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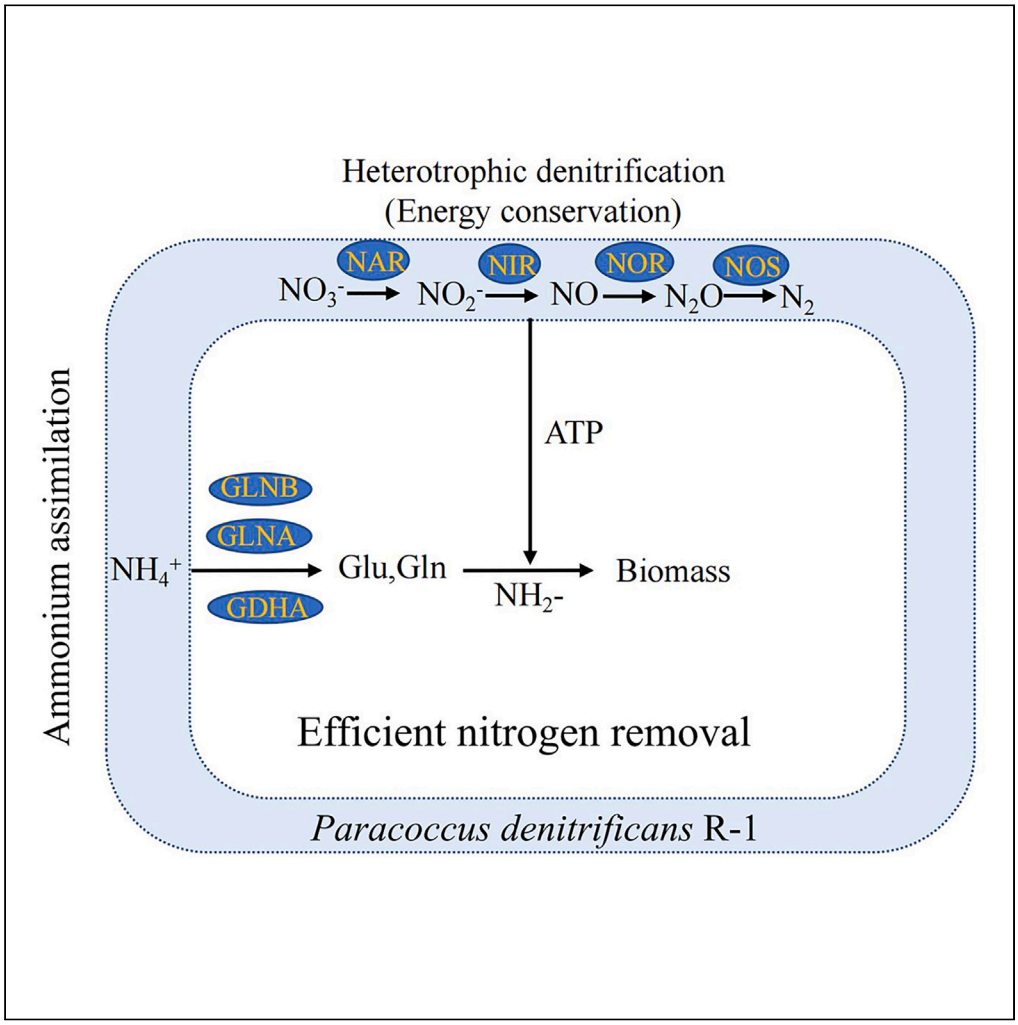

Wei Sun, Chunchen Hu, Jiapeng Wu, Mingken Wei, Jih-Gaw Lin, Yiguo Hong

yghong@gzhu.edu.cn

Highlights

Strain R-1 can remove NH_4^+ and NO_3^- under both anaerobic and aerobic conditions

Isotopic tracing analysis indicated strain R-1 consumed NH_4^+ through assimilation

Strain R-1 reduced NO_3^- through a respiratory process with electron donor oxidation

Genes for the ammonium assimilation and denitrification were complete in the genome

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Efficient nitrogen removal via simultaneous ammonium assimilation and heterotrophic denitrification of *Paracoccus denitrificans* R-1

Wei Sun,[1,2] Chunchen Hu,[1] Jiapeng Wu,[1] Mingken Wei,[2] Jih-Gaw Lin,[3] and Yiguo Hong[1,4,*]

SUMMARY

Although diverse microorganisms can remove ammonium and nitrate simultaneously, their metabolic mechanisms are not well understood. *Paracoccus denitrificans* R-1 showed the maximal NH_4^+ removal rate 9.94 mg $L^{-1}\cdot h^{-1}$ and 2.91 mg $L^{-1}\cdot h^{-1}$ under aerobic and anaerobic conditions, respectively. Analysis of the nitrogen balance calculation and isotope tracing experiment indicated that NH_4^+ was consumed through assimilation. The maximal NO_3^- removal rate of strain R-1 was 18.05 and 19.76 mg $L^{-1}\cdot h^{-1}$ under aerobic and anaerobic conditions, respectively. The stoichiometric consumption ratio of acetate to nitrate was 0.902 and NO_3^- was reduced to N_2 for strain R-1 through $^{15}NO_3^-$ isotopic tracing experiment, which indicated a respiratory process coupled with the oxidation of electron donors. Genomic analysis showed that strain R-1 contained genes for ammonium assimilation and denitrification, which effectively promoted each other. These findings provide insights into microbial nitrogen transformation and facilitate the simultaneous removal of NH_4^+ and NO_3^- in a single reactor.

INTRODUCTION

Nitrogen pollution is becoming increasingly severe in aquatic environments due to improper treatment of industrial wastewater, excessive discharge of domestic sewage, agricultural contamination, animal husbandry, and the metabolism of aquatic organisms.[1–3] Overloading nitrogen causes eutrophication of water bodies,[4] destroys ecosystem function, and threatens human health.[5] Microbiological nitrogen removal (MNR) is an economic and sustainable approach that will not lead to secondary pollution.[6] However, traditional nitrogen removal approaches involve two sequential reactions of aerobic nitrification coupled with anaerobic denitrification, which must be conducted in two separate pieces of equipment with independent conditions. This results in deficient performance when dealing with pollution with high concentrations of inorganic and organic nitrogen.[7] Thus, the high treatment costs have limited the development of MNR technology.

Fortunately, with the continuous development of MNR technology, diverse microorganisms have been isolated and identified for nitrogen removal from both engineered and natural ecosystems. Aerobic denitrifying bacteria (ADB) show higher growth rates and nitrogen removal efficiencies than autotrophic, traditional denitrifying bacteria,[8,9] and can simultaneously utilize various organic substrates[7]; therefore, aerobic denitrification technology overcomes the disadvantages of traditional nitrogen removal methods and exhibits prominent application value in the engineering process of wastewater treatment.[10]

To improve nitrogen removal efficiency, microbial strains that can simultaneously remove ammonium and nitrate have been isolated and identified from various environments. Most of these strains are ADB. The nitrogen removal mechanisms of these strains are often defined as heterotrophic nitrification and aerobic denitrification[11]; however, the evidence for this definition is insufficient. The nitrogen metabolic processes and mechanisms of ADB have not yet been clarified,[12] especially in regard to ammonium removal. Ammonium used as nitrogen source for the growth of most prokaryotes is very important and preferable,[13,14] but its removal is generally attributed to heterotrophic nitrification, and little attention has been paid to the role of microbial ammonium assimilation.[15,16] Cell growth and biomass accumulation are directly correlated with ammonium assimilation, which significantly affects nitrogen removal efficiency during aerobic denitrification.[16] Ammonium assimilation is an important driving force of aerobic denitrification.[6] According to current literature reports in this field, the mechanism responsible for the simultaneous removal of ammonium and nitrate by ADB remains unclear. Thus, the nitrogen metabolic mechanism of ADB warrants exploration as it will be beneficial for simultaneous removal of NH_4^+ and NO_3^- and its application in wastewater treatment.

[1]Institute of Environmental Research at Greater Bay Area, Key Laboratory for Water Quality and Conservation of the Pearl River Delta, Ministry of Education, Guangzhou University, Guangzhou 510006, P.R. China
[2]Guangdong Provincial Key Laboratory for Green Agricultural Production and Intelligent Equipment, College of Biological and Food Engineering, Guangdong University of Petrochemical Technology, Maoming 525000, P.R. China
[3]Institute of Environmental Engineering, National Chiao Tung University, 1001 University Road, Hsinchu City 30010, Taiwan
[4]Lead contact
*Correspondence: yghong@gzhu.edu.cn
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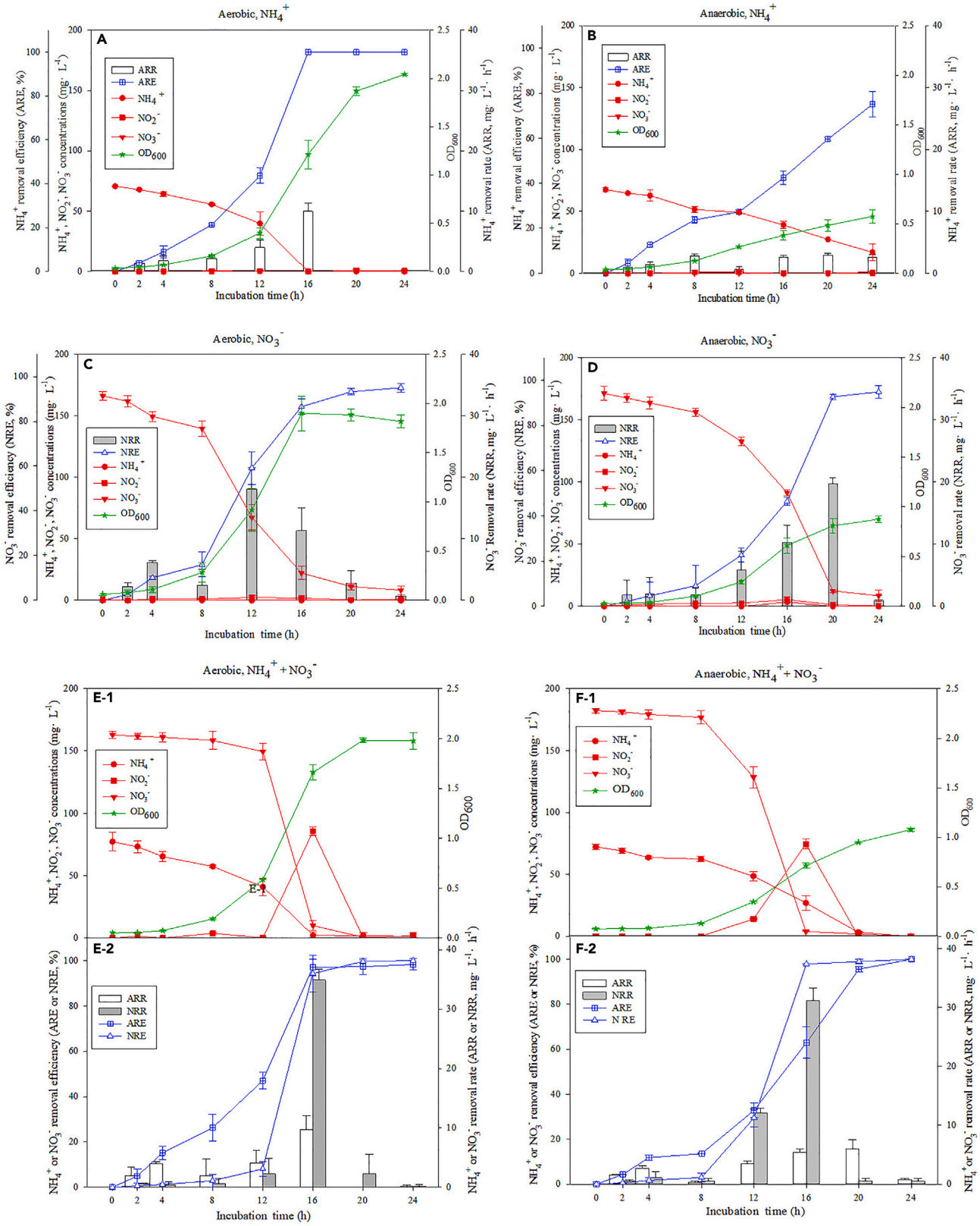
Aerobic, NH_4^+
A
ARR
ARE
NH_4^+
NO_2^-
NO_3^-
OD_{600}
NH_4^+ removal efficiency (ARE, %)
NH_4^+, NO_2^-, NO_3^- concentrations (mg· L^{-1})
OD_{600}
NH_4^+ removal rate (ARR, mg· L^{-1}· h^{-1})
Incubation time (h)
Anaerobic, NH_4^+
B
Aerobic, NO_3^-
C
NRR
NRE
NO_3^- removal efficiency (NRE, %)
NO_3^- Removal rate (NRR, mg· L^{-1}· h^{-1})
Anaerobic, NO_3^-
D
Aerobic, NH_4^+ + NO_3^-
E-1
E-2
NH_4^+ or NO_3^- removal efficiency (ARE or NRE, %)
NH_4^+ or NO_3^- removal rate (ARR or NRR, mg· L^{-1}· h^{-1})
Anaerobic, NH_4^+ + NO_3^-
F-1
F-2

Figure 1. The simultaneous removal of ammonium and nitrate by *Paracoccus denitrificans* R-1 under aerobic and anaerobic conditions

The various inorganic nitrogen concentrations, growth of strain R-1 and removal efficiencies and removal rates of ammonium and nitrate for R-1 in AM (A and B), DM (C and D), and ADM (E and F) under aerobic (A, C, and E) and anaerobic conditions (B, D, and F) using sodium acetate as electron donor. See also Figure S1 in supplemental information 1. The error bars epresent standard deviations of duplicate experiments.

We previously reported the genome sequence of an efficient ADB (named as *Paracoccus denitrificans* R-1), which was isolated from sewage treatment sludge in a Taiwan wastewater disposal plant.[15] In the present study, we investigated the performance and metabolic mechanisms of nitrogen removal for *Paracoccus denitrificans* R-1. The specific objectives were to (1) examine the NH_4^+ and NO_3^- simultaneous removal performance of strain R-1 by using growth characteristics and nitrogen removal performance when various inorganic nitrogen used as nitrogen resources under aerobic and anaerobic conditions and (2) clarify the metabolic pathways involved in the simultaneous removal of ammonium and nitrate by strain R-1 based on the production of metabolic intermediates during NH_4^+ and NO_3^- removal process, nitrogen balance analysis, stoichiometry between the carbon and nitrogen sources, thermodynamic analysis, ^{15}N-metabolic flux analysis, and metagenome analysis. A series of combined methodologies was used to elucidate the bacteria-mediated mechanism of simultaneous ammonium and nitrate removal, which will provide a deeper understanding of the nitrogen removal mechanism in ADB and promote its application in wastewater treatments.

RESULTS AND DISCUSSION

The simultaneous removal of ammonium and nitrate by strain R-1 under aerobic and anaerobic conditions

Effective electron donors for the removal of ammonium and nitrate

Paracoccus denitrificans R-1 strain was cultured in nitrification medium (AM), denitrification medium (DM), and nitrification and denitrification medium (ADM) supplemented with six different carbohydrates (formate, acetate, pyruvate, succinate, lactate, and glucose) under anaerobic conditions (supplemental information 1 Figure S4). The results showed that strain R-1 could use the selected carbohydrates to remove NH_4^+ from AM with an ammonium removal efficiency (ARE) of 17.73–52.42% (supplemental information 1 Figures S4A-1–S4A-6). ARE values higher than 50% were achieved with formate, acetate, and glucose as the carbon sources, and the growth curves of R-1 in AM were all linear with OD_{600} values of 0.28–0.62 (supplemental information 1 Figures S4A-1, S4A-2, and S4A-6). Comparatively, NO_3^- was almost completely reduced (with an nitrate removal efficiency [NRE] of almost 100%) within 12∼18 h in DM with acetate, pyruvate, succinate, lactate, and glucose, but not sodium formate, and the OD_{600} values of 0.62–1.67 were higher in DM than that in AM under the same conditions (supplemental information 1 Figures S4B-1–S4B-6). The ARE and NRE values were 80–99% and almost 100%, respectively, within 12∼18 h in ADM with acetate, pyruvate, succinate, lactate, and glucose, but not sodium formate; and the OD_{600} values of 1.02–1.68 were higher than those for AM and DM under the same conditions (supplemental information 1 Figures S4C-1–S4C-6). Moreover, the changing trends of NH_4^+ and NO_3^- removal curves were consistent along with incubation time, suggesting that NH_4^+ and NO_3^- were simultaneously removed from ADM (supplemental information 1 Figures S4C-1–S4C-6). Therefore, acetate, pyruvate, succinate, lactate, and glucose could be used as carbon sources to support the nitrogen metabolism of strain R-1.

The NH_4^+ removal performance and growth of strain R-1 under aerobic and anaerobic conditions

The removal of NH_4^+ and the growth of strain R-1 were analyzed in AM for 24 h under aerobic conditions as shown in Figure 1A. The concentration of NH_4^+ in AM decreased from 70.56 to 64.25 mg L^{-1} during 0–4 h, with an ammonium removal rate (ARR) of 1.79 mg $L^{-1}\cdot h^{-1}$ for NH_4^+. The content of NH_4^+ in AM decreased from 64.25 to 0 mg L^{-1} with the average ARR by R-1 was 5.35 mg $L^{-1}\cdot h^{-1}$ during 4–16 h. The highest ARR was 9.94 mg $L^{-1}\cdot h^{-1}$ at 16 h, which was close to the ARR of *Rhodococcus erythropolis* strain Y10 (9.69 mg $L^{-1}\cdot h^{-1}$),[6] but higher than the ARR of *Rhodococcus* sp. CPZ24 (3.1 mg $L^{-1}\cdot h^{-1}$), *Vibrio* sp. Y1-5 (2.65 mg $L^{-1}\cdot h^{-1}$), and *Pseudomonas mendocina* TJPU04 (4.69 mg $L^{-1}\cdot h^{-1}$).[17–19] These results indicated that strain R-1 has a strong NH_4^+ conversion ability. The growth curve with the OD_{600} values was consistent with a decrease in NH_4^+.

In contrast, the removal of NH_4^+ by strain R-1 and its growth in AM were obviously different under anaerobic conditions as shown in Figure 1B. The ARE reached 75.09% at 24 h, with the highest ARR of 2.91 mg $L^{-1}\cdot h^{-1}$ at 20 h, which was lower than the ARR under aerobic conditions. This may be due to the lack of available electron acceptors in AM under anaerobic conditions.[16] Energy derived only from the oxidation of organic carbon may be inefficient for ammonium assimilation during heterotrophic growth or respiration. A similar result reported that the removed NH_4^+ was mainly transformed into biological nitrogen through ammonium assimilation, with cell synthesis rates greatly inhibited under anaerobic conditions.[20] These results are not consistent with those of *Pseudomonas stutzeri* T13, which cannot utilize NH_4^+ as the sole nitrogen source to grow under anaerobic conditions.[16]

In addition, the accumulation of NO_2^- and NO_3^- was not observed in the AM of strain R-1 under aerobic or anaerobic conditions (Figures 1A and 1B), suggesting that NH_4^+ could support the growth and cell synthesis of strain R-1 as a preferable nitrogen source in the AM, which was in accordance with the previously reported strains, such as *Pseudomonas stutzeri* T13, *Ochrobactrum anthropic* LJ81, and *Rhodococcus erythropolis* Y10.[6,11,16] These results suggest that NH_4^+ was possibly removed through the assimilation pathway, but not through heterotrophic nitrification.

The NO_3^- removal performance and growth of strain R-1 under aerobic and anaerobic conditions

The kinetics of nitrate removal and growth of strain R-1 were analyzed in DM for 24 h under aerobic conditions, as shown in Figure 1C. The OD_{600} value of strain R-1 increased slowly during 0–4 h, and the nitrate removal rate (NRR) of strain R-1 reached 6.17 mg $L^{-1}\cdot h^{-1}$ at 4 h. The

OD_{600} value in DM then increased exponentially during 4–16 h, and the highest NRR was 18.05 mg $L^{-1}\cdot h^{-1}$ at 12 h. During 16–20 h, the OD_{600} value maintained stable, and the NRR decreased from 11.30 to 0.74 mg $L^{-1}\cdot h^{-1}$. Finally, the OD_{600} value decreased in the period 20–24 h, and the NRR of strain R-1 decreased to the lowest value of 0.74 mg $L^{-1}\cdot h^{-1}$, and the NRE reached 95.04% at 24 h.

The nitrate removal performance and bacterial growth in DM under anaerobic conditions are shown in Figure 1D. The NO_3^- content declined from 171.23 to 132.45 mg L^{-1}, and the NRR reached 5.59 mg $L^{-1}\cdot h^{-1}$ at 12 h. The NO_3^- content declined from 132.45 to 12.45 mg L^{-1} during 12–20 h and the highest NRR was 19.76 mg $L^{-1}\cdot h^{-1}$ at 20 h. The NRE reached 94.90% at 24 h under anaerobic conditions. The OD_{600} was 0.97 in the stable period (20–24 h), which was lower than the OD_{600} value observed under aerobic conditions (16 h, OD_{600} = 1.82). Similarly, *Pseudomonas stutzeri* T13 and *Paracoccus denitrificans* HY-1 have been reported to assimilate less nitrate into their biomass under anaerobic conditions than under aerobic conditions.[7,16] These results showed that strain R-1 could adopt NO_3^- as both a nitrogen source and an electron acceptor when it was supplied as the sole source under aerobic and anaerobic conditions. The maximal NRRs of strain R-1 in DM were 18.05 and 19.76 mg $L^{-1}\cdot h^{-1}$ under aerobic and anaerobic conditions, respectively, higher than stains of *Bacillus cereus* GS-5 (2.70 mg $L^{-1}\cdot h^{-1}$), *Paracoccus denitrificans* HY-1 (14.56 mg $L^{-1}\cdot h^{-1}$), and *Rhodococcus erythropolis* strain Y10 (5.43 mg $L^{-1}\cdot h^{-1}$),[6,7,21] which suggested that strain R-1 has a strong ability for NO_3^- conversion. During the whole NO_3^- removal process, NH_4^+ and NO_2^- were almost undetected, which was in accordance with the previously reported results for *Pseudomonas mendocina* X49 without NO_2^- accumulation.[22] However, *Pseudomonas stutzeri* T13 and *Rhodococcus erythropolis* strain Y10 were reported to accumulate NO_2^- during NO_3^- removal.[6,16] These differences may be due to the different NRRs among different ADB, different initial NO_3^- concentrations, or different detection times for the immediate products.

The synchronized dislodgement of NH_4^+ and NO_3^- and the growth of strain R-1 under aerobic and anaerobic conditions

The growth curve and simultaneous ammonium and nitrate removal by strain R-1 were determined in ADM cultured for 24 h under aerobic conditions, as shown in Figures 1E-1 and 1E-2. R-1 was in the lag phase in the first 4 h, with a slow increase in the OD_{600} from 0.05 to 0.07, which was accompanied by an ARE of 15.20% and no decrease in NO_3^- concentration at 4 h. The NH_4^+ concentration then decreased rapidly from 65.33 to 2.15 mg L^{-1} in the following 12 h, with the maximum ARR of 9.72 mg $L^{-1}\cdot h^{-1}$ between 12 and 16 h, and the OD_{600} of strain R-1 rapidly increasing from 0.07 to 1.66, which suggested that R-1 was in the logarithmic phase. At the same time, a rapid decline of the NO_3^- concentration was observed from 149.49 to 9.23 mg L^{-1} between 12 and 16 h, with a maximum NRR of 34.89 mg $L^{-1}\cdot h^{-1}$, and the NO_2^- content rapidly increased from 0.23 to 85.65 mg L^{-1}. In the final 8 h, the growth of strain R-1 reached the stationary phase, with a slow increase in OD_{600} from 1.66 to 1.97. Moreover, ARE and NRE were 98.37% and 100%, respectively, at 24 h and the NO_2^- concentration rapidly decreased from 85.65 to 0.24 mg L^{-1} during 16–20 h. The detection of NO_2^- at the logarithmic phase for strain R-1 could be due to the accumulation of intermediate products produced during denitrification, while microbiology autolysis may lead to a small quantity of NO_2^- reproduced after 20 h when infertile conditions occurred after nutrient depletion.[16,23]

The growth curve and simultaneous ammonium and nitrate removal by strain R-1 were determined in ADM cultured for 24 h under anaerobic conditions, as shown in Figures 1F-1 and 1F-2. The NH_4^+ concentration decreased slowly from 72.25 to 63.70 mg L^{-1} as it was used by a small amount of biomass, which increase from 0.02 to 0.04 in the initial 4 h under anaerobic conditions. The NH_4^+ concentration then rapidly decreased from 63.70 to 26.89 mg L^{-1} during 4–16 h, with an ARR of 3.98 mg L^{-1} h^{-1} from 8 to 12 h, and the OD_{600} increased from 0.04 to 0.61. Meanwhile, the NO_3^- content sharply declined from 179.29 to 3.95 mg L^{-1}, with a maximum NRR of 31.16 mg $L^{-1}\cdot h^{-1}$ from 12 to 16 h, while NO_2^- began to be detected from 8 h and reached a peak value of 74.52 mg L^{-1} at 16 h, with an average rate of increase of 9.32 mg $L^{-1}\cdot h^{-1}$. In the final 8 h, strain R-1 reached the stationary phase, with an increase in the OD_{600} value from 0.71 to 1.08, and a sharp reduction in the NO_2^- concentration from 74.52 to 0 mg L^{-1}. Meanwhile, the contents of NH_4^+ and NO_3^- decreased, with ARE and NRE of 100% at 24 h.

The maximal NRRs of strain R-1 in ADM were 34.89 and 31.16 mg $L^{-1}\cdot h^{-1}$ under aerobic and anaerobic conditions, respectively (Figures 1E-2 and 1F-2), which were obviously higher than that NRRs in DM with NO_3^- as the sole nitrogen source (Figures 1C and 1D), suggesting that the addition of NH_4^+ increased the denitrification rate and N_2 production for strain R-1 under both aerobic and anaerobic conditions. Similar results were found in studies of *Paracoccus denitrificans* HY-1 and *Rhodococcus erythropolis* strain Y10, which reported that the presence of NH_4^+ increased the NO_3^- denitrification rates.[6,7] Most prokaryotes can easily assimilate NH_4^+ as a preferable nitrogen source to synthesize biomacromolecule.[14] Thus, bacterial growth and reproduction are directly correlated with ammonium assimilation.[24] Increasing the initial NH_4^+ concentration supplied more plentiful nitrogen source for the cell synthesis of prokaryote, and then accelerated the biomass production through ammonium assimilation,[16] which provided sufficient material for NO_3^- denitrification. Although oxygen is absent under anaerobic conditions, NH_4^+ could also be effectively adopted as a nitrogen source through assimilation with NO_3^- as an electron acceptor, along with nitrate respiration to provide energy for assimilation.[16] Moreover, the aerobic and anaerobic denitrification efficiency enhanced by ammonium assimilation may be related to genes of the nitrogen metabolism pathway and transporters and genes associated with the other metabolism.[25]

The maximal ARR of strain R-1 was 5.92 mg $L^{-1}\cdot h^{-1}$ under anaerobic conditions in ADM (Figures 1F-2), which was two times higher than the ARR with NH_4^+ as the sole nitrogen source under anaerobic conditions (Figure 1B), suggesting that the existence of NO_3^- promotes the removal of NH_4^+ to biomass nitrogen. Similarly, the ARR increased from 3.75 to 5.70 mg $L^{-1}\cdot h^{-1}$ when adding NO_3^- to the novel denitrification bacteria *Ochrobactrum anthropic* LJ81.[11] Because cell synthesis belongs to a process of heterotrophic growth, organic carbon can serve as an electron donor to provide electrons for NO_3^- reduction and produce energy support for ammonium assimilation. Moreover, NO_3^- must be converted into improvable nitrogen sources via assimilative NO_3^- reduction for cell synthesis.[26] However, such a process may occur when NH_4^+ is exhausted, because strain R-1 prefers NH_4^+ over NO_3^- as a nitrogen source, similar to *Pseudomonas stutzeri* T13, *Rhodococcus erythropolis* Y10, *Acinetobacter haemolyticus* ZYL, and *Pseudomonas aeruginosa* P-1.[6,16,26,27]

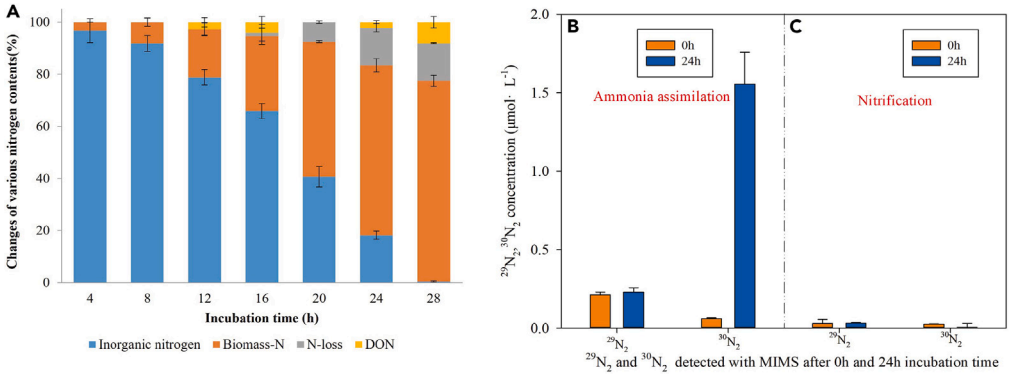

Figure 2. Ammonium removal mechanism of *Paracoccus denitrificans* R-1

(A) Ammonium removal process of strain R-1 based on nitrogen removal balance analysis. OD_{600} and the decrease of inorganic nitrogen (mainly NH_4^+), were detected during 0–28 h with samples taken every 4 h. Meanwhile, the increase of N-loss, dissolved organic nitrogen (DON), and biomass nitrogen (Biomass-N) were calculated.

(B and C) Ammonium removal process of strain R-1 based on $^{15}NH_4^+$ stable isotope tracer technique. (B) Verification of assimilation: ^{15}N-organic nitrogen (^{15}N-ON) as bacterial thallus of strain R-1 which might be labeled with $^{15}NH_4^+$ through ammonia assimilation in AM were digested with alkaline potassium persulfate at high temperature and was then used as substrate in DM. (C) Verification of nitrification: If $^{15}NO_2^-$ and $^{15}NO_3^-$ were labeled with $^{15}NH_4^+$ through nitrification in culture supernatant fluid in AM, they might be directly used as substrate in DM. Then $^{29}N_2$ and $^{30}N_2$ were detected with membrane injection mass spectrometer (MIMS) after 24 h culture under anaerobic conditions. See also Figure S2 and Table S1 in supplemental information 1. The error bars represent standard deviations of triplicate experiments in (A). The error bars represent standard deviations of duplicate experiments in (B) and (C).

The mechanism of ammonium removal by strain R-1 based on analysis of nitrogen balance and isotope tracing

Although heterotrophic nitrification accompanied by aerobic denitrification is widely accepted for ADB, some ADB may not follow this pathway. The nitrogen metabolic pathways for ADB are often deduced by conducting nitrogen intermediate product measurements corresponding to the key functional genes, as well as detection of the expressed enzyme activity and nitrogen balance calculations.[10,28] Due to the complex biological process of nitrogen transformation and the lack of reference genomes, the metabolic mechanisms whereby ADB utilize only one of the conventional methods remain elusive.[28] Therefore, it was necessary to integrate various methods to investigate the exact pathway of ammonium transformation by strain R-1.

The NH_4^+ metabolic pathway of strain R-1 was analyzed using a nitrogen balance experiment. Figure 2A presents the changes in different nitrogen species with time during the NH_4^+ removal process. Within 0–8 h, the inorganic nitrogen concentration decreased slowly, and the biomass-N increased from 3.33 to 8.16%. During 8–24 h, the inorganic nitrogen concentration decreased rapidly from 91.84 to 18.17%, and the biomass-N increased from 8.17 to 65.25%. During the final stage of the incubation, the NH_4^+ concentration decreased to 0.32 mg L^{-1}, with no accumulation of NO_2^- or NO_3^-, but a slight accumulation of dissolved organic nitrogen (DON) (supplemental information 1 Table S1). The variations in total nitrogen (TN) concentration in the entire incubation system showed that 77.06% NH_4^+ was transformed into biomass-N, 8.10% NH_4^+ was transformed into DON, and nitrogen loss was only 14.42% through gaseous or other forms (supplemental information 1 Table S1). These results suggest that the removal of NH_4^+ by strain R-1 occurred mainly through the assimilation pathway and not through nitrification. This result was similar to the results reported for *Paracoccus denitrificans* AC-3 and HY-1, which convert approximately 80.00% of NH_4^+ into intracellular nitrogen,[7,29] but was not consistent with the results reported for *Pseudomonas balearica* UFV3 and *Gordonia amicalis* UFV4, in which probably 55% of the NH_4^+ is converted into N_2 (g) and 45% is assimilated.[30] These results show that ammonium assimilation is the main NH_4^+-N transformation pathway in ADB.

However, the nitrogen balance detection method is based on nitrogen digestion, which unavoidably conceals the potential problems of nitrogen depletion during sample treatment. Moreover, the ammonium removed as a gas (N_2 or N_2O) through aerobic denitrification was mostly determined through deduction calculations from the detectable nitrogen, and the amount of gas transformed from ammonium has not been accurately detected in the reaction system thus far.[6,16,20] Thus, there is a "bottleneck" in the techniques used to detect various substrates during the nitrogen transformation process, and integrated techniques need to be conducted to determine the exact nitrogen metabolism pathways of ADB.

The $^{15}NH_4^+$ isotope tracer method was used to verify the NH_4^+ transformation pathway of strain R-1, as shown in Figures 2B and 2C. Verification of the $^{15}NH_4^+$ assimilation pathway was performed as follows: ^{15}N-NH_4^+ in AM→^{15}N-organic nitrogen in R-1 cells or biomass→^{15}N-NO_3^- obtained from the digestion of R-1 cells→$^{29}N_2$ or $^{30}N_2$ by reducing ^{15}N-NO_3^- by strain R-1 in DM (known as biomass group ^{15}N-ON). The culture supernatant was also used as a substrate for denitrification in DM to detect the presence of NO_3^- or NO_2^- (known as supernatant group ^{15}N-IN or $^{15}NO_3^-$ + $^{15}NO_2^-$). After 24 h of cultivation, the concentration of $^{30}N_2$ in the biomass group ^{15}N-ON increased from 0.06 to 1.56 μm·mol·L^{-1} (Figure 2B), while the content of $^{30}N_2$ in the supernatant group was unchanged (Figure 2C). The concentration of $^{29}N_2$ was unchanged in both groups. These results further demonstrate that $^{15}NH_4^+$ was transformed into ^{15}N-ON (biomass nitrogen) in strain R-1 through ammonium assimilation, but not through nitrification.

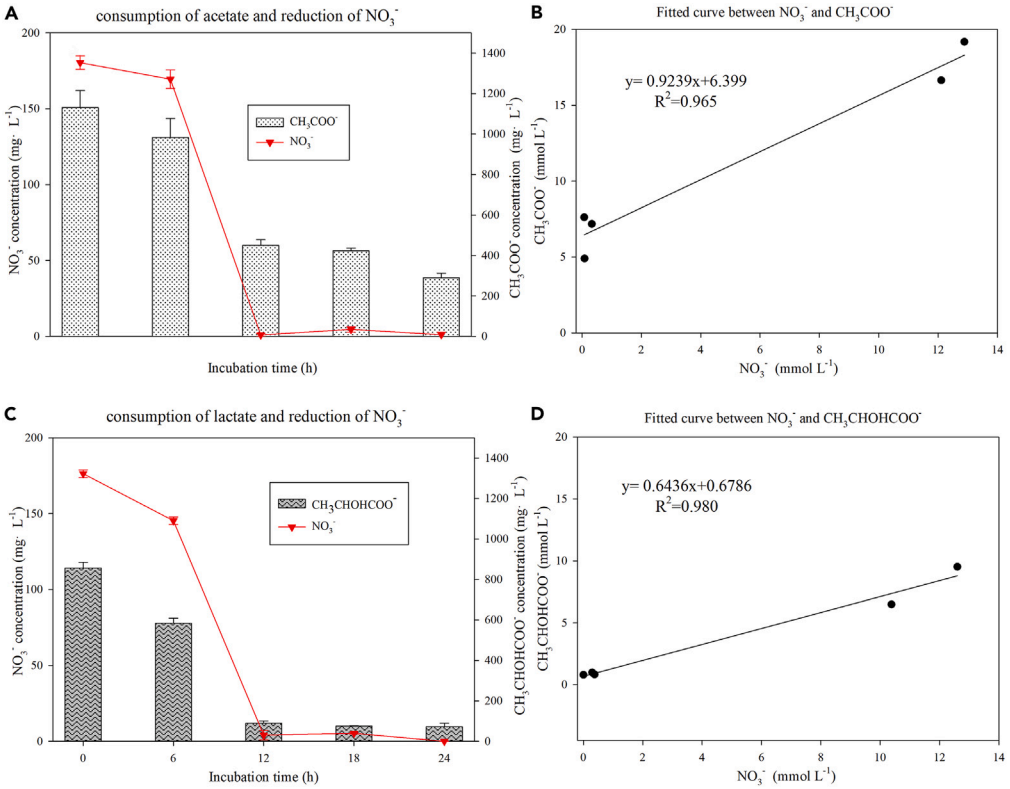

Figure 3. Stoichiometry of NO_3^- reduction coupled with the oxidation of electron donor by *Paracoccus denitrificans* R-1 under anaerobic conditions

(A) The consumption of acetate and the reduction of NO_3^- simultaneously detected.

(B) The fitted curves between NO_3^- reduction and acetate with different concentrations.

(C) The consumption of lactate and the reduction of NO_3^- simultaneously detected.

(D) The fitted curves between NO_3^- reduction and lactate with different concentrations. The error bars represent standard deviations of triplicate experiments. See also Figure S4 in supplemental information 1 and 2.

The removal mechanism of nitrate by strain R-1

The reduction of nitrate coupled to the oxidation of electron donors

To quantify the coupling relationship between the consumption of electron donors and the decrease in NO_3^- concentration, the oxidation of acetate or lactate and the reduction of NO_3^- were detected synchronously in DM with acetate or lactate as electron donors (Figure 3). The nearly complete disappearance of NO_3^- was accompanied by the consumption of acetate or lactate over time (Figures 3A and 3C). Moreover, a linear relationship was observed between the consumption of acetate or lactate and the decrease in NO_3^- concentration, with excess acetate or lactate and limited NO_3^- (Figures 3B and 3D). These results clearly show that the NO_3^- reduction by strain R-1 depends entirely on the consumption of electron donors.

The dissimilatory reduction of nitrate is an important nitrogen removal process that is usually required to provide electron donors for energy production. The redox potentials of various electron donors are related to the different efficiencies of nitrate reduction and energy conservation. The stoichiometric equation of acetate oxidation related to NO_3^- reduction was expressed by the metrological Equation 1,[31] using the cell molecular formula $C_5H_7NO_2$ proposed by Hoover and Porgess.[32] The molar ratio of oxidized acetate and NO_3^- reduced in the reaction process was calculated to be 0.902, which is slightly higher than the theoretical value of 0.819 (Equation 1). This result indicated that acetate was sufficiently oxidized for energy generation, and nitrate was eventually completely reduced to N_2, which was similar to Fe (II) oxidation coupled with nitrate reduction.[33] According to the chemical equation between glucose and NO_3^- [31], we speculated that the stoichiometric equation between lactate and nitrate is the metrological Equation 2. The experimental results showed that the lac/N (ratio of lactate to nitrate, mol/mol) molar ratio was 0.691 between lactate and NO_3^- consumed in the reaction process, which was lower than the theoretical value of 0.811.

$$0.819CH_3COOH + NO_3^- + NH4^+ \rightarrow 0.068C_5H_7NO_2 + HCO_3^- + 0.466N_2 + 0.301CO_2 + 0.902H_2O \quad \text{(Equation 1)}$$

$$0.811C_3H_6O_3 + NO_3^- + 0.932H^+ \rightarrow 0.203C_5H_7O_2N + 0.399N_2 + 1.419CO_2 + 2.189H_2O \quad \text{(Equation 2)}$$

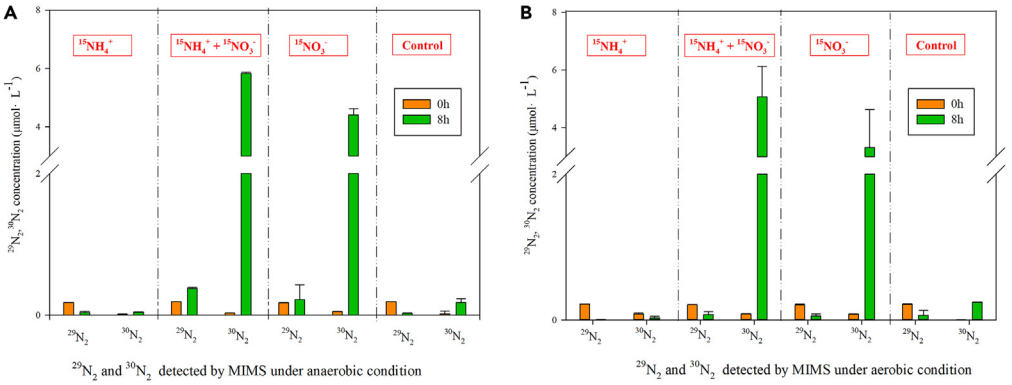

Figure 4. Ammonium and nitrate removal mechanism based on stable isotope tracer techniques

$^{15}NH_4^+$, $^{15}NO_3^-$, or $^{15}NH_4^+$ + $^{15}NO_3^-$ as substitute nitrogen sources were respectively added into the AM, DM, and ADM under (A) anaerobic and (B) aerobic conditions. Blank control experiments were included to test the effects of the medium in the absence of $^{15}NH_4^+$ and $^{15}NO_3^-$. The error bars represent standard deviations of duplicate experiments. See also Figure S3 in supplemental information 1.

Under aerobic conditions, the values of OD_{600} all reached approximately 2.0, both in AM and ADM with acetate as the electron donor (Figures 1A and 1E−1), suggesting that bacterial growth was not significantly different between the two media. Owing to the absolute advantage of O_2 in terms of redox potential, the growth of strain R-1 depends mainly on aerobic respiration with O_2 as an electron acceptor under aerobic conditions. Although nitrate reduction also occurred, it is possible that it was not the main pathway for energy conservation to support growth. However, the OD_{600} value was significantly higher in ADM (1.1) than in AM (0.5) under anaerobic conditions, with acetate as the electron donor (Figures 1B and 1F-1), suggesting that the nitrate reduction process was able to generate energy to support bacterial growth.

Thus, strain R-1 displayed remarkable characteristics of anaerobic nitrate respiration, which can conserve energy using nitrate as a terminal electron acceptor under anaerobic conditions. NO_3^- is transformed to N_2 through denitrification by receiving reducing force originated from the oxidation of acetate or lactate with the electron transport chain. Thermodynamic calculations also indicated that the oxidation of acetate or lactate, coupled with nitrate reduction and electron transmission, exhibited great potential to generate enough energy to maintain the growth and metabolism of strain R-1 (supplemental information 2).

The pathway of reduction of nitrate confirmed by ^{15}N isotope tracing

Isotope tracing experiments were designed to verify the NO_3^- removal mechanism under anaerobic and aerobic conditions (Figure 4). $^{30}N_2$ was generated with a net increase of 5.78 μmol L^{-1} in ADM with $^{15}NH_4^+$ and $^{15}NO_3^-$ added and 4.36 μmol L^{-1} in DM with $^{15}NO_3^-$ added under anaerobic conditions (Figure 4A), which was higher than 4.98 and 3.24 μm·mol·L^{-1}, respectively, observed under aerobic conditions (Figure 4B). $^{29}N_2$ remained at a low level in all of the aforementioned experiments, and $^{30}N_2$ was not detected in the control experiments without isotope-labeled nitrogen sources. These results suggested that strain R-1 removed NO_3^- through denitrification under both anaerobic and aerobic conditions. Moreover, the production of $^{30}N_2$ was higher in ADM than in DM under both anaerobic and aerobic conditions (Figures 4A and 4B), indicating that the presence of NH_4^+ promoted the transformation of NO_3^-. However, $^{30}N_2$ was not directly produced in AM with $^{15}NH_4^+$ added as the only nitrogen source, which further demonstrated that NH_4^+ was removed by ammonium assimilation, but not nitrification by strain R-1. These results are consistent with the aforementioned physiological and biochemical results of the strain R-1 nitrogen transformation process.

The genetic basis of nitrogen transformation by strain R-1

The nitrogen transformation processes and related functional genes (or enzymes) of strain R-1 were revealed using genomic analysis. Kyoto Encyclopedia of Genes and Genomes (KEGG) annotation indicated that multiple nitrogen metabolic pathways were encoded in the genome of strain R-1, including denitrification, ammonia assimilation, and nitroalkane oxidation (supplemental information 1 Figure S5). The enzyme names and the corresponding serial numbers shown in Figure S5 were listed in supplemental information 1 Table S2. In nitrogen dissimilation pathways, nitrate is reduced to nitrite by dissimilatory nitrate reductase, followed by the transformation of nitrite into ammonia by dissimilatory nitrite reductase (Nir S). Hydroxylamine and formadine are converted to ammonia by hydroxylamine reductase and formamidase, respectively. Ammonium is assimilated into glutamate by glutamine synthetase (GlnA), glutamate synthase (GltB), and glutamate dehydrogenase (GdhA) via the nitrogen assimilation pathway. In the nitrification pathway, only nitrite oxidoreductase (NxrA and NxrB) was detected, which oxidizes nitrite into nitrate, whereas ammonia monooxygenase (Amo) and hydroxylamine oxidase (Hao), which are crucial enzymes for the oxidation of ammonia to hydroxylamine and hydroxylamine to nitrite, respectively, were not detected in the genome of strain R-1. Additionally, nitroalkane monooxygenase, which catalyzes nitrite formation, was encoded in the genome of strain R-1. The intermediate NO_2^- can then be transformed into gaseous nitrogen or assimilated into R-1 cells.

Genome functional annotation revealed the presence of key enzymes involved in the transformation of NO_3^- and NO_2^- into N_2, including nitrate reductase (Nar GHI), Nir S, nitric oxide reductase (Nor B), and nitrous oxide reductase (Nos Z). Periplasmic nitrate reductase (Nap),

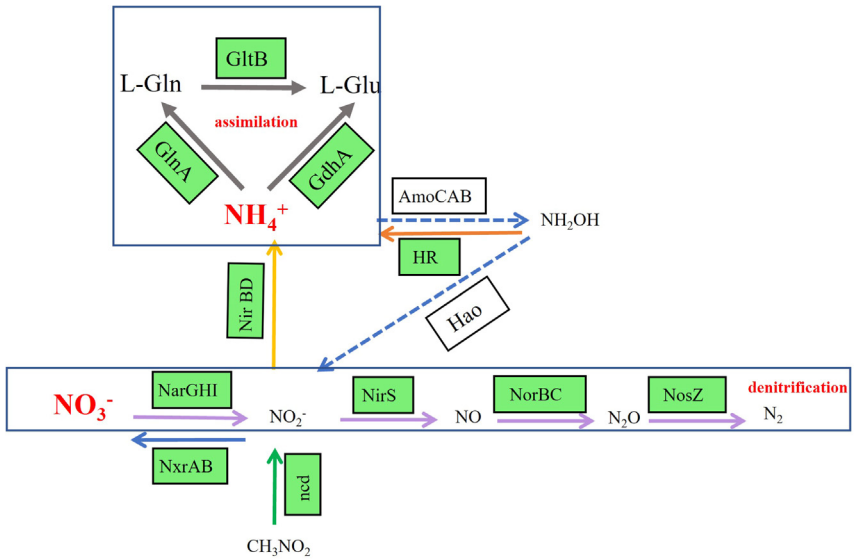

Figure 5. Nitrogen metabolism pathways of *Paracoccus denitrificans* R-1 annotated in KEGG database

The solid line arrow indicated the existence of the pathway, and the dotted line arrow indicated the inexistence of the pathway. (Nrt, nitrate/nitrite transporter; NarGHI, nitrate reductase; NorBC, nitric oxide reductase gene; NosZ, nitrous oxide reductase; GlnA, glutamine synthetase; GltB, glutamic acid synthetase; GdhA, glutamate dehydrogenase; NirS, nitrite reductase; Hcp, hydroxylamine reductase; Ncd2, nitrate monooxygenase; AmoCAB, ammonia monooxygenase; Hao, hydroxylamine oxidase). See also Figure S5 and Table S2 in supplemental information 1.

which is mainly expressed under aerobic conditions, is involved in aerobic denitrification.[34,35] Nevertheless, a new electron transport chain, as a hypothesis for aerobic denitrification in the presence of Nar and Nir, may transmit electrons to NO_3^- under aerobic conditions without the restriction of the presence or absence of oxygen.[34,36] Therefore, the presence of NarG, NirS, NorB, and NosZ in strain R-1, based on metagenomics analysis (supplemental information 1 Figure S5), further confirmed that strain R-1 could execute the complete denitrification pathway ($NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$) to degrade NO_3^- under both aerobic and anaerobic conditions (Figure 5).

The existence of N-related genes may provide direct evidence of the number of N-metabolism enzymes in bacteria. The presence of *amo*A in the genome of *Halomonas* sp. *strain* B01 indicates that it can conduct heterotrophic nitrification at high salt concentrations.[37] *Exiguobacterium mexicanum* SND-01 has been proposed to possess a heterotrophic nitrification pathway via the *hao* gene.[38] In contrast, Amo and Hao enzyme activities were undetected in *Rhodococcus erythropolis* Y10, suggesting that NH_4^+ is transformed by strain Y10 via ammonium assimilation, but not by nitrification.[6] Based on metagenomics analysis, the presence of *gln*A, *glt*B, and *gdh*A and absence of *amo*A confirmed that strain R-1 could utilize NH_4^+ as a nitrogen source via ammonium assimilation for cell propagation (supplemental information 1 Figure S5). Ammonium can be transformed into glutamate via the glutamate dehydrogenase (GDH) pathway to promote cell propagation, which may take place preferentially in ADB at high ammonium concentrations.[12] It has been speculated that the expression of the GDH-related genes *gln*A (glutamate-ammonia ligase) and *glt*B (glutamate synthase large subunit) may be pivotal for the excellent nitrogen removal performance of strain R-1, which coincides with the novel ADB *Acinetobacter* sp. TAC-1.[39] Ammonium assimilation is beneficial for the growth and propagation of bacteria and is convenient for the absorption and utilization of inorganic nitrogen.[24] Therefore, the gene metagenomics and annotation results both indicated that strain R-1 could assimilate ammonium and complete denitrification (Figure 5), which was consistent with the above physiological and biochemical results of nitrogen transformation process for strain R-1.

The biotechnological significance of understanding the mechanism of nitrogen removal by strain R-1

In summary, the overall mechanism of the simultaneous removal of NH_4^+ and NO_3^- by strain R-1 is summarized in the model shown in Figure 6. First, ammonium assimilation provides a nitrogen source for strain R-1, which supplies a nitrogen nutrient substance for cell biosynthesis. Second, the reduction in nitrate dissimilation in strain R-1 provides energy for cell growth and metabolism. In general, ammonium removal in bioreactors is frequently attributed to heterotrophic nitrification, but the significant role of ammonium assimilation has often been neglected because of the lack of understanding of the mechanism of microbial ammonium removal.[16,28] Our findings indicate that ammonia assimilation is an important pathway for nitrogen removal from bioreactors.

Our research showed that strain R-1 can effectively remove ammonium and nitrate under both aerobic and anaerobic conditions, which inspired us to use strain R-1 to enhance nitrogen removal from biological reactors. In the aerobic stage, strain R-1 can effectively remove ammonium mineralized from organic matter, while some nitrate and nitrite may be lost due to aerobic denitrification. In the anaerobic or hypoxic stage, strain R-1 mainly removes nitrate. By strengthening both the aerobic and anaerobic stages, nitrogen removal efficiency can be greatly improved. The results of this study will facilitate the development of culturable ADB as prospective candidates for nitrogen pollution management and biological remediation in various environments.

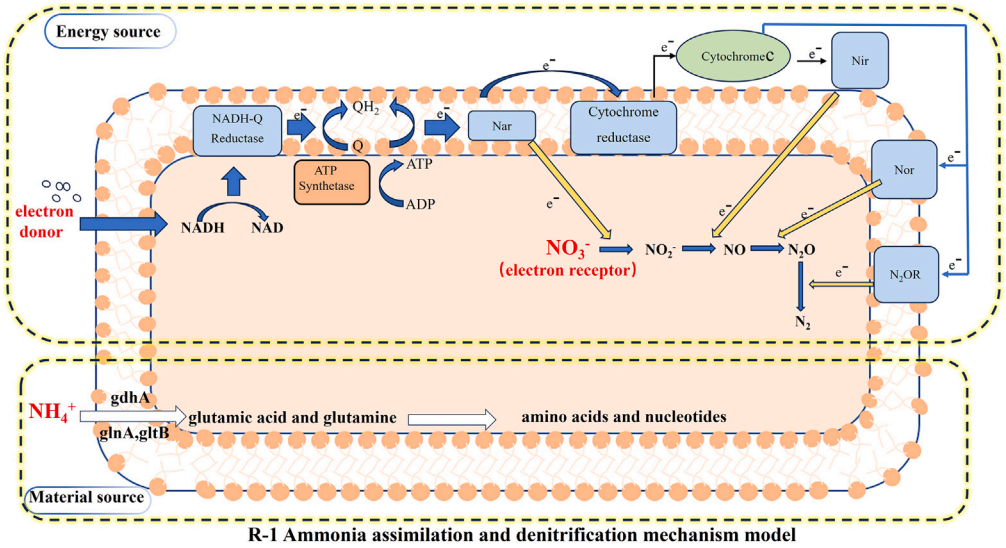

Figure 6. Ammonia assimilation and denitrification mechanism model for *Paracoccus denitrificans* R-1

The ammonium assimilation provides a nitrogen substance for cell biosynthesis of strain R-1. The nitrate reduction in dissimilation of strain R-1 provides energy for cell growth and metabolism. See also Figures 1–5 and Tables S1 and S2 in supplemental information 1.

Conclusions

In this study, the highly efficient ADB strain *Paracoccus denitrificans* R-1 exhibited prominent capacity for simultaneous ammonium and nitrate removal under aerobic and anaerobic conditions. Evidence provided through combined analysis of nitrogen balance, ^{15}N isotope tracing and metagenomic techniques demonstrated that strain R-1 utilizes NH_4^+ through assimilation, rather than heterotrophic nitrification, and it synchronously removes nitrate through denitrification under aerobic and anaerobic conditions. Moreover, ammonium assimilation and nitrate denitrification effectively promoted each other. In addition, nitrate reduction by strain R-1 was shown to be a biochemical or electron transport process coupled with the oxidation of electron donors through stoichiometric and thermodynamic analyses. In conclusion, our results provide novel insights into the nitrogen removal mechanism of ADB, which may be helpful in establishing a theoretical basis for the practical application of simultaneous ammonium and nitrate removal processes in the dispose of wastewater with high contents of various inorganic nitrogen in bioreactors.

STAR★METHODS

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  - Nitrogen removal processes of R-1 under aerobic and anaerobic conditions
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  - Ammonium removal based on isotope tracing analysis
  - Analysis of nitrate and ammonium removal mechanism based on isotope tracing
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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2024.110599.

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AUTHOR CONTRIBUTIONS

Y.H. designed research; C.H. and J.W. conducted the field campaign and performed the isotopic measurements; W.S. and C.H. analyzed data; W.S. and C.H. drafted the paper and wrote the paper with input from all coauthors; J.-G.L. and M.W. reviewed and revised the paper.

DECLARATION OF INTERESTS

The authors declare no competing interest.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Critical commercial assays		
NH_4Cl	sigma	CAS:12125-02-9
KNO_3	sigma	CAS: 7757-79-1
$Na_2HPO_4 \cdot 7H_2O$	sigma	CAS: 7782-85-6
KH_2PO_4	sigma	CAS: 7778-77-0
$CH_3COONa \cdot 7H_2O$	sigma	CAS: 127-09-3
formate	sigma	CAS: 141-53-7
succinate	sigma	CAS: 150-90-3
pyruvate	sigma	CAS: 127-17-3
lactate	sigma	CAS: 312-85-6
glucose	sigma	CAS: 14431-43-7
$^{15}NH_4^+$	Shanghai Institute of Isotopes	CAS: 39466-62-1
$^{15}NO_3^-$	Shanghai Institute of Isotopes	CAS: 31432-45-8
$ZnCl_2$	sigma	CAS: 7646-85-7
HiPure soil DNA kit	Magen	D3142-01
End Prep Enzyme Mix	Baiolaibo	NGS Fast DNA Library Prep Set for Illumina
Deposited data		
The raw reads of the genome sequence of *Paracoccus denitrificans* R-1	This study	Bioproject: CP087986, CP087987, and CP087988
Software and algorithms		
Kyoto Encyclopedia of Genes and Genomes data library (KEGG)	https://www.kegg.jp/kegg/	https://www.kegg.jp/kegg/
Sigmaplot 12.5	https://systatsoftware.com/sigmaplot/	https://systatsoftware.com/sigmaplot/
Other		
NanoDrop Lite	Thermo Fisher Scientific	https://www.thermofisher.com/order/catalog/product/ND-LITE-PR
Illumina sequencing	Illumina	NovaSeq PE150 platform

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources should be directed to and will be fulfilled by the lead contact, Yiguo Hong (yghong@gzhu.edu.cn).

Materials availability

This study did not generate new unique reagents.

Data and code availability

- The genome sequence of *Paracoccus denitrificans* R-1was deposited in GenBank under the accession numbers CP087986, CP087987, and CP087988.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

METHOD DETAILS

Strain, medium, and cultivation

Paracoccus denitrificans R-1 was isolated from a sewage treatment sludge in the Xinfeng Wastewater Disposal Plant.[15] The denitrification medium (named as DM) used in this study was composed of $KNO_3$1.2 g L^{-1}, $Na_2HPO_4{\cdot}7H_2O$ 7.9 g L^{-1}, KH_2PO_4 1.5 g L^{-1}, $CH_3COONa{\cdot}7H_2O$ 4.7 g L^{-1}, and trace element solution 2 mL L^{-1}, as reported previously.[40] The above DM medium was respectively added NH_4Cl 0.3 g L^{-1} (named as AM) or NH_4Cl 0.3 g L^{-1} + KNO_3 1.2 g L^{-1} (named as ADM) as replacement of KNO_3, which was used to test the strain R-1 capability for removal performance of ammonium or simultaneous ammonium and nitrate.

The R-1 bacterial solution was added to the DM and cultured for 12 h until the logarithmic phase of bacterial growth was attained. The culture was centrifuged, the supernatant was disposed, and the bacterial precipitate was rinsed with sterile water for three times. Then, 1 mL of the DM solution was added to form a bacterial suspension, which was used as the seed liquid. The equation OD600 (seed liquid) × V1 = 100 mL * 0.05 was used to calculate the inoculation volume, V_1, which ensured that the optical density at 600 nm (OD_{600}) of strain R-1 was consistently 0.05 in the culture media for the experimental groups at the initial cultivation stage.

The prepared seed culture was added to a 250 mL conical flask with 100 mL sterile medium and incubated for 24 h at 30°C with a rotational speed of 150 rpm for aerobic cultivation (Supplemental information 1 Figure S1A). The conical flasks were replaced with anaerobic bottles to ensure an anaerobic culture system (Supplemental information 1 Figure S1B). Anaerobic culture technologies were based on those reported in a previous study.[41] High-purity helium (He) was subjected to sterile filtration before use. The prepared medium was split into 100 mL anaerobic bottles sealed with septa and flushed with sterilized high-purity He for 10 min. After inoculation with seed liquid, the bottles were cultured at 30°C. The cultures were sampled at different time points (2 and 4 h or 0, 6, 12, 18, and 24 h). The biomass was determined by measuring the OD_{600}, and the concentrations of NH_4^+, NO_2^-, and NO_3^- were determined using a rapid spectrophotometry method.[42,43]

Effect of different electron donors on strain R-1 growth and nitrogen removal under aerobic and anaerobic conditions

To explore the coupling of the growth and nitrogen transformation of strain R-1 with the oxidation of electron donors, R-1 cells were cultured in AM, DM, and ADM containing different electron donors (formate, acetate, succinate, pyruvate, lactate, and glucose) with an initial C/N ratio of 9.65. The concentrations of electron donors were determined by ion chromatography, as described in a previous report.[44] The stoichiometric equation for denitrification with the selected carbon sources was deduced by measuring the content of electron donors and nitrates as acceptors in the determination system. The above test was all conducted in triplicate.

Nitrogen removal processes of R-1 under aerobic and anaerobic conditions

The nitrogen removal kinetics of R-1 were explored in AM, DM, and ADM using sodium acetate as an electron donor under aerobic and anaerobic conditions. The ammonium or NRE(ARE or NRE) and rate (ARR or NRR) were determined according to the formulae: ARE or NRE (%) $=\frac{(A-B)}{A}\times 100$ and ARR or NRR ($mg{\cdot}L^{-1}{\cdot}h^{-1}$) $=\frac{(C-D)}{T}$, where A is the initial ammonium or nitrate concentration, B is the observed ammonium or nitrate concentration, C and D are the observed ammonium or nitrate concentrations between adjacent sampling times, and T is the time phase between adjacent samplings. The above analysis was all conducted in duplicate.

Nitrogen balance analysis of ammonium removal

Nitrogen balance analysis was used to identify the NH_4^+ removal pathway. The seed liquid of strain R-1 was injected into 250 mL triangular flasks including 100 mL of AM, which then cultured for 28 h at 30°C and 150 rpm. Bacterial suspensions were sampled every 4 h for determining the biomass concentration, OD_{600}, and TN concentration of the culture. The contents of NH_4^+, NO_2^-, NO_3^-, and TN' in the liquid supernatant were determined after centrifugation (8,000 × g, 10 min), and the DON concentration was calculated as the TN concentration minus the sum of the inorganic nitrogen concentrations. Biomass N was calculated as TN minus TN' concentrations.[20] Nitrogen loss (N-loss) was determined as the initial TN concentration minus the final TN concentration after cultivation in non-centrifuged medium. The above test was all conducted in triplicate.

Ammonium removal based on isotope tracing analysis

The seed liquid of strain R-1 was injected into 250 mL triangular flasks including 100 mL of AM, with NH_4^+ replaced with the stable isotope $^{15}NH_4^+$. After 24 h of culture, R-1 cells and supernatants were collected by centrifugation. The R-1 cells were washed three times with ultrapure water and transferred to a clean colorimetric tube. After the addition of an alkaline potassium persulfate solution, the cells were digested at 121°C for 30 min. If $^{15}NH_4^+$ assimilation occurred during AM cultivation, the organic nitrogen in the R-1 cells was labeled with ^{15}N (^{15}N-ON) and released as ^{15}N-labeled inorganic nitrogen after digestion. The digested solutions were adjusted to pH 7 and then transferred to a 60 mL serum bottle, which was then used as the nitrogen source in DM and inoculated with R-1 seed liquid for 24 h of cultivation (Supplemental information 1 Figure S2A). Meanwhile, the supernatants in AM obtained after centrifugation were directly used as a nitrogen source in DM (known as ^{15}N-IN), which were added to the serum bottle and then inoculated with the seed liquid of strain R-1 (Supplemental information 1 Figure S2B). After 24 h of cultivation, a MIMS was used to determine the generation of $^{29}N_2$ and $^{30}N_2$ in DM.[42] The variation in $^{30}N_2$ concentration after cultivation in these two groups of experiments was used to determine whether NH_4^+ was assimilated into the organic nitrogen of R-1 cells or nitrified to NO_2^- and NO_3^- in the supernatants. The above test was all conducted in duplicate.

Analysis of nitrate and ammonium removal mechanism based on isotope tracing

$^{15}NH_4^+$ (200 μmol L^{-1}), $^{15}NO_3^-$ (200 μmol L^{-1}), or $^{15}NH_4^+$ (200 μmol L^{-1}) + $^{15}NO_3^-$ (200 μmol L^{-1}) were respectively added into the AM, DM, and ADM as substitute nitrogen sources under aerobic and anaerobic conditions (Supplemental information 1 Figure S3). Sterilized media were injected into 60 mL serum bottles with no headspace and aerated with high-purity He for 10 min. The seed liquid of R-1 was inoculated into serum bottles sealed with butyl rubber stoppers and cultured at 30°C. The culture systems used for the aerobic experiments comprised 30 mL of culture medium and 30 mL of headspace to ensure dissolved oxygen in the media. The experimental settings were the same as those used for the anaerobic experiments described above. Blank control experiments were included to test the effects of the medium in the absence of $^{15}NH_4^+$ and $^{15}NO_3^-$. At 0 and 8 h, 2 mL of 50% $ZnCl_2$ solution was injected using a syringe to inactivate the bacterial cells in the culture systems, which were then transferred to a dark environment for 24 h of precipitation. A MIMS was used to determine the contents of $^{29}N_2$ and $^{30}N_2$ produced in the culture systems, as previously described.[45] The above test was all conducted in duplicate.

Genome sequencing and gene annotations to nitrogen transformation pathways

Total genomic DNA was extracted from strain R-1 using a HiPure soil DNA kit (Magen, Guangzhou China). DNA was fragmented into 300–350 bp segments to construct a paired-end library. The end repair of sequences (including phosphorylation of the 5′ end and the addition of A to the 3′ end) was carried out using End Prep Enzyme Mix (NGS Fast DNA Library Prep Set for Illumina, Baiolaibo, Beijing China), and sequencing adapters were added to both ends. Magnetic beads were used to purify the fragments.[46] The NovaSeq PE150 platform (Illumina, San Diego, CA, USA) was used to conduct paired-ended sequencing (Azenta Biotech, Suzhou, China) after mixing the DNA libraries labeled with different indices. Sequencing and raw data processing was performed as described in detail in a previous report.[15] Sequencing data were analyzed by Azenta Biotech (Suzhou, China). The key genes encoding important enzymes closely related to bacterial nitrogen metabolism were recognized by annotation using the Kyoto Encyclopedia of Genes and Genomes data library (KEGG, https://www.kegg.jp/kegg/).