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Characterization of the Nitrogen Removal Potential of Two Newly Isolated *Acinetobacter* Strains under Low Temperature

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Abstract: Excess nitrogen and phosphorus in the water causes several ecological problems for nutrients. Biological nitrogen removal is an economical and efficient way to prevent excessive nitrogen in the environment. For most areas of China, temperatures are usually lower than 20 °C except during the summertime. It is necessary to discover microbes that can efficiently remove nitrogen at low temperatures. In this study, two *Acinetobacter* strains were isolated from a sample in a wastewater tank in Taizhou for their capabilities to remove NO_3^-–N and NO_2^-–N at 15 °C. Heterotrophic nitrification experiments showed that both strains could efficiently remove nitrogen from the culture medium. The maximum removal rates of NH_4^+–N were 3.15 mg/L·h and 4.74 mg/L·h for heterotrophic nitrification by the strains F and H, respectively. Strain H grew faster and removed both nitrite and nitrate more efficiently than strain F. Genome sequencing showed that strains F and H could be classified into *Acinetobacter johnsonii* and *Acinetobacter bereziniae*, respectively. NO_2^-–N (100 mg/L) was completely removed in 3 days by strain H. The maximum NO_3^-–N removal rate was 3.53 mg/L·h for strain F. When strain H was cultured in a broth with 200 mg/L NO_3^-–N, 97.46% of NH_4^+–N (200 mg/L) was removed in 5 days, and the maximum NH_4^+–N removal rate was 4.04 mg/L·h. Genomic sequence analysis showed that both the strains lacked genes involved in the denitrification pathway that transforms NO_3^- into N_2. This implies that nitrate or nitrite is removed through the nitrogen assimilation pathway. Genes responsible for nitrate assimilation are clustered together with molybdopterin cofactor biosynthesis genes. Strain H contains fewer resistance genes and transfer elements. All the above data demonstrate that strain H is a promising candidate for nitrogen removal at lower temperatures. But there is still a lot to be done to systematically evaluate the potential of *A. bereziniae* strain H in treating wastewater at a pilot scale. These include the long-term performance, environmental tolerance, and nitrogen removal efficiency in wastewater. And the application of these *Acinetobacter* strains in diverse wastewater treatment settings might require careful optimization and real-time monitoring.

Keywords: biological nitrogen removal; *Acinetobacter* strains; wastewater treatment; low temperature; heterotrophic nitrification

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1. Introduction

Excessive nitrogen compounds can cause serious problems in wastewater processing and eutrophication of the receiving water. These problems threaten ecological safety and sustainable development [1]. People have invested considerable financial resources in controlling and reducing the nitrogen level in wastewater in recent decades. Compared to physical and chemical treatment approaches, biological treatment is becoming a favorable choice due to its high efficiency, lack of secondary pollution, low cost, and environmental friendliness. Traditional biological nitrogen removal processes usually include the separate

use of autotrophic nitrifiers and heterotrophic denitrifiers. This approach has to be applied differently under anaerobic and aerobic conditions. This results in high facility costs, long time consumption, and high energy consumption [2,3].

Thiosphaera pantotropha was the first isolate capable of combined heterotrophic nitrification and aerobic denitrification (HN-AD) [4]. This allows the processing of nitrification and denitrification simultaneously within one cell [5]. Since then, a large number of HN-AD bacteria have been isolated and characterized. Strains from the genera *Paracoccus*, *Bacillus*, *Pseudomonas*, *Alcaligenes,* and *Acinetobacter* have been widely reported as HN-AD strains for their higher growth rate, stronger environmental adaptability, and ability to utilize organics [6–10]. HN-AD strains may remove nitrogen through different bioconversion pathways, such as assimilation, dissimilation, nitrification, and denitrification [11]. Systematic characterization of HN-AD bacteria is key to evaluating their practical application potential. However, the total nitrogen (TN) removal rate of HN-AD bacteria is often affected by the high accumulation of nitrite when ammonia and nitrate simultaneously serve as the nitrogen source [12,13]. In particular, nitrite accumulation is toxic to some environmental bacteria and limits the stability of nitrogen removal application [14]. Therefore, it is significantly important to discover and characterize novel microorganisms to remediate multiple nitrogen pollutants without nitrite accumulation.

Presently, most reported nitrogen-removing microorganisms are mesophilic, performing nitrification, and denitrification at optimal temperatures between 25 °C and 37 °C [15,16]. In most areas of China, temperatures lower than 20 °C occur over long periods throughout the year. Lower temperatures significantly limit the capabilities and applications of these HN-AD bacteria [17]. Thus, it is necessary to isolate and fully characterize microorganisms that can efficiently remove nitrogen at lower temperatures.

Herein, we report the isolation of two metabolically versatile *Acinetobacter* sp. strains (strains H and F) from a pharmaceutical and chemical wastewater treatment plant in winter. The nitrogen removal performances of strains F and H for high concentrations of ammonium, nitrate, and nitrite were investigated under low-temperature conditions (15 °C). Moreover, the genomes of both strains F and H were sequenced, and the nitrogen metabolism pathways and genetic risks were analyzed and compared.

2. Materials and Methods

2.1. Strain and Culture Conditions

Strains H and F were isolated from wastewater treatment tanks in winter in Taizhou, China. Luria–Bertani Agar (LA, containing 10 g tryptone, 5 g yeast extract, and 10 g NaCl per liter, pH 7.2) and bromothymol blue agar (BTB, containing 1.0 g KNO_3, 1.0 g KH_2PO_4, 1.0 g K_2HPO_4, 8.5 g sodium succinate, 0.5 g $FeCl_3$, 0.2 g $CaCl_2$, 1.0 g $MgSO_4$, 1 mL bromothymol blue (1% in ethanol), and 15 g agar per liter) were obtained. The media used for nitrification and denitrification assays are listed in Table 1.

Table 1. Media used for nitrification and denitrification assays.

No.	Name	Composition (/L)	Reference
1	NM medium	7.0 g K_2HPO_4, 3.0 g KH_2PO_4, 0.1 g $MgSO_4{\cdot}7H_2O$, 1.0 g $(NH_4)_2SO_4$, 0.05 g $FeSO_4{\cdot}7H_2O$ and 10 g CH_3COONa	[18]
2	DM medium	7.0 g K_2HPO_4, 3.0 g KH_2PO_4, 0.1 g $MgSO_4{\cdot}7H_2O$, 1.8 g KNO_3 (DM-1) or 0.986 g $NaNO_2$ (DM-2), 0.05 g $FeSO_4{\cdot}7H_2O$ and 10 g CH_3COONa	
3	SND medium	7.0 g K_2HPO_4, 3.0 g KH_2PO_4, 0.1 g $MgSO_4{\cdot}7H_2O$, 1.0 g $(NH_4)_2SO_4$, 1.8 g KNO_3 (SND-1) or 0.986 g $NaNO_2$ (SND-2), 0.05 g $FeSO_4{\cdot}7H_2O$ and 10 g CH_3COONa	

A nitrification medium (NM) was used to determine the ammonium removal ability. The denitrification medium (DM) was used to determine the denitrification ability of nitrate (or nitrite). Simultaneous nitrification and denitrification medium (SND) was used to assess the simultaneous nitrification and aerobic performance with ammonium and nitrate (or nitrite). The initial pH of all media was set at 7.2, and all the above media were sterilized for 20 min at 0.11 MPa and 121 °C.

2.2. Temperature Characteristics

To determine the optimum growth temperature, the isolate was cultured in a 150 mL DM medium in 250 mL flasks inoculated with 5 mL preculture at 10, 15, 20, 30, and 37 °C. After 7 d of stationary cultivation, 3 mL of the culture was sampled to examine the cell optical density (OD_{600}).

2.3. Estimation of Nitrogen Removal Capacity

A single colony of strain F (or strain H) was inoculated into 50 mL of DM medium and cultured at 150 r/min and 15 °C for 24 h. The precultured strain was centrifuged at 10,000 r/min, washed once with sterilized water, and resuspended in sterilized water. Then, the cells were inoculated into 50 mL of NM, DM-1, DM-2, SND-1, or SND-2 and incubated at 15 °C under aerobic conditions at 150 r/min. Different medium samples were taken and measured to investigate the cell density (OD_{600}), ammonium, nitrate, nitrite, and total nitrogen concentrations at 24 h intervals. All experiments were conducted in triplicate.

2.4. Gas Detection in Sealed Serum Bottles

A total of 300 mL glass serum bottles were filled with 100 mL basal medium and inoculated with 5 mL bacterial suspensions of the isolate in triplicate. These bottles were then fully aerated with pure oxygen gas, tightly sealed with a rubber septum, and cultivated at 15 °C and 150 r/min. A system without a bacterial inoculum was used as a control. Gas samples (10 mL) were collected periodically using a gas-tight syringe to detect N_2 and N_2O using gas chromatography.

2.5. Analytical Methods

The cell density was monitored by OD_{600} using a spectrophotometer. Before analysis, all bacterial samples were appropriately diluted with sterile water to achieve an OD_{600} reading between 0.2 and 0.7.

Ammonium, nitrate, nitrite, and total nitrogen were detected according to the standard methods of the National Environmental Bureau of China [19]. All the samples were centrifuged at 10,000 rpm for 5 min before analysis. Ammonium nitrogen was analyzed using Nessler's reagent spectrophotometry. Nitrate nitrogen was calculated by subtracting the background absorbance value at 275 nm twice from the absorbance value at 220 nm using the UV spectrophotometric method. Nitrite nitrogen was determined using the N-(1-naphthyl)-ethylenediamine method. Total nitrogen was calculated by subtracting the background absorbance value at 275 nm twice from the absorbance value at 220 nm using the alkaline potassium persulfate digestion-UV spectrophotometric method.

2.6. Statistical Analysis and Graphical Work

The efficiency of N removal = $(C_0 - C_1)/C_0 \times 100\%$, where C_0 and C_1 are the N concentrations at the start and finish time points; rate of N removal = $(C_A - C_B)/(T_B - T_A)$, where C_A and C_B ($mg \cdot L^{-1}$) were the N concentrations at times T_A and T_B, respectively, and T_A was the start time and T_B was the end time of the experiment [20]. Statistical analysis and graphical work were carried out by using Excel and GraphPad Prism 5.0. The results are presented as the means ± SDs (standard deviation of means).

2.7. Genome Sequence and Analysis

The cell cultures of both strains were sent to a sequencing company on dry ice. The sequencing DNA libraries were constructed with a TruSeq^{TM} DNA Sample Prep Kit according to the manufacturer's instructions. Paired-end sequencing was carried out on the Illumina NovaSeq platform. The clean data were assembled with SPAdes (v3.12.0) in a de novo approach [21]. The ORFs were predicted with GeneMarkS (v4.32) and annotated with NCBI NR (v20171010). The comparison of genomes was carried out with Mauve (V2.3.1). A phylogenetic tree was constructed using Mega software (v10.0.5). This whole-genome shotgun project of strains F and H has been deposited at DDBJ/ENA/GenBank under the accession numbers JAOWAJ000000000 and JAOWAI000000000, respectively.

3. Results

3.1. Isolation and Characterization of Strains F and H

After the enrichment of the denitrification bacteria with 1/10 (V/V) DM-1, the wastewater samples were spread on bromothymol blue agar plates and incubated at 15 °C for 5 days. Twelve candidate colonies with a larger blue color surrounding the zone were picked, and the nitrate removal ability was further compared in DM-1 media. Strains F and H, which have circular and salient colonies with regular edges and smooth surfaces (Figure 1A), showed the highest efficiency in removing nitrate from the culture broth.

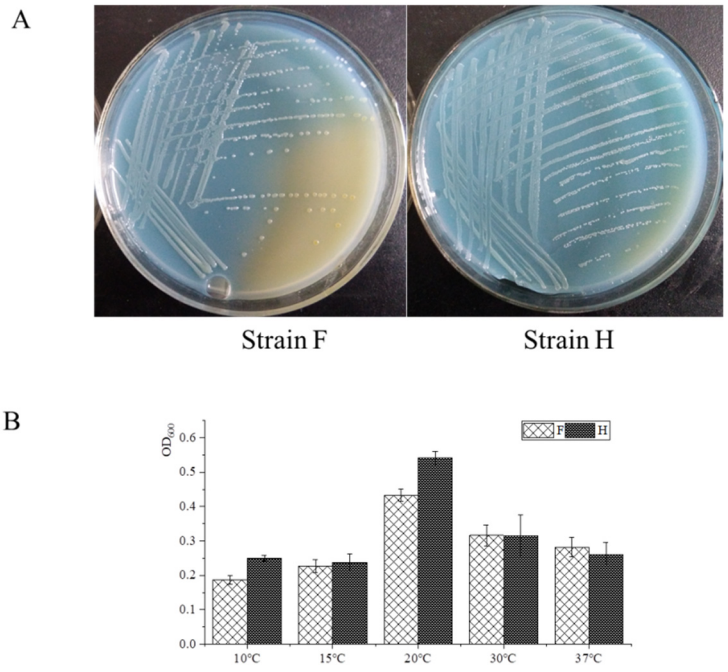


Figure 1. Characterization of strains F and H. (**A**) Colonies on bromothymol blue agar plates; (**B**) growth of both strains at different temperatures. Values are shown as the means ± SDs for three replicates.

Partial *gyrB* sequences of strains F and H were obtained via PCR amplification with primer pairs and sent for Sanger DNA sequencing. The *gyrB* fragments of strains F and H showed 99.88% and 100% similarities to those of *Acinetobacter johnsonii* XBB1 and *Acinetobacter bereziniae* KCTC 23199, respectively (data not shown). These two strains belonged to the genus *Acinetobacter*.

The optimum growth temperature for these two strains was investigated by monitoring their growth in DM-1 media for 7 days at 10, 15, 20, 30, and 37 °C. The optical density (OD_{600}) values of the culture broth were recorded at 24 h intervals from the samples of each strain. Under most temperatures, strain H grew faster than strain F (Figure 1B). Both strains F and H achieved the highest OD_{600} values at 20 °C. Obviously, 20 °C was the optimum

growth temperature for both strains. To investigate their potential under low temperatures, all following experiments were carried out at 15 °C.

3.2. Evaluation of the Removal Capability of Ammonium

The ammonium removal ability was examined using $(NH_4)_2SO_4$ as the sole nitrogen source at 15 °C. The curves of cell growth and nitrogen concentration are illustrated in Figure 2. It was obvious that strain H grew much faster than strain F. Strain H reached a higher cell density than strain F. After an approximately 1 day long lag phase, the cells grew rapidly into the logarithmic phase, with an obvious increase in the OD_{600} value at 4 days (from 0.45 to 3.27) and 3 days (0.31 to 4.06) for strains F and H, respectively. The cell growth rates were consistent with the NH_4^+–N removal rates during the logarithmic phase. For stain F, 91.24% of NH_4^+–N was reduced at 4 d, and the maximum removal rate was 3.15 mg NH_4^+–N/L/h between 2 d and 3 d. Moreover, for stain H, 97.51% of NH_4^+–N was removed at 3 days, and the maximum removal rate was 4.74 mg NH_4^+–N/L/h between 1 day and 2 days. The maximum nitrification rate of strain H was much higher than that previously reported for *Acinetobacter indicus* ZJB20129 (3.66 mg/L/h) [22].

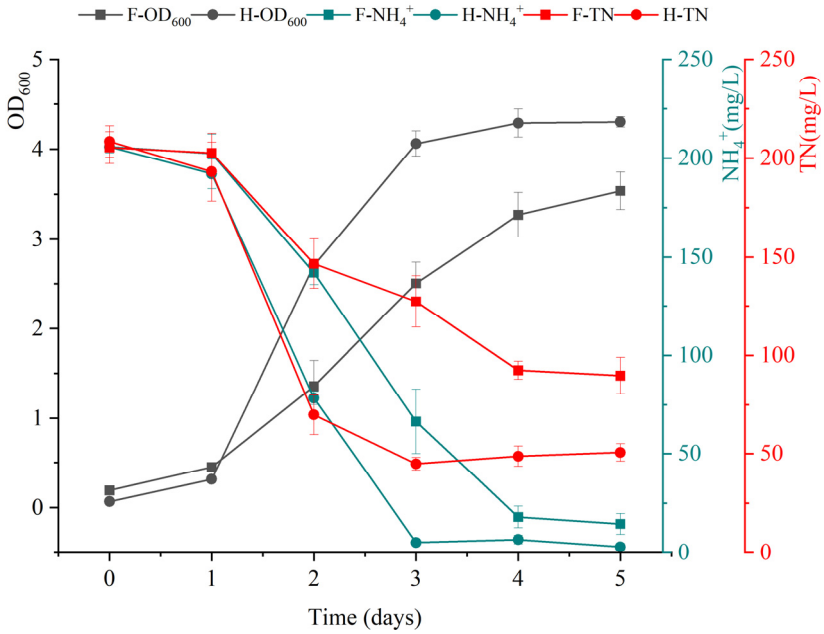


Figure 2. The removal capability of NH_4^+–N at 15 °C. Values are shown as the means ± SDs for three replicates.

During the NH_4^+–N removal process, no obvious accumulation of NO_3^-–N and NO_2^-–N was detected for either strain. This was similar to that reported for *Acinetobacter junii* YB [23], *Acinetobacter* sp. Y16 [24], and *Acinetobacter* sp. JR1 [9]. For *A. indicus* ZJB20129, 5.26 mg/L NO_3^-–N accumulated at 24 h (its logarithmic phase was 12 h to 36 h).

Nitrogen is important for cell growth and the metabolism of nitrogen-containing metabolites. The extracellular total nitrogen concentration of the strain H culture decreased from 208.368 mg/L to 44.832 mg/L at 3 d. For strain F, the extracellular TN decreased from 205.368 mg/L to 92.474 mg/L at 4 d. When the total nitrogen cost was calculated in the logarithmic phase, the total cell nitrogen recovery rates were 39.05 mg/L·OD and 39.62 mg/L·OD. This was similar to that of *Acinetobacter indicus* ZJB20129 (the total cell nitrogen recovery rate was approximately 37.08 mg/L·OD, which was calculated using the intracellular TN and cell density values). This result implied that most of the NH_4^+–N was assimilated by the cells.

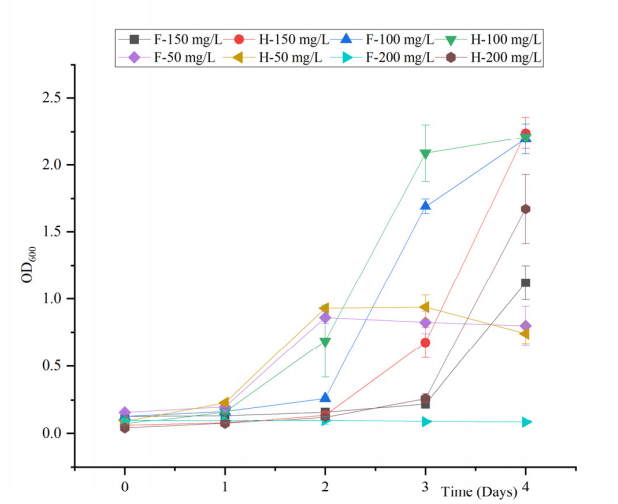
In the stationary phase, extracellular TN concentrations were 89.729 mg/L and 50.646 mg/L for strains F and H, respectively. The TN removal efficiencies of strains H and F were 78.48%

and 54.97%, respectively. Both were higher than that of *Pseudomonas tolaasii* Y-11 (30.5%) [18]. There was no accumulation of NO_3^- or NO_2^-. The remaining TN was probably composed of hydroxylamine and some free amino acids.

3.3. Evaluation of the Removal Capability of Nitrite and Nitrate

The nitrite tolerance of these two strains was examined by culturing them in a DM-2 medium with different concentrations of NO_2^-–N. The results are presented in Figure 3A. A high concentration of NO_2^- led to a longer lag phase. Strain F showed nearly no growth under 200 mg/L of NO_2^-–N. Strain H showed better tolerance to NO_2^- than strain F. Strain H even grew under 200 mg/L NO_2^-–N after a longer lag phase. Both strains grew well under 100 mg/L of NO_2^-. Therefore, in the following experiments with NO_2^-–N, its concentration was set to 100 mg/L.

A



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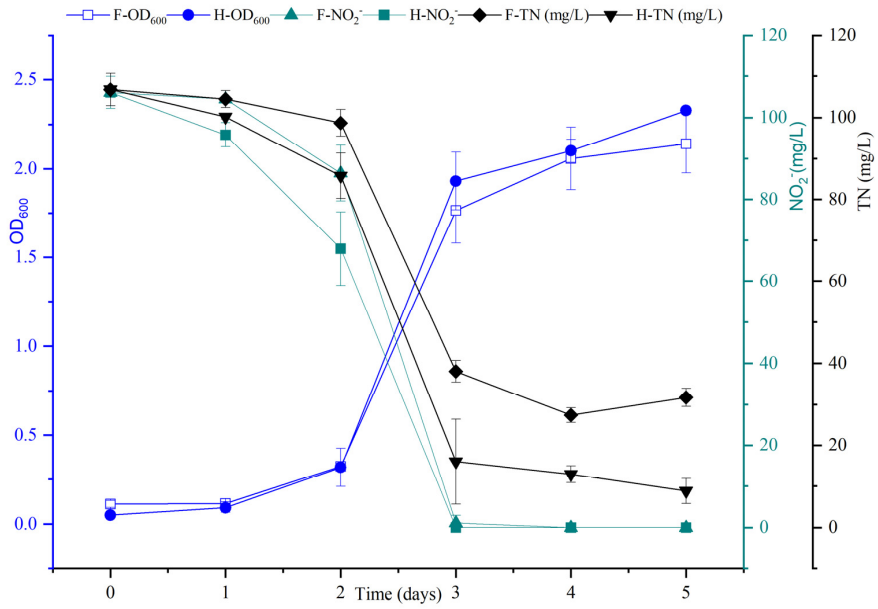


Figure 3. *Cont.*

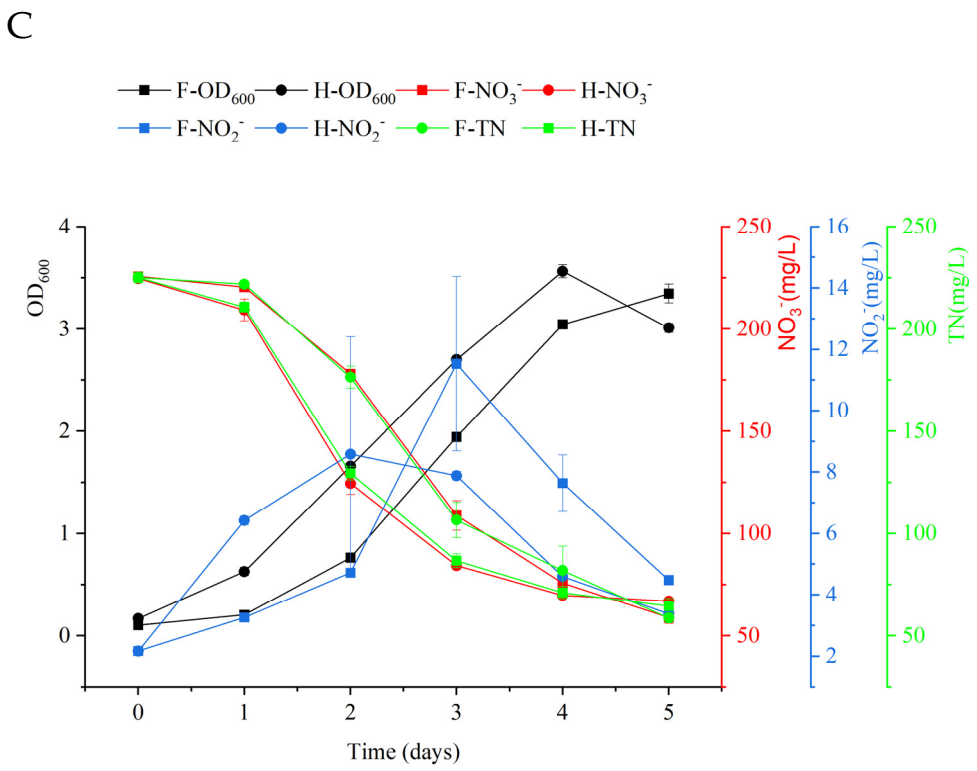


Figure 3. The removal capability of nitrite and nitrate at 15 °C. (**A**) The growth curves of both strains under different NO_2^-−N concentrations. The degradation capability of both strains using (**B**) nitrite and (**C**) nitrate as sole nitrogen sources. Values are shown as the means ± SDs for three replicates.

The removal capability of NO_2^-−N was assayed using $NaNO_2$ as the sole nitrogen source at 15 °C (Figure 3B). The initial NO_2^-−N concentration was 106 mg/L. Both strains showed a longer lag phase (0 to 2 days). Then, the cells grew rapidly into the logarithmic phase, with an obvious increase in the OD600 value at 3 days (from 0.32 to 1.76) and 3 days (0.31 to 1.93) for strains F and H, respectively. Strain F achieved a 98.93% nitrite removal efficiency at 3 days. The nitrite removal efficiency of strain H was 100%. No nitrate or ammonia accumulation was detected during this experiment in either strain. After 4 d of cultivation, the total nitrogen removal efficiencies of strains F and H reached 74.35% and 87.88%, respectively. The total cell nitrogen recovery rates were 40.24 mg/L•OD (F) and 40.82 mg/L•OD (H). The values were very similar to those obtained from NH_4^-−N assimilation.

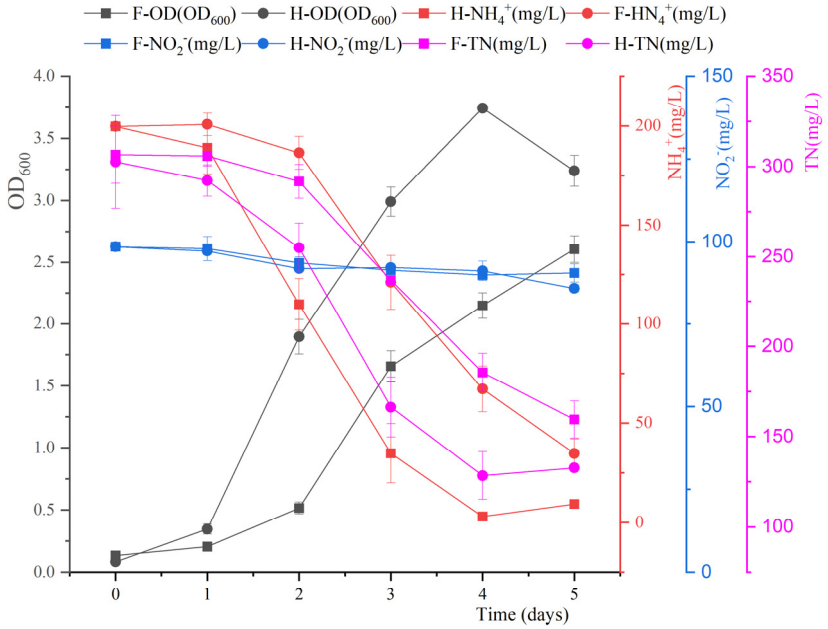
The removal capability of NO_3^-−N was assayed using $NaNO_3$ as the sole nitrogen source at 15 °C (Figure 3C). Both strains showed a shorter lag phase (less than 1 day). Both strains grew rapidly into the logarithmic phase with an obvious increase in the OD_{600} value (strains F from 0.204 to 3.045, H from 0.627 to 3.563) at 4 d. Strain F achieved 51.79% nitrate removal efficiency at 3 d. Strain H achieved 62.47% nitrate removal efficiency at 3 d. The maximum nitrate removal rates of strain H and F were 3.53 mg/L/h between 1 d and 2 d and 2.89 mg/L/h between 2 d and 3 d, respectively. A small degree of nitrite accumulation was detected in the culture broth of both strains. No ammonia accumulation was detected. After 5 days of cultivation, the total nitrogen removal efficiencies of strains F and H reached 73.92% and 71.33%, respectively. The total cell nitrogen recovery rates were 49.27 mg/L•OD (F) and 47.54 mg/L•OD (H). These values were slightly higher than those obtained from NH_4^+−N and NO_2^-−N assimilation.

The above results showed that strain H had a stronger capability to remove nitrate and nitrite than strain F.

3.4. Characterization of Simultaneous Removal of Ammonium–Nitrite and Ammonium–Nitrate

To evaluate the capability of simultaneous removal of ammonium–nitrate and ammonium–nitrite, an SND medium containing 200 mg/L ammonium nitrogen, 230 mg/L nitrate nitrogen, or 100 mg/L nitrate nitrogen was used. Both strains showed similar growth patterns in both media. The growth curves for the different SND media are presented in Figure 4.

A



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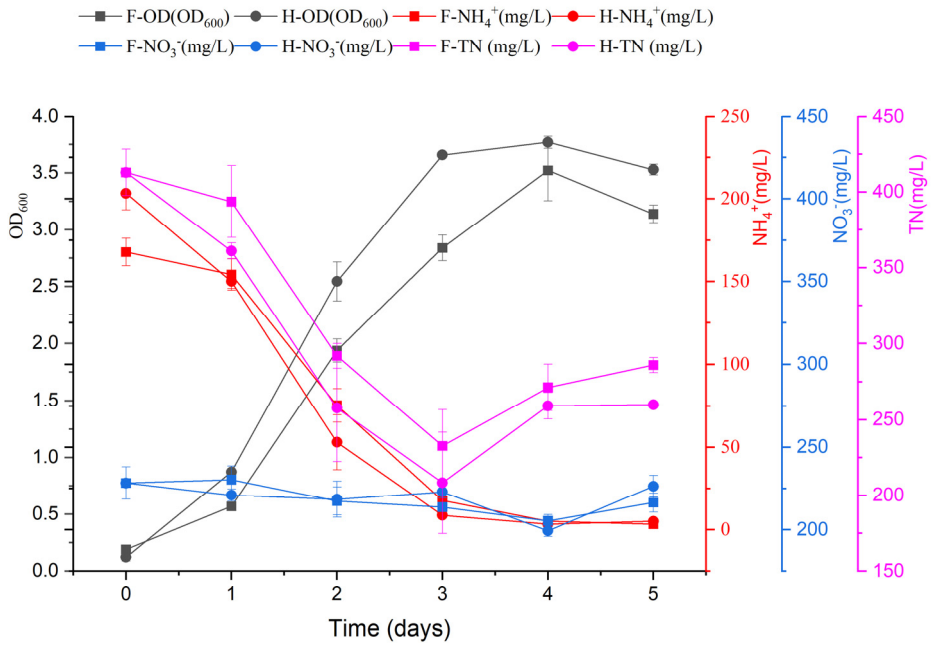


Figure 4. The removal capability of ammonium–nitrite and ammonium–nitrate at 15 °C. (**A**) Nitrogen with NH_4^+−N and NO_2^-−N; (**B**) nitrogen with NH_4^+−N and NO_3^-−N.

Strain H grew faster in both media containing nitrate and nitrite than strain F. For both strains, nitrate or nitrite was nearly unconsumed under these simultaneous nitrification and denitrification conditions.

When the culture broth contained 100 mg/L NO_2^-−N, 82.62% of NH_4^+−N was reduced at 5 d, and the maximum removal rate of strain F was 2.48 mg NH_4^+−N/L/h between 2 d and 4 d (Figure 4A). In addition, 95.35% of NH_4^+−N was removed at 5 d, and the maximum removal rate was 3.21 mg NH_4^+−N/L/h between 1 d and 3 d for strain H. Accumulation of NO_3^-−N was not detected. The total cell nitrogen recovery rates were 59.45 mg/L•OD (F) and 47.57 mg/L•OD (H). They were much higher than those obtained from NH_4^+−N and NO_2^-−N assimilation.

When the culture broth contained 200 mg/L NO_3^-−N, 97.96% of NH_4^+−N was reduced at 5 d, and the maximum removal rate of strain F was 3.28 mg NH_4^+−N/L/h between 2 d and 4 d (Figure 4B). In addition, 97.46% of NH_4^+−N was removed at 5 d, and the maximum removal rate was 4.04 mg NH_4^+−N/L/h between 1 d and 3 d for strain H. Additionally, there was no accumulation of NO_3^-−N in the culture broth of either strain. The total cell nitrogen recovery rates were 42.51 mg/L•OD (F) and 42.14 mg/L•OD (H). These values were very similar to those obtained from NH_4^+−N assimilation.

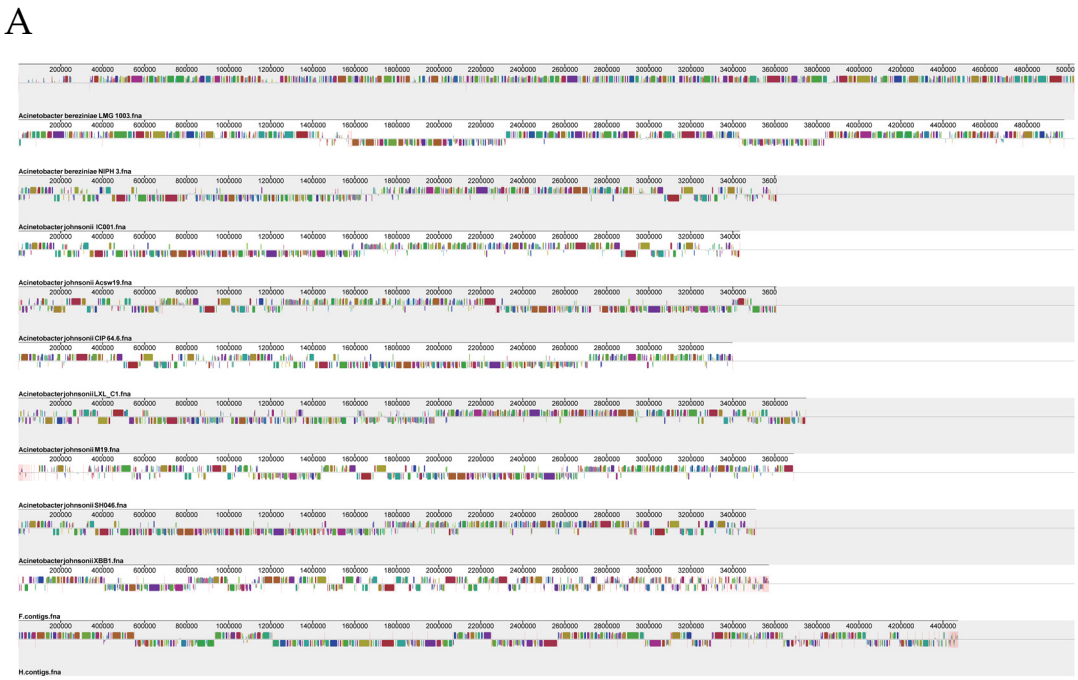
The growth rate of strain F in a medium containing 100 mg/L NO_2^-−N was significantly slower than that of strain H. This was consistent with the above results, and strain F was more susceptible to nitrite than strain H.

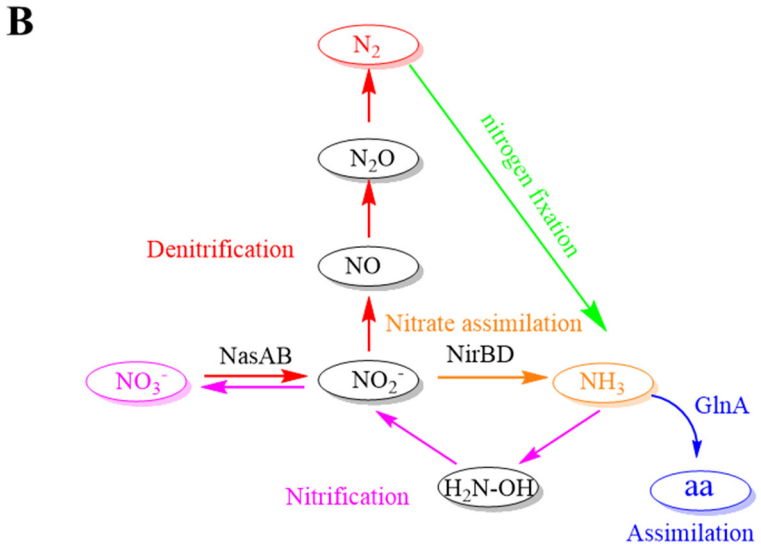
3.5. Genetic Investigation of the Nitrogen Removal Potential in Strains F and H

Usually, specific primers are employed to investigate HAO-, NAP-, and NIR-encoding genes in HN-AD strains (for *napA*, V17m:5′-TGGACCATGGGCTTCAACC-3′, napA4r:5′-CCTCGCGCGCGGTGCCGCA-3′; for *nirS*, cd3Af:5′-GTSAACGTSAAGGARACSGG-3′, R3cd:5′-GASTTCGGRTGSGTCTTGA-3′; for HAO, haoF1:5′-TGCGTGGARTGYCAC-3′, haoR3:5′-AGRTARGAKYSGGCAAA-3′) [23,25,26]. However, target fragments often fail to amplify due to nonspecific amplification or mismatches. In this study, we sent both strains for NGS sequencing. The draft genome sequence was annotated using automatic software. Genome comparison with the genomes of neighbor species showed that strain F was similar to *Acinetobacter johnsonii* XBB1, so it was named *Acinetobacter johnsonii* F. Strain H was similar to *Acinetobacter bereziniae* strain XH901, so it was named *Acinetobacter bereziniae* H (Figure 5A). The genome conservation distance matrix of strains F and H is listed in Table 2. Strain F was very similar to *Acinetobacter johnsonii* XBB1 and M19. Strain H was similar to *Acinetobacter bereziniae* NIPH 3. Similarly to the reported *Acinetobacter bereziniae* species, strain H had a larger genome size of approximately 4.1 Mb.

Table 2. Genome conservation distance matrix.

Strain/ Strains	KCTC 23199	LMG003	NIPH 3	IC001	ACws19	CIP 64.6	LXL-C1	M19	SH046	XBB-1	F	H
KCTC23199	0.000	0.022	0.163	0.523	0.518	0.520	0.520	0.514	0.510	0.514	0.504	0.132
LMG 1003	0.022	0.000	0.175	0.531	0.526	0.527	0.528	0.522	0.515	0.522	0.513	0.147
NIPH 3	0.163	0.175	0.000	0.527	0.526	0.526	0.528	0.523	0.513	0.522	0.513	0.110
IC001	0.523	0.531	0.527	0.000	0.190	0.207	0.198	0.204	0.199	0.185	0.188	0.529
ACws19	0.518	0.526	0.526	0.190	0.000	0.185	0.177	0.175	0.185	0.162	0.161	0.520
CIP.64.6	0.520	0.527	0.526	0.207	0.185	0.000	0.189	0.200	0.199	0.187	0.190	0.523
LXL-C1	0.520	0.528	0.528	0.198	0.177	0.189	0.000	0.184	0.193	0.166	0.170	0.523
M19	0.514	0.522	0.523	0.204	0.175	0.200	0.184	0.000	0.196	0.105	0.080	0.517
SH046	0.510	0.515	0.513	0.199	0.185	0.199	0.193	0.196	0.000	0.181	0.178	0.515
XBB-1	0.514	0.522	0.522	0.185	0.162	0.187	0.166	0.105	0.181	0.000	0.083	0.516
F	0.504	0.513	0.513	0.188	0.161	0.190	0.170	0.080	0.178	0.083	0.000	0.508
H	0.132	0.147	0.110	0.529	0.520	0.523	0.523	0.517	0.515	0.516	0.508	0.000





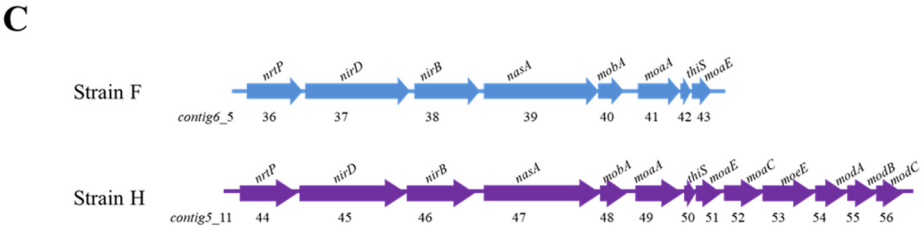


Figure 5. *Cont.*

D

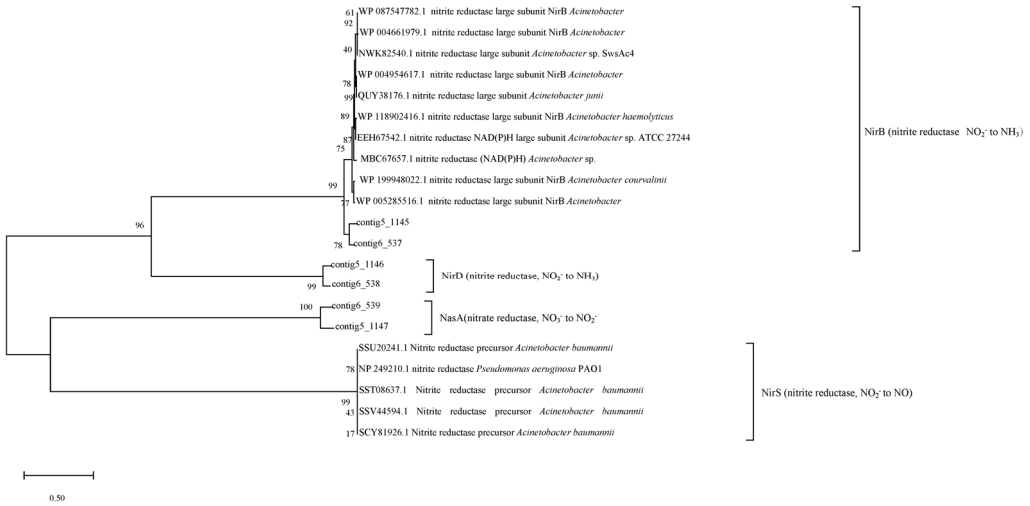


Figure 5. Genetic potential for nitrogen removal from both strains. (**A**) Comparitve genome alignment of both strains with their neighboring species; (**B**) the putative nitrogen metabolism pathways of strains F and H; (**C**) the nitrogen assimilation gene clusters in both strains; (**D**) the phylogenetic analysis of nitrite reductases in *Acinetobacter*.

An analysis of nitrogen metabolism-related genes showed that only nitrate and nitrite reductase-encoding genes (*nir* and *nas*, respectively) were found in both genomes. The putative nitrogen metabolism pathway was identified based on the genome sequence analysis (Figure 5B). Only the nitrate and nitrite reductase-encoding genes involved in nitrate assimilation were found in the genome. N_2 and N_2O emissions were not detected in any of the cultures. Therefore, we proposed that there was no aerobic denitrification system for either strain.

The nitrate reductase system is usually responsible for NO_3^-−N assimilation in microbes. As shown in the figure (Figure 5B), nitrate and nitrite reductases can reduce NO_3^-−N to NH_3 with the help of cofactors. NH_3 can usually be incorporated into the biosynthesis of amino acids by GlnA under the control of the GlnR regulator. In *A. johnsonii* H and *A. bereziniae* F, the genes encoding the nitrate reductase system were clustered (Figure 5C). This cluster contained genes encoding a nitrate transporter, a nitrate reductase, two nitrite reductases, and enzymes involved in cofactor molybdopterin biosynthesis. Interestingly, the cluster in *A. johnsonii* H was larger than that in *A. johnsonii* F. More molybdopterin biosynthesis-related genes were located downstream of the nitrate reductase-encoding genes.

The reported partial *napA* aa sequence (QCY54495.1, from *Acinetobacter hemolyticus*) was used for blast in the GenBank database to check the distribution of homologous genes in *Acinetobacter* nitrite reductase [27]. NP_249210.1 nitrite reductase [*Pseudomonas aeruginosa* PAO1] was also used to blast the protein database to identify homologous proteins for the Acinetobacter genus. The two groups of proteins were combined to construct a phylogenetic tree (Figure 5D). Except for *Acinetobacter baumannii*, no other *Acinetobacter* was homologous to *nirS*. Nitrite reductase subunits NirD and B together can convert nitrite to ammonium. The bacterial NirD contains a single Rieske domain, which is a [2Fe-2S] cluster-binding domain involved in electron transfer. The [2Fe-2S] cluster-binding domains are commonly found in Rieske nonheme iron oxygenase (RO) systems, such as naphthalene and biphenyl dioxygenases. In RO systems, the N-terminal Rieske domain of the alpha subunit acts as an electron shuttle that accepts electrons from a reductase or ferredoxin component and transfers them to the mononuclear iron in the alpha subunit C-terminal domain to be used for catalysis. NAD(P)H-nitrite reductase has a large subunit for energy production and conversion. The nitrate reductase contains a

molybdopterin-binding (MopB) domain. In many bacterial species, molybdopterin is in the form of a dinucleotide, with two molybdopterin dinucleotide units per molybdenum. Molybdenum is present in most of these enzymes in the form of molybdopterin, a modified pterin ring with a dithiolene side chain, which is responsible for ligating Mo. The MopB domain is found in a wide variety of molybdenum- and tungsten-containing enzymes, including several forms of nitrate reductase (Nap, Nas, and NarG). The molybdopterin oxidoreductase Fe4S4 domain is found in a number of reductase/dehydrogenase families, which include the periplasmic nitrate reductase precursor and the formate dehydrogenase alpha chain.

3.6. Pathogenic Potential and Antibiotic Resistance Gene Risk Analysis of Both Strains

The assembled sequences were submitted to the database of the Center for Genomic Epidemiology (CGE) for analysis: KmerResistance 2.2 "https://cge.food.dtu.dk/services/KmerResistance/ (accessed on 13 November 2022)", PathogenFinder 1.1 "https://cge.food.dtu.dk/services/PathogenFinder/ (accessed on 13 November 2022), and PlasmidFinder 2.1 "https://cge.food.dtu.dk/services/PlasmidFinder/ (accessed on 13 November 2022). Strain H contains a beta-lactamase [*Acinetobacter bereziniae*] encoded by contig6_1300, which is a blaOXA-355 (GenBank access No.: KF29758) homologous gene. Moreover, blaOXA-355 homologous genes were found in both genomes. However, two macrolide resistance-related genes, *msrE* (GenBank access No.: FR751518) and *mphE* (GenBank access No.: DQ839391), were found in the genome of strain F. They are neighbored on the assembled contig 69 of strain F. The gene annotation of contig_69 is listed in Table 3. Contig69_3191 was annotated as a plasmid replication–initiation protein. This implied that the assembled contig 69 sequence of strain F probably belonged to a plasmid (Table 3). BLAST in GenBank with this contig showed nearly 100% identity with the plasmid pXBB1-9 of *Acinetobacter johnsonii* XBB-1. This plasmid in strain *A. johnsonii* F was probably acquired from other *Acinetobacter johnsonii* strains via horizontal gene transfer.

Table 3. Gene distribution in the contig of the putative plasmids.

	Length (aa)	Hit_Name	Hit_Description	e Value
contig69_3190	179	SEM34325.1	Hypothetical protein SAMN05216500_1256 [*Acinetobacter* sp. DSM 11652]	2.90×10^{-71}
contig69_3191	305	SEM34356.1	Protein involved in the initiation of plasmid replication, partial [*Acinetobacter* sp. DSM 11652]	3.80×10^{-156}
contig69_3192	69	WP_058952608.1	Hypothetical protein [*Acinetobacter johnsonii*]	1.30×10^{-32}
contig69_3193	536	WP_057082130.1	Hypothetical protein, partial [*Acinetobacter pittii*]	1.60×10^{-303}
contig69_3194	249	WP_086166669.1	Hypothetical protein [*Acinetobacter* sp. ANC 4654]	1.60×10^{-112}
contig69_3195	78	WP_086183153.1	Hypothetical protein [*Acinetobacter* sp. ANC 4558]	3.90×10^{-25}
contig69_3196	250	WP_075041359.1	Hypothetical protein [*Acinetobacter radioresistens*]	1.50×10^{-139}
contig69_3197	68	WP_075041358.1	Hypothetical protein [*Acinetobacter radioresistens*]	6.00×10^{-30}
contig69_3198	583	KQE20847.1	Hypothetical protein APD38_11590 [*Acinetobacter pittii*]	0
contig69_3199	79	ALJ89856.1	Hypothetical protein AN415_8035 (plasmid) [*Acinetobacter baumannii*]	7.00×10^{-38}
contig69_3200	95	WP_068553177.1	Hypothetical protein [*Acinetobacter pittii*]	6.80×10^{-48}
contig69_3201	495	WP_032002906.1	Sodium-independent anion transporter [*Acinetobacter baumannii*]	3.10×10^{-261}
contig69_3202	283	WP_065033555.1	Universal stress protein [*Acinetobacter baumannii*]	1.50×10^{-154}
contig69_3203	203	WP_065033561.1	Resolvase [*Acinetobacter baumannii*]	1.30×10^{-101}
contig69_3204	294	WP_043974506.1	Mph(E)/Mph(G) family macrolide 2′-phosphotransferase [*Acinetobacter* sp. NBRC 110496]	4.70×10^{-164}
contig69_3205	491	WP_031976859.1	Msr family ABC-F type ribosomal protection protein [*Acinetobacter baumannii*] EXC89516.1	2.80×10^{-270}

4. Conclusions and Discussion

Ammonium at a high content has become a major contaminant in domestic wastewater treatment [28]. Two newly isolated bacteria, *A. johnsonii* strain F and *A. bereziniae* strain H, can efficiently remove nitrogen at low temperatures (15 °C). Overall, the rapid growth, efficient NH_4^+−N removal capability, and lack of NO_3^-−N/NO_2^-−N accumulation of the two strains showed great potential for NH_4^+–N removal at low temperatures under aerobic conditions. The maximum NH_4^+–N removal rate of strain H was 4.74 mg/L·h under 15 °C. This probably is the highest reported removal rate at low temperatures. Even when strain H was cultured in broth with 200 mg/L NO_3^-−N, up to 97.46% of NH_4^+−N (200 mg/L) was removed in 5 days, and the maximum NH_4^+−N removal rate was 4.04 mg/L·h. *A. bereziniae* strain H showed greater potential for biological nitrogen removal.

Due to the well-documented toxicity of nitrite to some bacteria and the energy consumption of denitrification, when using NO_2^-−N as the sole nitrogen source, a long lag time duration was necessary for adaptation due to the cellular toxicity of nitrite [18]. When the culture broth contained 100 mg/L NO_2^-−N, 95.35% of NH_4^+−N was removed at 5 d, and the maximum removal rate reached 3.21 mg NH_4^+−N/L/h between 1 d and 3 d for strain H. As we mentioned above, a high amount of nitrite nitrogen can inhibit the bacterial growth, and thus repress their nitrogen removal activities. Strain H showed better NO_2^- tolerance. And no NO_2^- was accumulated during the utilization of NO_3^-−N. These properties increased the potential of strain H to treat wastewater at low temperatures.

The genomic sequence of strain F was similar to that of *A. johnsonii* XBB-1. A putative plasmid containing an antibiotic resistance gene (macrolide 2′-phosphotransferase encoding gene) was detected in strain F. Homologous analysis showed nearly 100% identity with the plasmid pXBB1-9 from *A. johnsonii* XBB-1. This probably brings the horizontal transfer risks of genes during environmental applications.

Overall, strain H was efficient and safe for N removal applications to treat wastewater in winter. For the safety of the environmental release of bacteria, genome sequence analysis is necessary for the systematic evaluation of the application potential of strains. Further investigation of the functional genes of *A. bereziniae* strain H involved in N removal will provide parts or devices for the construction of robust hosts to treat wastewater using synthetic biology approaches. *A. bereziniae* strain H can also be developed as a chassis for wastewater treatment engineering. However, there is still a lot of need for more investigation in the future. Because iswastewater are diverse, the efficiency of nitrogen removal by this strain may be highly dependent on specific environmental conditions. So, the tolerance of strain H should be tested in the future. It will be necessary to systematically evaluate the potential of *A. bereziniae* strain H in treating real wastewater at a pilot scale, including the long-term performance, environmental tolerance, and nitrogen removal efficiency.

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Institutional Review Board Statement: This article does not contain any study with human participants or animals performed by any of the authors.

Data Availability Statement: Whole Genome Shotgun projects have been deposited at DDBJ/ENA/GenBank under the accession JAOWAI000000000 (strain H) and JAOWAJ000000000(strain F). All data generated or analyzed during this study were included in this manuscript. The strains are available by email from the corresponding author.

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