⁺ in the extracellular environment. The NH₄⁺ carrier protein AmtB then transports NH₄⁺ into the interior of cells for nitrogen assimilation metabolism when the intracellular NH₄⁺ level is insufficient (28, 29). Thus, the two possible reasons for extracellular NH₄⁺ production by *P. stutzeri* in the e-BNF system are the following: (i) *P. stutzeri* in the e-BNF system might produce far more NH₄⁺ than the NH₄⁺ assimilation demand for biomass growth, and (ii) the NH₄⁺ transport activity of AmtB protein might be inhibited by the redox potential of the working electrode in the e-BNF system.

NH₄⁺ assimilation inhibitor addition was used to investigate whether the extracellular NH₄⁺ production was attributed to the first possible reason listed above. In the absence of the NH₄⁺ assimilation inhibitor, extracellular NH₄⁺ production by *P. stutzeri* was found only in the culture in the e-BNF system and not in the culture with lactate as the electron donor (Fig. 3A), with the biomass increasing by ~9.13 mg in the e-BNF group and ~11.29 mg in the lactate group. NH₄⁺ assimilation inhibitors can block the biomass increases and allow all NH₃ produced to diffuse into the extracellular medium. In the presence of the NH₄⁺ assimilation inhibitor, as shown in Fig. 3A, the total NH₄⁺ yields of *P. stutzeri* in the e-BNF system were more than 4 times greater than those of microbial nitrogen fixation with lactate. Compared to the biomass increases in the absence of NH₄⁺ assimilation inhibitor, the NH₄⁺ yield of *P. stutzeri* in the e-BNF group was indeed ~3 times higher than the NH₄⁺ assimilation demand for biomass growth. Thus, the first reason is valid.

The AmtB experiments (Fig. 3B and C) were used to see whether extracellular NH₄⁺ production is also due to the second possible reason listed above. Neither *amtB1* nor *amtB2* had a significant difference in abundance between the e-BNF and lactate groups (Fig. 3B), and the mutant study revealed that the AmtB protein still worked in wild-type *P. stutzeri* of the e-BNF group (Fig. 3C). Thus, the extracellular NH₄⁺ production could not be explained by the above-described second possible reason.

To date, unlike the outward electron transfer mechanisms, only two inward electron transfer mechanisms from electrodes to bacteria have been confirmed with direct experimental evidence (33). One is direct electron transfer via outer membrane cytochromes of microbes, and the other is indirect electron transfer via electron shuttles such as flavin. Previous e-BNF studies on the generation of H₂ strictly did not belong to extracellular electron transfer (Table S2). Rago et al. (10) found that their mixed cultures might transfer electrons with cytochrome c, type IV pili, and flavoproteins, since the genes encoding these proteins were upregulated. In our study, H₂ did not accumulate during the incubation at a poised potential of −0.3 V (versus standard hydrogen electrode [SHE]), and multiple lines of evidence (Fig. 4) demonstrated that the soluble endogenous electron shuttle (most likely phenazine-1-carboxylic acid) mediated the electron transfer from the electrode to both attached and planktonic *P. stutzeri*. Considering the low solubility, high explosibility, high catalyst cost, high energy consumption, and low faradaic efficiency (Table S2) of H₂ generation, electron uptake mediated by soluble endogenous electron shuttles is a superior alternative for future applications.

The electron transfer pathway in the inner membrane of *P. stutzeri* was investigated using electron transport blockers (Fig. 5). Up to 1,000 μM rotenone (blocking complex I) and dicumarol (inhibiting electron transport between complex I and the quinol pool) had slight and transitory effects, suggesting that complex I is not indispensable to the inward electron transfer pathway of *P. stutzeri*. Quinacrine (200 μM), which blocks the FAD and FMN centers of complex II (34, 35), showed obvious and unrecoverable inhibitory effects in both the e-BNF group and the lactate group. Thus, complex II might be important in both the extracellular electron uptake and the metabolic activity of *P. stutzeri*. Antimycin A (50 μM), which cuts off the electron transfer between complex III and ubiquinone, had obvious inhibitory effects on inward electron transfer, while the same dosages of it only showed slight effects on the lactate group, demonstrating that
complex III and quinone were indispensable in the inward extracellular electron transfer of *P. stutzeri*. Dicyclohexylcarbodiimide (300 μM), which inhibits the ATPase and blocks the proton channel, had strong inhibitory effects on inward electron transfer in the e-BNF group. Although 300 μM dicyclohexylcarbodiimide did not block the metabolic activity when we used lactate as the electron donor, 600 μM could suppress 98% of the metabolic activity in the lactate group. That means the inward electron uptake from extracellular electrodes was more sensitive to dicyclohexylcarbodiimide than that from the intracellular lactate, suggesting that inward electron uptake from extracellular electrodes was a more energy-intensive process than that from lactate.

A proposed mechanism of electron uptake from the electrode to *P. stutzeri* is shown in Fig. 6. Electron uptake at the attached/planktonic cell-electrode interface is performed by the soluble endogenous electron shuttle phenazine-1-carboxylic acid. The phenazine-1-carboxylic acid should accumulate to a certain concentration to begin the electron uptake, which resulted in the 2-day lag period of the current curve. Phenazine-1-carboxylic acid transports electrons to the inner membrane. Electrons then are transferred to the respiratory chain with complex III to generate the reducing power (NADH and FADH₂) via the proton motive force and donated downhill to the membrane-bound Rnf complex (19, 36) and the nitrogenase enzyme complex to reduce N₂ to NH₃.

This work reveals that *P. stutzeri* A1501 can produce extracellular NH₄⁺ in the e-BNF system with extracellular electron uptake. The extracellular NH₄⁺ production ability without inhibitors and the endogenous phenazine-mediated electron transfer mechanism of *P. stutzeri* optimize the performance of e-BNF systems by addressing the unsustainability of NH₄⁺ assimilation inhibitor addition, the complexity of genetic manipulation, and the difficulties in the application of the H₂-mediated route in previous e-BNF technologies. Thus, the e-BNF system provides an environmentally friendly and sustainable alternative to enhance the fertility of a nitrogen-deficient environment using e-BNF coupled with the bioaugmentation of *P. stutzeri*, specifically in some infertile areas, such as the desert and subsoil, which lack dissolved organic carbon or plants as electron donors for the nitrogen fixation of endophytic diazotrophs.

Moreover, this study indicates that a diazotroph can obtain electrons from an...
extracellular electron-donating partner to drive N₂ fixation. *P. stutzeri* is abundant in diverse soils and sediments, while the typical electron-donating partners, electroactive microbes such as *Geobacter* spp., are also ubiquitous in these environments. The linear sweep voltammogram (Fig. S5) showed that *Geobacter* spp. generated anodic current and *P. stutzeri* generated cathodic current in a potential range of −0.4 V to 0.2 V (versus SHE), which was within the range of natural soil and sediment redox potential, suggesting that the electron flows from *Geobacter* spp. to *P. stutzeri* can be generated in nature (37). Thus, this finding enlightens us that diazotrophs might be able to conduct nitrogen fixation by forming a syntrophic partnership with electroactive microbes, and future works will explore some novel nitrogen-fixing modes of the syntrophic microbial communities in the complicated natural environment.

**MATERIALS AND METHODS**

**Reactor configuration and microbial inoculation.** An H-shaped two-chamber e-BNF system with a liquid volume of 60 ml and a headspace volume of 90 ml for each chamber was constructed as described in Logan and Regan (38). A proton exchange membrane (Nafion 117; DuPont, USA) was used to separate the two chambers. Carbon cloth (projected area, 4 cm by 4 cm) connected to a 0.5-mm titanium wire was used as the working electrode and the counter electrode, and a saturated calomel electrode (SCE) was used as the reference electrode. The e-BNF system was autoclaved at 121°C for 30 min, and then the working chamber was filled with modified sterile medium K containing (in 1 liter deionized water) 2.0 g of NaHCO₃, 0.4 g of KH₂PO₄, 0.1 g of K₂HPO₄, 0.1 g of NaCl, 0.2 g of MgSO₄·7H₂O, 0.01 g of MnSO₄·H₂O, 0.01 g of Fe₂(SO₄)₂·H₂O, and 0.01 g of Na₂MoO₄·H₂O, and the initial pH of the solution was adjusted to 6.9. The counter chamber was filled with sterile PBS (8.8 g/liter Na₂HPO₄, 4.56 g/liter NaH₂PO₄, and 0.13 g/liter KCl, pH 7.0). Both the working chamber and counter chamber were purged with an N₂ atmosphere containing 0.5% oxygen.

*P. stutzeri* A1501 and the *amtB1-amtB2* double deletion mutant were kindly provided by Min Lin (Chinese Academy of Agricultural Sciences, Beijing, China) and were cultured in medium K (39) at 30°C in which NaHCO₃ was omitted while 10 ml/liter sodium lactate and 0.4 g/liter (NH₄)₂SO₄ were supplied, unlike the case of the above-described modified medium K. *P. stutzeri* at the logarithmic phase then was collected, washed with PBS at least three times, and inoculated into the working chamber. The working electrode was poised at −0.3 V versus a standard hydrogen electrode (SHE) using a multichannel potentiostat (1040C; Chenhua, China). The current data were normalized to the projected area of the working electrode. The experimental group was named N₂ + −0.3 V + *P. stutzeri* A1501. Four different controls were set: (i) reactors inoculated with dead *P. stutzeri* (i.e., N₂ + −0.3 V + *P. stutzeri* A1501 [dead]), (ii) reactors purged with Ar instead of N₂ (i.e., Ar + −0.3 V + *P. stutzeri* A1501 [dead]), (iii) reactors without any bacteria (i.e., N₂ + −0.3 V), and (iv) reactors in an open circuit (i.e., N₂ + *P. stutzeri* A1501). The dead-cell control was prepared by autoclaving *P. stutzeri* with equivalent biomass to the live-cell treatment at 121°C for 30 min. Each treatment was performed at least in triplicate at 30°C.

**Electrochemical measurements.** CV of the e-BNF system was performed in the range of −0.8 to 0.4 V at a scan rate of 1 mV/s using a CHI660E instrument on day 6 of operation (Chenhua, China). DPV was conducted in a 25-ml three-electrode test vial with a glassy carbon working electrode, a platinum plate auxiliary electrode, and a saturated calomel reference electrode. The parameters were set as a recording range of −0.8 to 0.4 V, a scanning rate of 5 mV/s, a pulse height of 50 mV, a pulse width of 300 ms, a step height of 2 mV, and a step time of 500 ms. Five electron transport blockers (quinacrine, rotenone, dicyclohexylcarbodiimide) were used to investigate the electron transport components of the electron transfer pathway in the inner membrane with the working electrode poised at −0.3 V (versus SHE) as previously described (34). The effects of all electron transport blockers on the e-BNF group were first investigated with 50 µM dosage. If small dosages of blockers already had obvious and unrecoverable effects on the current uptake, we stopped increasing the dosage. If the small dosages could not obtain obvious effects, we increased the dosage gradually until obvious effects appeared or the dosage reached 1,000 µM. We then used the smallest inhibitory dosages of each blocker in the e-BNF group to conduct the control experiment with lactate to determine whether these dosages influence the extracellular electron transfer or metabolic activity. Some experiments were carried out with dialysis bags (molecular mass cutoff of 1 KDa) to physically isolate cells and the electrode (40).

**Nitrogen fixation characterization.** The cathode was centrifuged at 8,000 rpm, and then the supernatant was passed through a 0.22-µm-pore-size filter to detect extracellular NH₄⁺ production using the indophenol method (41). Calculations of faradic efficiency representing the percentage of electrical energy used for N₂ reduction were described in Soundararajan et al. (12).

The concentrations of the total nitrogen on the first day and last day were analyzed with a portable colorimeter (DR/3900; Hach Company, USA) using the nitrogen total reagent set (Test ’N Tube) (11). The nitrogenase activity was determined by using the acetylene-ethylene assay (39). The e-BNF system was purged with an Ar atmosphere containing 0.5% oxygen after inoculation with *P. stutzeri*, and then C₂H₂ was injected into the headspace to reach a vol/vol gas phase concentration of 10% C₂H₂. The nitrogenase activity was expressed as nanomoles of ethylene per hour per milligram of protein. To detect the biomass in the working chamber, the protein was extracted and measured as previously described (42, 43).

The headspaces of the e-BNF system were repeatedly evacuated (0.1 kPa) and filled with high-purity...
helium (99.999%, 120 kPa) five times, and finally, the pressure was adjusted to 101.3 kPa. Twenty milliliters of 15N-labeled N2 gas (purity, >98.5%; Aladdin, China) was purged into the e-BNF system. The electrolyte containing bacteria was collected by a freeze-drying process after 13 days and then used to determine the 15N/(m/z = 29)/14N/(m/z = 28) ratio using the Thermo Fisher Scientific MAT 253 Plus gas bench isotope mass spectrometer.

**Microbial analyses.** The morphology of the P. stutzeri A1501 biofilm was characterized by confocal laser scanning microscopy (CLSM) (LSM880; Zeiss, Germany) after staining the working electrode using a LIVE/DEAD BacLight viability kit (L7012; Thermo Fisher Scientific, USA) (44).

**Quantitative real-time PCR (RT-qPCR) was used to quantify the expressions of genes associated with nitrogen fixation (nifA, nifD, nifK, and nifH) and ammonium transport (amtB1 and amtB2). The primers used for RT-qPCR are listed in Table 1.** The total RNA was extracted using the RNeasy minikit (Qiagen Inc., USA) and purified to remove DNA using the TURBOB DNA-free kit (Thermo Fisher Scientific, USA). The reactions were carried out in a final volume of 20 μl containing 10 μl of iTaq Universal SYBR green supermix (Bio-Rad, Hercules, USA) and 0.6 μl each of reverse and forward primers (1 μmol/liter). The amplification and detection of specific products were performed using the following procedure: 95°C for 5 min, followed by 40 cycles of 95°C for 15 s and 55°C for 30 s, and finally 72°C for 30 s. The 16S RNA gene was used as the internal standard, and the gene expression levels were normalized as described by Zhan et al. (26).

**Electron shuttle detection.** The H2 was detected using a gas chromatograph (GC240; Shimadzu, Japan) equipped with a Porapak Q column (5 mm by 3 mm) and a thermal conductivity detector. The detailed conditions were set as in a previous study (45). After the e-BNF system reached its maximum current density, the electrolyte of the working chamber was collected and centrifuged at 5,000 × g for 10 min, and 1 ml of the supernatant was freeze-dried. Two hundred microliters of 80% methanol with 0.1% formic acid was added to the freeze-dried supernatant and centrifuged at 15,000 × g for 5 min at 4°C after incubation on ice for 5 min. The supernatant was diluted to a final concentration containing 60% methanol and then passed through a 0.22-μm-pore-size filter. The electrolyte extracts were injected at a flow rate of 0.2 ml/min for LC-MS/MS analysis with a mass spectrometer (QE-HF; Thermo Fisher, USA) using a Hyperil gold column (100 mm by 2.1 mm, 1.9 μm) maintained at 40°C. The positive polarity mode eluents were solution A (0.1% formic acid in water) and solution B (100% methanol). The eluents for the negative polarity mode were solution C (5 mM ammonium acetate, pH 9.0) and solution B. A solvent gradient was set as 2% B from 0.0 to 1.5 min, 2% to 100% B from 1.5 to 12.0 min, 100% B from 12.0 to 14.0 min, and then an immediate switch to 2% B for the period from 14.0 to 16.0 min. The spectrometer was operated in the positive/negative polarity mode with a spray voltage of 3.2 kV, a capillary temperature of 320°C, a sheath gas flow rate of 35 arb, and an auxiliary gas flow rate of 10 arb. The LC-MS/MS data were processed using the Thermo Fisher Compound Discoverer 3.1 software. Peaks were matched using mzCloud and ChemSpider databases to obtain accurate qualitative results.

**SUPPLEMENTAL MATERIAL**

Supplemental material is available online only.

**SUPPLEMENTAL FILE 1**, PDF file, 0.7 MB.

**ACKNOWLEDGMENTS**

This work was financially supported by the National Natural Science Foundation of China (no. 41877052 and 41925028).

We thank Da Song and Xunan Yang at Guangdong Institute of Microbiology for facilitating the amino acid sequence alignment.
REFERENCES


