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Comparative Genome Analysis of Two Heterotrophic Nitrifying *Pseudomonas putida* Strains Isolated from Freshwater Shrimp Ponds in Soc Trang Province

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Abstract: Nitrogen compounds, especially ammonia, are widely produced in aquaculture systems during cultivation. Ammonia has been investigated as a model compound for use by heterotrophic nitrifying bacteria. *Pseudomonas* TT321 and *Pseudomonas* TT322, isolated from shrimp pond water in Soc Trang province, Vietnam, are identified by comparing them with 31 of the closest genomes sequences from the NCBI nucleotide database. The genome sizes of strains TT321 and TT322 were 5,566,241 bp and 5,563,644 bp, respectively. No plasmids were evident in these strains. Genome analysis revealed that TT321 and TT322 belonged to *Pseudomonas putida* and shared a common ancestor with 33 genomes. Analysis based on the comparison of genomes showed that three genes, carbamate kinase (*arc*C), glutamine synthetase (*Glul*), and aminomethyltransferase (*amt*), are involved in three metabolic pathways. These pathways are: (i) arginine and proline metabolism, (ii) alanine, aspartate and glutamate metabolism, and (iii) glycine, serine and threonine metabolism. These genes may play important roles in ammonia reduction and support bacterial growth via ammonia assimilation.

Keywords: *Pseudomonas putida*; heterotrophic nitrifying activity; ammonia assimilation; freshwater shrimp ponds; nitrogen compounds

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1. Introduction

Pseudomonas are ubiquitous and versatile bacteria that belong to the subclass Gamma of the Proteobacteria [1]. *Pseudomonas* is a complex genus of rod-shaped, Gram-negative bacteria that demonstrate a wide range of metabolic mechanisms and consequently are present in various niches [2]. Whereas some *Pseudomonas* sp. are models of interest in medical research due to their pathogenicity in animals and plants, other soil and water *Pseudomonas* spp., such as *Pseudomonas putida*, are capable of metabolizing pollutants, and are thus studied for their metabolic capability and potential bioremediation applications [3].

Modern shrimp farming methods result in the discharge of a significant amount of nutrients into the aquatic surroundings, especially in the case of nitrogen (N) [4]. The majority of nitrogen input comes from protein-rich shrimp feeds, of which around 20% is converted to harvested shrimp, while the remaining nitrogen accumulates as ammonia (NH_3^+), nitrite (NO_2^-), nitrate (NO_3^-), or is taken up by phytoplankton and subsequently settles as sediment [4,5]. This results in anoxic sludge that significantly hinders shrimp production, as well as nutrient-rich discharge that may cause the eutrophication of surrounding waterways [6]. As the shrimp industry aims to move on from unsustainable

practices, reducing toxic N components within the pond and in the wider environment is crucial to future developments.

Nitrifying bacteria are chemolithotrophs that acquire energy through the oxidation of inorganic nitrogen compounds such as ammonia or nitrite [7]. Genera of nitrifying bacteria such as *Nitrosomonas* and *Nitrobacter* have been considered as potential candidates to alleviate the nitrogen eutrophication problem [8]. However, as autotrophs, their relatively low growth rate is a limiting factor for large-scale applications. Alternatively, several heterotrophs have recently been discovered and highlighted as potential bioremedial agents, having demonstrated their ability to remove accumulating inorganic nitrogen compounds [9,10]. Among these heterotrophs, some *Pseudomonas* species were recently shown to utilize ammonia in wastewater [11–13]. Notably, among these species, *Pseudomonas putida* was found to be capable of both high growth and rapid ammonia removal [13].

Pseudomonas putida strains TT321 and TT322 were recently isolated from water samples of shrimp farms in Vietnam. They can remove ammonia via simultaneous heterotrophic nitrification and denitrification in aerobic conditions [14].

To understand the classification, gene transfer events and the presence of genes related to ammonia conversion and suggest the molecular mechanisms of nitrogen utilization by TT321 and TT322, we present a comparative whole-genome analysis of *P. putida* strains TT321 and TT322 with 31 other *Pseudomonas* bacterial reference genomes. The complete genomes of the two strains were sequenced and the genes involved in ammonia metabolism were identified. This work helps to explain the mechanisms by which these strains could thrive in ammonia-rich environments.

2. Materials and Methods

2.1. DNA Extraction, Genome Sequencing and Annotation

TT321 and TT322 were isolated from shrimp pond water in Soc Trang province, Vietnam [14]. DNA samples of TT321 and TT322 were extracted using AllPrep® Bacterial DNA/RNA/Protein Kit (Qiagen, Germantown, MD, USA) following the manufacturer's instructions. Total extracted DNA was used for sequencing using the Nextera DNA Flex Library Prep Kit (Illumina, San Diego, CA, USA), to generate 550 bp paired-end sequences at 1st Base, Selangor, Malaysia, on the Illumina HiSeq2500 platform (Illumina, San Diego, CA, USA) which produced paired-end reads (2 × 300 bp).

2.2. QC and De Novo Assembly

Sequence adaptors and reads with low quality scores were removed from paired-end Illumina sequences using bbduk of the BBTools Packages (https://jgi.doe.gov/data-and-tools/bbtools/, accessed on 25 September 2020). QC reads were assembled de novo using SPAdes [15]. The 3.11.1. QUAST tool (Algorithmic Biology Lab, St. Petersburg Academic University of the Russian Academy of Sciences) was then used to assess the quality of the genome assemblies [16]. The resulting scaffolds of each dataset were manually inspected for sequence coverage and sequence length. Scaffolds that were shorter than 1000 bp and/or with coverage lower than the mean of scaffolds were removed, as these resembled sequence contaminants.

2.3. Identity of Assembled Genomes

The TT321 and TT322 genomes were blasted against the NCBI nucleotide database (https://www.ncbi.nlm.nih.gov/, accessed on 25 September 2020).

2.4. Annotation of Selected Genome Sequences

The combination of 31 complete genome sequences obtained from the NCBI database and the two genomes of the *Pseudomonas* isolates were used for re-annotation [17] via the Rapid Annotation program using the Subsystem Technology (RAST) server (https://rast.nmpdr.org, accessed on 15 October 2020) following default parameters. This step avoids the possible deviation of different annotation methods.

2.5. Phylogenetic Tree Analysis

Two different methods were used to construct phylogenetic trees. The first used the 16S rRNA sequences of the 33 genomes to reveal relationships via the Neighbor-Joining (NJ) method of MEGA 7 [18]. The second method used the Neighbor-Joining algorithm by BPGA version 1.3 [19] based on pan genomes.

2.6. Comparative Genome Analysis

The whole genome alignment was constructed using the Alignment algorithm implement in Mauve version 2.4 [20] following the default parameters. In order to provide information about core genomes, pan-genomes, and accessory genomes in *Pseudomonas*, the Bacterial Pan Genome Analysis Pipeline (BPGA) (Indian Institute of Chemical Biology, CSIR—https://iicb.res.in, accessed on 21 January 2021) was used.

2.7. Genomic Island Prediction

IslandViewer 4 [21] was used to predict genomic islands (GIs) based on the IslandPick, IslandPath-DIMOB, SIGI-HMM, and Islander methods.

Subsystem core gene information related to nitrogen metabolism of the 33 studied genomes was returned by RAST. These genes were then selected to analyze the ammonia conversion of the two strains (TT321 and TT322).

3. Results and Discussion

3.1. Genome Features

The general features of the 33 genomes containing TT321, TT322 and another 31 *Pseudomonas* genomes are presented in Table 1. Short contigs and sequences of TT321 and TT322 with potential contamination were removed to obtain 130 contigs with N50 values of 199.8 Kbp and 228.2 Kbp, respectively. The genomes of *Pseudomonas putida* TT321 and *Pseudomonas putida* TT322 were 5,566,241 bp and 5,563,644 bp, respectively. The TT321 genome contains 66 contigs, with the largest being 497.1 Kbp. The TT322 genome contains 64 contigs, with the largest being 497.1 Kbp. The GC contents of these two genomes were the same, at 61.8%. The CDS numbers in the TT321 and TT322 genomes were 5210 and 5234, respectively. There were no plasmid sequences detected in TT321 or TT322. There are 393 subsystems, based on SEED subsystems analysis, and 13 genes involved in nitrogen metabolism, which were identified in TT321 and TT322 (Supplementary Table S1). The genomic size of the *Pseudomonas* bacteria in this study ranges from 5.5 Mbp to 7.1 Mbp (Table 1). *Pseudomonas putida* TT321 and *Pseudomonas putida* TT322 have the smallest genome size among the 33 genomes. This suggests the limit of horizontal transfer genes in these two strains. This study is consistent with other studies where a larger genome size was demonstrated to lead to more horizontally transferred genes than a small genome size [22–24].

Table 1. Genome characteristics of the 33 *Pseudomonas* strains used in this study.

Accession [a]	Organism	Size (bp)	GC [b] (%)	CDSs [c]	No. Plasmids [d]	No. Contigs [e]	Subsystems [f]
This study	*Pseudomonas putida* TT321	5,566,241	61.8	5210	0	62	393
This study	*Pseudomonas putida* TT322	5,563,644	61.8	5234	0	64	393
NC_016830.1	*Pseudomonas fluorescens* F113	6,845,832	60.8	6249	0	1	410
NC_012660.1	*Pseudomonas fluorescens* SBW25	6,722,539	60.5	6162	0	1	413
NC_017530.1	*Pseudomonas putida* BIRD-1	5,731,541	61.7	5297	0	1	393
NC_009512.1	*Pseudomonas putida* F1	5,959,964	61.9	5436	0	1	395
NC_010322.1	*Pseudomonas putida* GB-1	6,078,430	61.9	5541	0	1	397

Table 1. *Cont.*

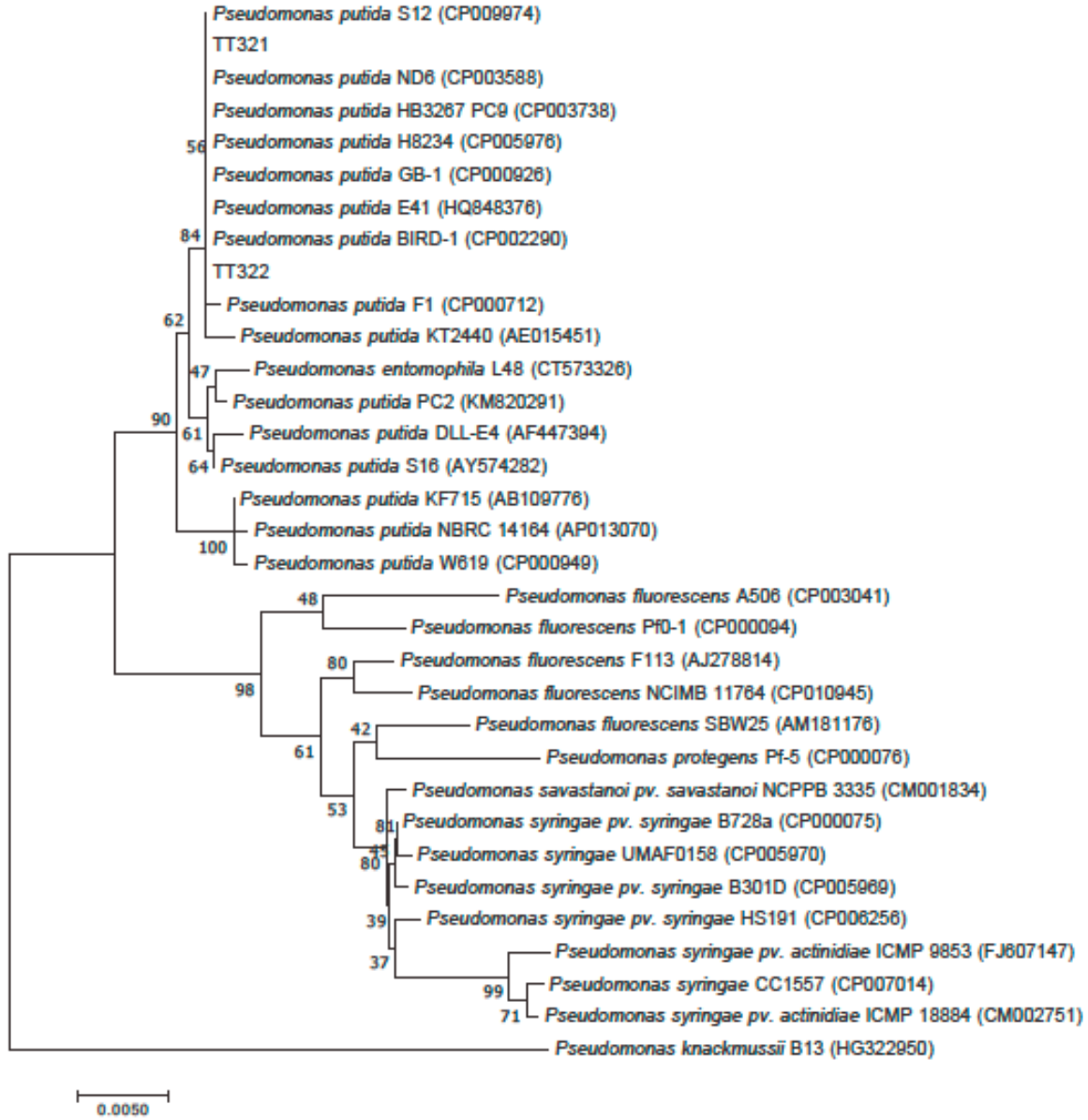
Accession [a]	Organism	Size (bp)	GC [b] (%)	CDSs [c]	No. Plasmids [d]	No. Contigs [e]	Subsystems [f]
NC_021491.1	*Pseudomonas putida* H8234	6,870,827	61.6	6593	0	1	409
NZ_CP007620.1	*Pseudomonas putida* DLL-E4	6,484,062	62.5	6252	0	1	399
NC_002947.4	*Pseudomonas putida* KT2440	6,181,873	61.5	5716	0	1	402
NC_021505.1	*Pseudomonas putida* NBRC 14164	6,156,701	62.3	5556	0	1	402
NZ_CP011789.1	*Pseudomonas putida* PC2	5,808,624	63.2	5232	0	1	390
NC_015733.1	*Pseudomonas putida* S16	5,984,790	62.3	5701	0	1	404
NC_010501.1	*Pseudomonas putida* W619	5,774,330	61.4	5357	0	1	405
NZ_CP005969.1	*Pseudomonas syringae pv. syringae* B301D	6,094,819	59.2	5413	0	1	389
NC_007005.1	*Pseudomonas syringae pv. syringae* B728a	6,093,698	59.2	5395	0	1	389
NC_008027.1	*Pseudomonas entomophila* L48	5,888,780	64.2	5209	0	1	397
NC_017911.1	*Pseudomonas fluorescens* A506	5,962,570	60.0	5505	1	1	400
NZ_CP010945.1	*Pseudomonas fluorescens* NCIMB 11764	6,998,154	59.0	6564	0	1	413
NC_007492.2	*Pseudomonas fluorescens* Pf0-1	6,438,405	60.5	5840	0	1	410
NZ_HG322950.1	*Pseudomonas knackmussii* B13	6,162,905	65.6	5887	0	1	387
NC_004129.6	*Pseudomonas protegens* Pf-5	7,074,893	63.3	6412	0	1	416
NZ_CP024085.1	*Pseudomonas putida* E41	6,093,023	62.2	5433	0	1	407
NC_019905.1	*Pseudomonas putida* HB3267	5,875,750	62.6	5475	1	1	401
NZ_AP015029.1	*Pseudomonas putida* KF715	6,583,377	61.9	6072	7	1	419
NC_017986.1	*Pseudomonas putida* ND6	6,085,449	61.8	5632	2	1	401
NZ_CP009974.1	*Pseudomonas putida* S12	5,798,534	61.8	5269	1	1	394
NZ_CP008742.1	*Pseudomonas savastanoi pv. savastanoi* NCPPB 3335	6,016,828	58.1	5800	0	1	383
NZ_CP007014.1	*Pseudomonas syringae* CC1557	5,758,024	58.6	5244	0	1	378
NZ_CP018202.1	*Pseudomonas syringae pv. actinidiae* ICMP 9853	6,439,609	58.7	6046	0	1	390
NZ_CP011972.2	*Pseudomonas syringae pv. actinidiae* ICMP 18884	6,555,569	58.4	6183	1	1	387
NZ_CP006256.1	*Pseudomonas syringae pv. syringae* HS191	5,950,211	59.0	5271	1	1	389
NZ_CP005970.1	*Pseudomonas syringae* UMAF0158	5,787,986	59.3	5145	1	1	384

[a] NCBI RefSeq accession number. [b] Percentage of G and C in the total nucleotide of a sequence. [c] Number of protein-coding DNA sequences (CDSs). [d] Number of plasmids. [e] Number of contig. [f] Number of subsystems based on RAST Annotation Server on the SEED.

3.2. Phylogenetic Tree Analysis

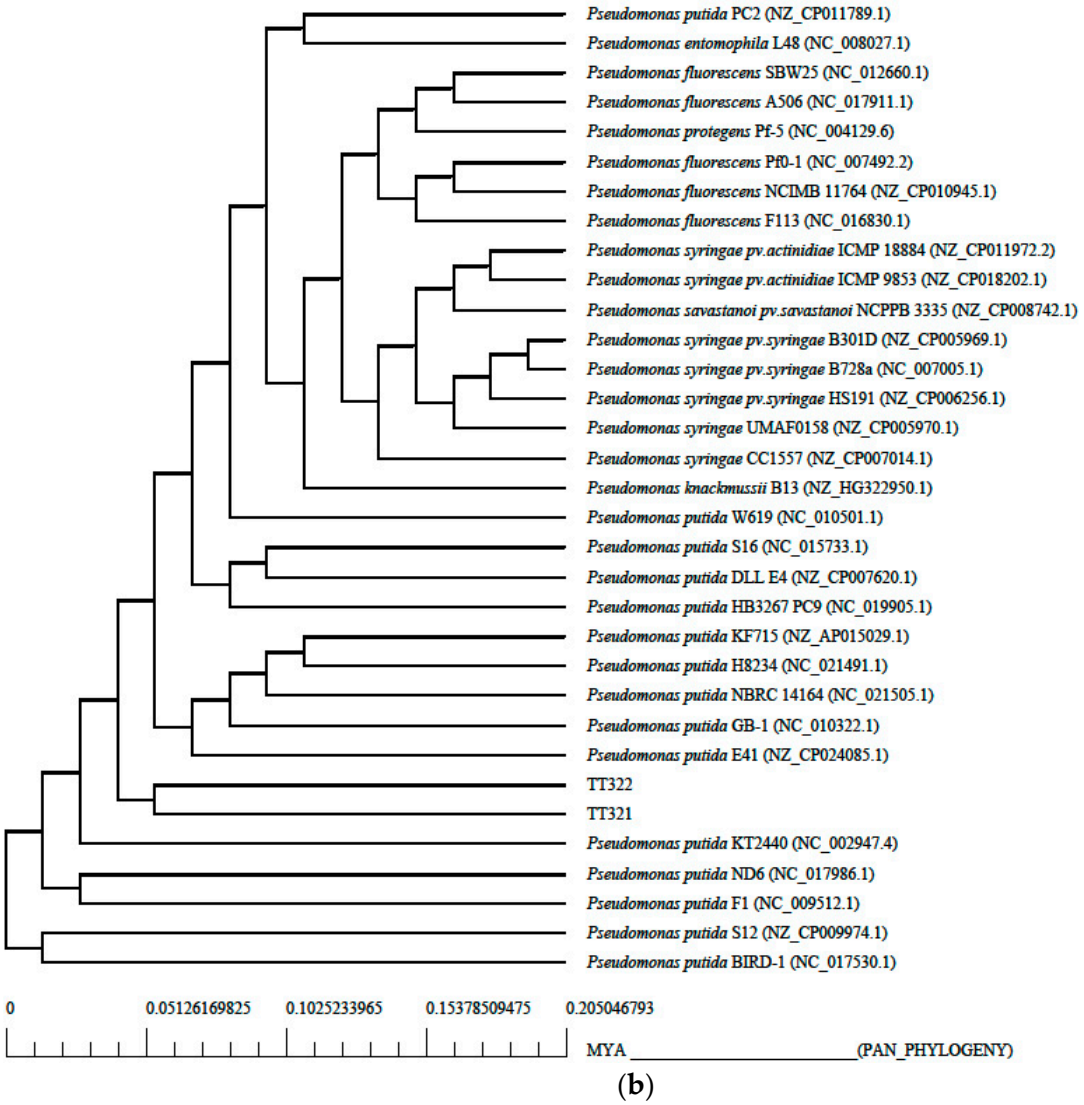
16S rRNA sequences demonstrated that they do not contain enough phylogenetic information to distinguish closely related bacteria [15,25,26]. In order to attain a higher phylogenetic resolution, an analysis based on pan-genomes was constructed. The phylogenetic analysis based on amplified 16S rRNA sequences (Figure 1a) (universal 16S rRNA primer) showed that the TT321 and TT322 strains were close to *Pseudomonas putida* (the highest similarity values to *Pseudomonas putida* BIRD-1 (CP002290) were 98.5% and 98.9%, respectively). Further support for the classification of TT321 and TT322 was based on

phylogenetic tree analysis of pan-genomes (Figure 1b). The phylogenetic tree analysis based on the 16S rRNA sequences and pan-genomes showed that TT321 and TT322 were monophyletic. All members of the *Pseudomonas fluorescens* group and *Pseudomonas syringae* group shared two specific clades, while these two groups were clustered together in the same clade. This clade illustrated the close relationship between strains of *Pseudomonas putida*. Interestingly, TT321 and TT322, based on pan-genome analysis, have not revealed the closest phylogenetic neighbors with a specific member of *Pseudomonas putida*. Therefore, the relationship between TT321 and TT322 was close and belonged to *Pseudomonas putida* based on the phylogenetic tree analysis of the 16S rRNA genes and pan-genomes of 33 collected genome sequences. This branch did not share the same ancestor with any strains in 31 reference genomes.



(**a**)

Figure 1. *Cont.*

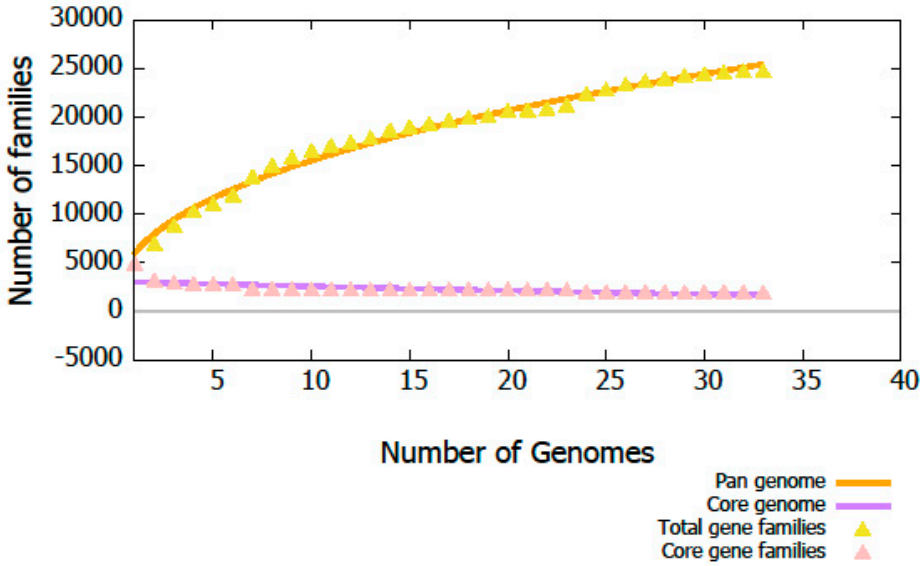


(**b**)

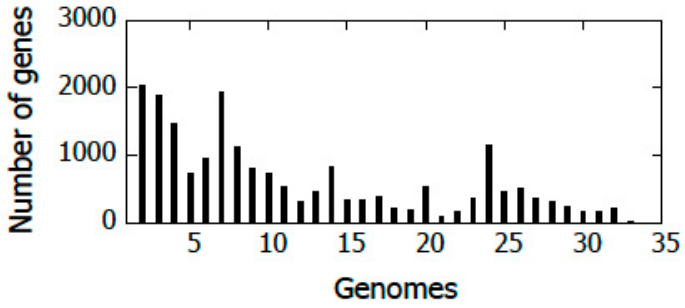
Figure 1. Phylogenetic tree analysis of the 33 *Pseudomonas* genomes. (**a**) Phylogenetic tree based on 16S rRNA sequences; (**b**) phylogenetic tree based on pan-genomes.

3.3. Genome Annotation and Gene Repertoire

The core genome analysis of *Pseudomonas* showed that the total gene families did not reach saturation (Figure 2a). The decrease in the number of core gene families (Figure 2a) and number of new genes (Figure 2b) indicated that most of the core genes and new genes were present in the 33 genomes. Smaller numbers of new genes were found with the increased number of the studied genomes. The number of core genes was 1929, which accounted for approximately 40% of the total genes for each of TT321 and TT322, while the number of new genes in TT321 and TT322 were 1 and 2 (Supplementary Table S2), respectively.



(a)



(b)

Figure 2. Pan-genome analysis based on the 33 studied genomes. (**a**) Core-pan plot; (**b**) number of new genes.

3.4. Genome Islands (GIs) of Pseudomonas

TT321 included 28 integrated genomic islands (Supplementary Table S4), which carry 381 genes (Figure 3), while TT322 included 27 integrated genomic islands (Supplementary Table S5), which carry 379 genes (Figure 4). Some of the genes carried by GIs on TT321 and TT322 are related to transporter and transcription, while most of them encode hypothetical proteins. The range of the GI size of TT321 is from 4297 to 47,386 bp, and for TT322 is from 4744 to 47,386 bp (Supplementary Tables S4 and S5).

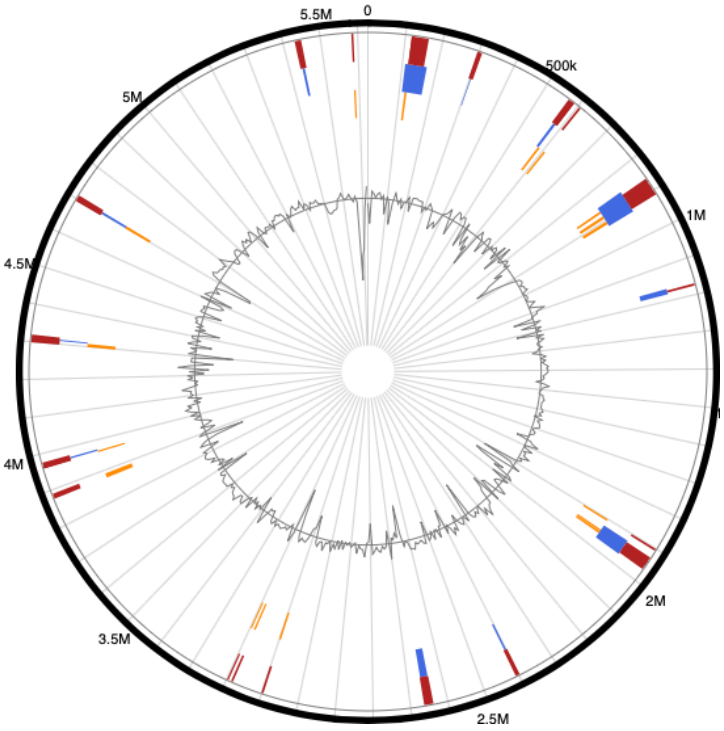


Figure 3. Circular map of the *Pseudomonas putida* TT321 genome. Genome islands (GIs) are highlighted by color within the circular map for prediction methods. Orange and blue were predicted by SIGI-HMM and IslandPath-DIMOB, respectively. Red regions were predicted GIs by at least one method (integrated GIs). GC contents (%) of the genomic sequence are shown by the black line plot.

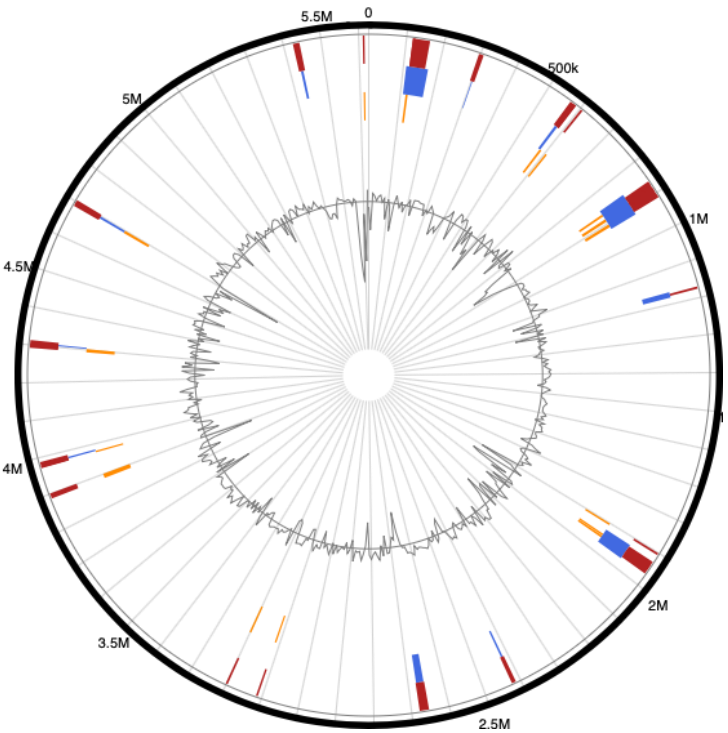


Figure 4. Circular map of the *Pseudomonas putida* TT322 genome. Genome islands (GIs) are highlighted by color within the circular map for prediction methods. Orange and blue were predicted by SIGI-HMM and IslandPath-DIMOB, respectively. Red regions were predicted GIs by at least one method (integrated GIs). GC contents (%) of the genomic sequence are shown by the black line plot.

3.5. The Core Genomic Repertoire Involved in Ammonia Conversion

Genome analysis has been used to investigate functions and applications in nature. *Pseudomonas putida* is involved in natural processes involving the degradation of xenobiotic and biogenic pollutants [27]. Additionally, most *Pseudomonas putida* strains are utilized as non-pathogenic bacteria [28]. TT321 and TT322 have been shown to utilize inorganic nitrogen sources for growth [14]. In this study, genes involved in ammonia conversion and ammonia formation were revealed. There are no homologues for ammonia monooxygenase identified in TT321 and TT322. In these two strains, the ammonia reductase activities may be based on three nitrogen metabolic pathways involving three enzymes: carbamate kinase, glutamine synthetase, and aminomethyltransferase. Carbamate kinase converts ammonia to carbamoyl phosphate and contributes directly to arginine and proline metabolism. Glutamine synthetase converts ammonia to glutamine. This reaction is irreversible, but glutamine is then hydroxylzed by a different mechanism via glutaminase [29]. Both glutamine synthetase and glutaminase were found in TT321 and TT322. All sequences of *Pseudomonas* genomes encode glutamine synthetase (*gs*) to regulate the pool of glutamine and glutamate in a cell, and glutaminase is present in all 33 genomes. Aminomethyltransferase catalyzes ammonia to the aminomethyl moiety of glycine [30]. The three enzymes involved in ammonia formation contain histidine ammonia-lyase, aspartate ammonia-lyase, and nitrite reductase. Histidine ammonia-lyase catalyzes histidine to ammonia and urocanic acid. Aspartate ammonia-lyase catalyzes aspartate to ammonia and fumarate. Nitrite reductase catalyzes nitrite to ammonia via the denitrification process. All of these enzymes were found in the studied genomes.

The ammonia assimilation components are mostly conserved among the 33 analyzed genomes. The Rapid Annotation using Subsystem Technology (RAST) analysis showed that almost all *Pseudomonas* strains lack the complete denitrification process from nitrate to molecular nitrogen, with the exception of *Pseudomonas fluorescens* F113 and *Pseudomonas fluorescens* NCIMB 11764. The annotation results also indicated the absence of arginase 1 (Supplementary Table S6), which leads to an incomplete urea cycle. This is likely because arginase 1 is involved in the last step (to form ammonia) in the urea cycle and was found in almost all studied strains, with the exception of *Pseudomonas syringae* UMAF0158, *Pseudomonas protegens* Pf-5, *Pseudomonas fluorescens* A506, *Pseudomonas syringae pv. syringae* B728a, *Pseudomonas syringae pv. syringae* B301D and *Pseudomonas fluorescens* SBW25. This implies that TT321 and TT322 could use nitrogen energy efficiently for ammonia assimilation.

Based on the genomic data analysis, a hypothesis on the possible pathways of ammonia conversion by TT321 and TT322 is proposed in Figure 5, which was adapted from [26,31]. All enzymes shown in Figure 5 were found in the 33 genomes studied. Four processes are known to be involved in the formation of ammonia: from nitrate by a denitrification process; from the histidine catalyzed by the histidine ammonia-lyase; from aspartate via the aspartate ammonia-lyase; and from glutamate by the glutamate hydrogenase (GDH). Three processes to decompose ammonia may be used in TT321 and TT322 as follows: to carbamoyl phosphate via carbamate kinase; to glycine, serine and threonine metabolism via aminomethyltransferase; and to glutamine via glutamine synthetase.

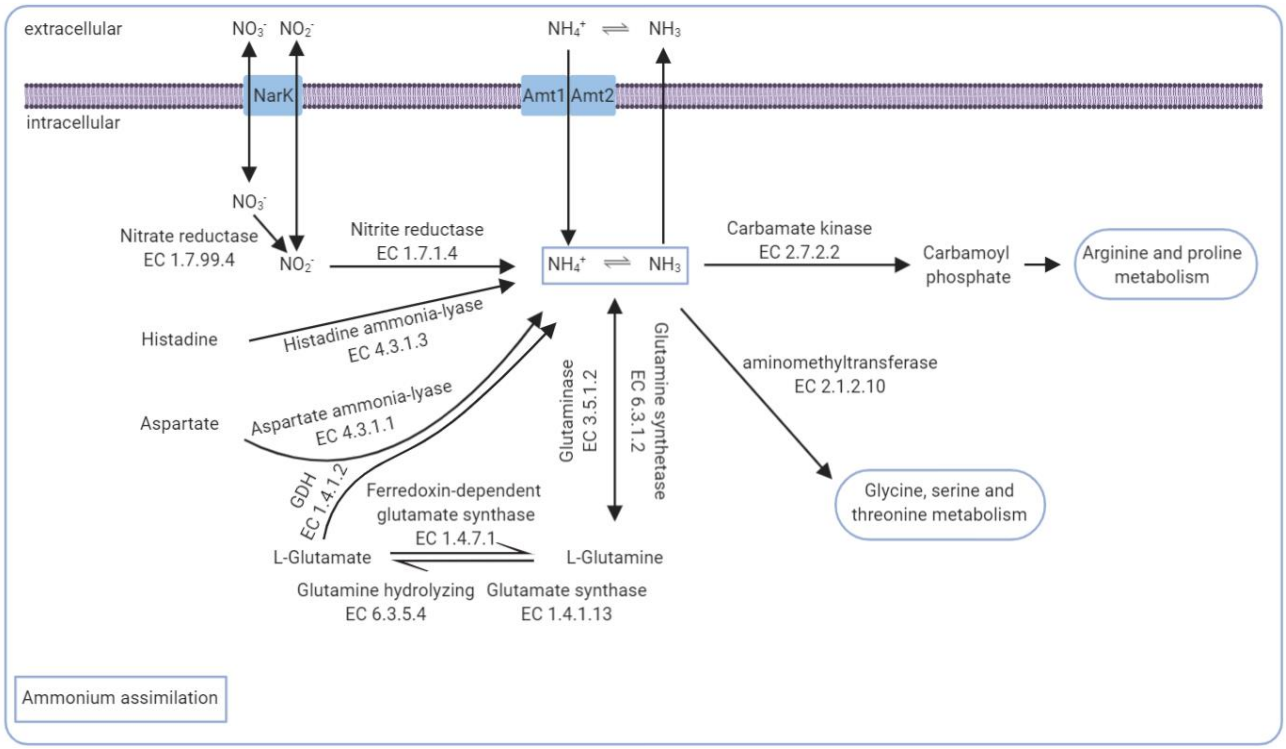


Figure 5. A hypothesis of ammonia conversion in *Pseudomonas putida* TT321 and *Pseudomonas putida* TT322. The ammonia conversion was adapted from [26,31]. All enzymes were found in studied genomes.

4. Conclusions

This study provides novel information about the conversion of ammonia by *Pseudomonas* TT321 and TT322ss as well as using ammonia as a nitrogen source to grow with L-glutamine and L-glutamate metabolism. Ammonia assimilation in *Pseudomonas putida* was increased with the involvement of two more pathways comprising arginine and proline metabolism, and glycine, serine and threonine metabolism.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fermentation8070336/s1, Table S1: The gene sequences involved in nitrogen metabolism in 33 genomes; Table S2: BPGA analysis; Table S3: The number of Genome Islands (GIs) in 33 genomes; Table S4: The integrated genomic islands on TT321; Table S5: The integrated genomic islands on TT322; Table S6: The presence of arginase, carbamoyl phosphate synthetase I and carbamate kinase in 33 genomes.

Author Contributions: T.T.T. performed the experiments, analyzed the data, and wrote the manuscript. N.J.B. completed phylogenetic tree analysis and edited the manuscript. R.v.G. provided feedback and edited the manuscript. N.T.N. collected the samples and submitted sequences. P.M.T.C. completed the BPGA analysis. H.H.C. designed the experiments and modified the manuscript. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: TT321 accession number VCPR00000000; TT321 accession number VCPS00000000.

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Conflicts of Interest: The authors declare no conflict of interest.

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