Liu *et al. BMC Microbiology* (2025) 25:104
https://doi.org/10.1186/s12866-025-03808-9

BMC Microbiology

RESEARCH Open Access

Nitrogen metabolism of the highly ureolytic bacterium *Proteus penneri* S99 isolated from the rumen

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Abstract

Background The model rumen-dominant ureolytic bacterium *P. penneri* S99 exhibits high urease activity. It was cultivated using ammonia, urea, amino acids, or their combination as nitrogen sources. To identify differences in gene expression, the transcript abundances of various genes involved in nitrogen metabolism were analyzed by harvesting mRNA from cells during the exponential growth phases on different nitrogen sources.

Results *P. penneri* S99 can utilize ammonia, urea, or amino acids as the sole nitrogen sources for growth and shows a preference for utilizing urea. It exhibits similar growth rates and maximum biomass on ammonia and urea, but showed higher growth rates and maximum biomass on amino acids. Transcriptome sequencing analysis revealed different transcription patterns in response to different nitrogen sources. The urease gene expression was detected in all three different nitrogen sources, and complete hydrolysis of urea was also observed when other nitrogen sources were added to the medium containing urea. The regulation of urease in *P. penneri* S99 was characterized by constitutive expression, not by urea. The growth of *P. penneri* S99 on ammonia, ammonium acid, and urea was similar, with the only observed difference being an increase in urease transcript abundance.

Conclusions The transcription patterns of nitrogen metabolism genes offer insights into how nitrogen is utilized in the rumen.

Keywords Rumen, Urease, *Proteus penneri* S99, Ammonia assimilation, Transcriptome

Background

The ultimate end products of the digestion and metabolism of true protein and non-protein nitrogen (urea) in the diet of ruminant animals are ammonia, which serves as an important source for rumen bacteria to synthesize microbial protein [1, 2]. Microbial protein is the major metabolizable nitrogen for ruminant milk and meat production. Furthermore, ammonia is an important precursor of bacterial nitrogen. Nitrogen plays a crucial role in the formation of vital components within cells, including amino acids, nicotinamide adenine dinucleotide, pyrimidines, purines, and amino sugars [3]. Thus the efficiency

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of ammonia assimilation significantly impacts bacterial protein synthesis and feed utilization efficiency [4].

Ammonia assimilation enzymes in bacterial domains are conserved, with their transcriptional regulation influenced by the environmental niche [5, 6]. The process of nitrogen assimilation is regulated by three enzymes: GDH (*gdh*A), a redox-dependent enzyme that facilitates the direct integration of ammonium into α–ketoglutarate; GS (*gln*A and *gln*N), an ATP-dependent enzyme responsible for synthesizing glutamine; and GOGAT, an enzyme composed of two subunits encoded by *glt*B and *glt*D genes, involved in the synthesis of glutamate. This enzymatic mechanism functions in a cyclical manner within the GS-GOGAT pathway, facilitating the interconversion of glutamate and glutamine [7]. Urea is hydrolyzed by urease into ammonia and carbamate, with carbamate spontaneously decomposing to generate two molecules of ammonia and carbonic acid [8]. The assimilation of ammonia molecules occurs via either the GDH or GS-GOGAT pathway.

Urea is frequently employed as an economical substitute for feed proteins in the ruminant diet, serving as a non-protein nitrogen source [9]. Ureolytic bacteria play a major role in rumen urea hydrolysis and nitrogen metabolism [10]. However, urea hydrolysis mediated by urease occurs rather rapidly, resulting in poor efficiency in the utilization of urea-nitrogen in ruminants. The diversity and ecological distribution of ureolytic bacteria in the rumen have been extensively studied to improve urea nitrogen utilization, but their regulatory mechanism in nitrogen metabolism has not been thoroughly investigated [11, 12].

Current research indicates that different ureolytic bacteria have varying preferences for nitrogen sources and nitrogen metabolism patterns. *Ruminococcus albus* 8 can utilize urea, ammonia, or peptides for growth but cannot use amino acids [4]. Its nitrogen metabolism genes and enzyme activities are regulated by the nitrogen source and ammonia levels. *Succinivibrio dextrinosolvens* Z6 can grow on urea, ammonia, and amino acids, with the maximum biomass achieved under amino acid cultivation conditions [13]. The activity of nitrogen metabolism enzymes differs among various nitrogen sources. We isolated and identified 28 strains of ureolytic bacteria from rumen, with *Proteus penneri* S99 being identified as the dominant high urease activity ureolytic bacterium [14]. The difference in the S99 urease gene cluster from those reported in published studies suggests the possibility of new or alternative nitrogen metabolism pathways. This study used transcriptome analysis to study the responses of nitrogen metabolism genes to various nitrogen sources in *P. penneri* S99. This study provides a theoretical basis for analyzing rumen nitrogen metabolism and improving the efficiency of urea nitrogen utilization.

Methods

Bacteria and culture conditions

The *P. penneri* S99 strain used in this study was previously isolated in our laboratory. *P. penneri* S99 cultures were grown at 39℃ in an anaerobic chamber with an atmosphere consisting of 85% N_2, 10% CO_2, and 5% H_2. The base medium consisted of (100 ml) 5 ml of clarified rumen fluid, 0.05 g glucose, 0.05 g cellulose, 15 ml solution 4 (3 g/l K_2HPO_4), 15 ml solution 5 (0.6 g/l $CaCl_2$, 3 g/l KH_2PO_4, 6 g/l NaCl, 0.6 g/l $MgSO_4{\cdot}7H_2O$), 0.1 ml pfennig trace element (300 mg/L H_3BO_3, 100 mg/l $ZnSO_4{\cdot}7H_2O$, 30 mg/l $MnCl_2{\cdot}4H_2O$, 20 mg/l $CoCl_2{\cdot}6H_2O$, 30 mg/l $Na_2MoO_4{\cdot}2H_2O$, 10 mg/l Na_2SeO_3, 20 mg/l $NiCl_2$, 10 mg/l $CuCl_2{\cdot}2H_2O$, 150 mg/l $FeCl_2{\cdot}4H_2O$), 5 ml hemin (0.05 mg/ml), 0.1 ml resazurin (0.1%), 0.31 ml volatile fatty acid mix (17 ml/l acetic, 6 ml/l propionic, 4 ml/l n-butyric, 1 ml/l n-valeric, 1 ml/l isovaleric, 1 ml/l isobutyric, 1 ml/l 2-methyl butyric acids), 0.8 g $NaHCO_3$, 0.05 g L-cysteine HCl, and 59.49 ml ddH_2O, with the only variation being in the chemical form of nitrogen provided. The medium nitrogen sources used included 7 different combinations: urea, $(NH_4)_2SO_4$, amino acid mixture (Coolaber, Beijing, China), urea + $(NH_4)_2SO_4$, urea + amino acid mixture, $(NH_4)_2SO_4$ + amino acid mixture, and urea + $(NH_4)_2SO_4$ + amino acid mixture. Additionally, a nitrogen source equivalent to an NH_4^+ concentration of 18.8 mM was included.

P. penneri S99 was activated in a medium with different nitrogen sources (1% inoculation amount). It was then incubated anaerobically at 39℃ for 12 h and subcultured on the same medium (1% inoculation amount) through subsequent generations. The optical density (OD600nm) was determined by a spectrophotometer at 0, 3, 6, 9, 12 and 24 h, respectively. At the same time, 1.5 ml bacterial culture was collected and immediately centrifuged (12,000 g, 4℃, 5 min). An equal volume of 25% metaphosphoric acid was then added to the supernatant and it was frozen at -80℃ for chemical analysis. In addition, the bacterial culture was collected during the logarithmic growth phase and immediately centrifuged at 12,000 g for 5 min at 4℃. The sediment pellets were then frozen at -80℃ for RNA extraction. Three replicates were set per process.

Chemical analyses

The NH_3-N was determined using the Berthelot alkaline phenol-hypochlorite method [15]. The urea-N was determined using the diacetyl monoxime method kit (Nanjing Jiancheng Co., Nanjing, China). The total free amino acid was determined using ninhydrin colorimetry method kit (Nanjing Jiancheng Co., Nanjing, China). Statistical significance ($P < 0.05$) was tested with Wilcoxon rank-sum test. A line graph illustrating the differences in growth

and substrate utilization among various nitrogen sources was generated using GraphPad Prism 8 [16].

RNA extraction and cDNA synthesis

The total RNA of the bacteria was extracted using the MolPure® Bacterial RNA Kit (Yeasen Biotechnology (Shanghai) Co., Ltd., Shanghai, China) and operated according to the provided instructions. The concentration and purity of the purified RNA were detected using Nanodrop 2000, while the integrity of RNA was assessed using Agilent 2100. The total amount of RNA required for a single library construction is 2 ug, with a concentration of ≥ 100ng/μL and an OD260/280 ratio between 1.8 and 2.2. Using Hieff NGS® MagSP rRNA Removal Kit (Prokaryote) with purification beads Kit (Yeasen Biotechnology (Shanghai) Co., Ltd., Shanghai, China), we removed the rRNA and added fragmentation buffer to randomly break down mRNA into small fragments of about 200bproteus by using Hifair® AdvanceFast 1st Strand cDNA Synthesis Kit (No Dye) (Yeasen Biotechnology (Shanghai) Co., Ltd., Shanghai, China), reverse transcription synthesis of cDNA is performed. The cDNAs were paired-end (2 × 150 bp) sequenced on the Illumina Hiseq system.

Transcriptome statistical analysis

Quality control of raw data was performed using FastQC[1] in HTQC software [17]. After removing the low-quality sequences, the sequences are pruned using Sickle software and SeqPrep2 program. High-quality sequences were compared to the genome of *P. penneri* S99 using the Burrow-Wheeler Aligner (BWA) and DIAMOND [18]. The predicted coding genes were compared with six mainstream databases (NR [19], Swiss-Prot [20], Pfam [21], COG [22], GO [23], KEGG [24]) for functional annotation. The gene expression levels were analyzed quantitatively by RSEM [25]. After obtaining the read counts of genes through gene expression analysis, DESeq was used to identify and analyze the differentially expressed genes between samples or groups [26]. *P. penneri* S99's whole genome sequencing and gene annotation can be found in the previously published article [14], where nitrogen metabolic pathways are mapped by Figdraw [27].

Results

P. Penneri S99 nitrogen metabolism pathway

We determined that the genome of *P. penneri* S99 is 3.94 Mbp, with a G + C content of 37.84%. It has a total of 3442 coding sequences (Fig. 1). Genome analysis revealed that *P. penneri* S99 encodes key genes involved in nitrogen metabolism and ammonia assimilation, as illustrated in Fig. 2. The study identified two subunits of GOGAT (large subunit *gltB* and small subunit *gltD*), as well as type I GS (*glnA*), adenylate transferase (*glnE*), NADPH-dependent GDH (*gdhA*), NADH-dependent GDH (*gdh*), urease (*ureD*, *ureA*, *ureB*, *ureC*, *ureE*, *ureF*, ure*G*), high affinity ammonium transporter (*amtB*), nitrogen regulatory protein PII1 (*GlnB*), nitrogen regulatory protein PII2 (*GlnK*) and prokaryotic two-component nitrogen regulatory system genes (*glnL*, *glnG*).

P. Penneri S99 nitrogen utilization

The growth of *P. penneri* S99 varied among different nitrogen sources. The growth rate was highest when amino acids were the sole nitrogen source, resulting in a greater maximum cell biomass (OD600 = 0.26) compared to urea (OD600 = 0.18) and ammonia (OD600 = 0.16) as the exclusive nitrogen sources (Fig. 3A). When urea (OD600 = 0.22) and ammonia (OD600 = 0.24) were supplemented along with amino acids, or when a mixture of urea, ammonia, and amino acids (OD600 = 0.23) was used as nitrogen sources, the growth rate and maximum biomass of *P. penneri* S99 exhibited similarity to those observed when amino acids served as the sole nitrogen source (Fig. 3A). When urea and ammonia were the sole nitrogen sources, the growth rate of *P. penneri* S99 was comparable to its maximum cell biomass (Fig. 3A). When a mixture of urea and ammonia was used as nitrogen sources, the growth rate and maximum cell biomass (OD600 = 0.18) of *P. penneri* S99 remained similar to those observed when urea and ammonia were the only nitrogen sources (Fig. 3A). *P. penneri* S99 has the ability to utilize urea, ammonia, and amino acids as its sole nitrogen sources for optimal growth. After fermenting for 24 h, urea is completely decomposed (Fig. 3B), while there is a reduction in ammonia by 20% (Fig. 3C), and a reduction in amino acids by 50% (Fig. 3D).

Complete hydrolysis of urea was observed after 24 h of fermentation when ammonia, amino acids, or a combination thereof were introduced into the urea medium (Fig. 4A BD). When ammonia, urea, or a mixture of ammonia and urea are added to the amino acid medium, the hydrolysis of amino acids occurs slowly with an increase in fermentation time, indicating a preference for utilizing urea (Fig. 4B CD). When urea, amino acids, or a combination of both were added to the ammonia medium, the ammonia levels remained stable for 6 h before fermentation and gradually increased afterwards. The amount of ammonia decreased slowly after 3 h of fermentation when amino acids were added to the ammonia medium, and then increased after 24 h (Fig. 4A CD). The presence of ammonia in the environment is preferred for growth when it is available, possibly because the transportation of amino acids requires energy.

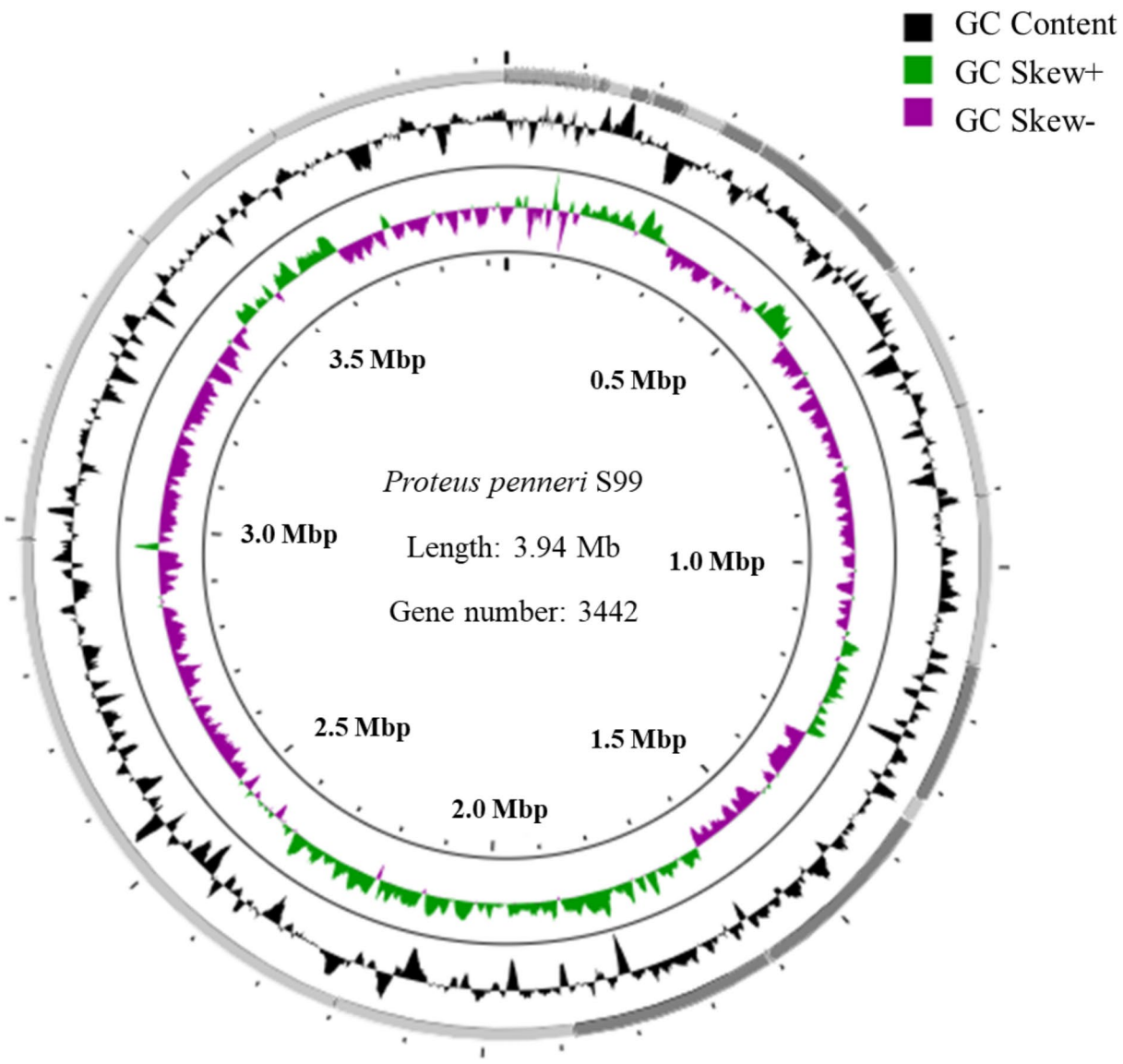

Fig. 1 Circle diagram showing the genome of *P. penneri* S99. From the outside to the inside are Contigs, GC-content and GC-skew

P. Penneri S99 gene expression quantity

There was no difference in the overall gene expression and distribution of *P. penneri* S99 among different nitrogen sources (Fig. 5A). *P. penneri* S99 exhibited 4022 genes with shared expression across three distinct nitrogen sources. Specifically, 20 genes were expressed in response to urea, while the expression of 21 specific genes was induced by amino acids. Additionally, ammonia triggered the expression of 12 specific genes (Fig. 5B). Compared to ammonia, 387 genes were upregulated, while 464 genes were downregulated in urea (Fig. 5C). In comparison with amino acids, urea led to the upregulation of 255 genes and the downregulation of 179 genes (Fig. 5C). Conversely, when compared to amino acids, ammonia resulted in the upregulation of 642 genes and the downregulation of 395 genes (Fig. 5C).

Transcript abundances of nitrogen utilization genes

The expression levels of urease genes (*ureD*, *ureA*, *ureB*, *ureC*, *ureE*, *ureF*, and *ureG*) and GDH genes (*gdhA* and *gdh*) were found to be upregulated in the presence of urea compared to ammonia. Notably, a significant difference was observed in the expression level of the *ureD* and *ureC* gene ($P < 0.05$). On the other hand, downregulation was observed in GS gene (*glnA*), GOGAT genes (*gltB* and *gltD*), adenylate transferase gene (*glnE*), high-affinity ammonium transporter gene (*amtB*), nitrogen regulatory proteins genes (*GlnK* and *GlnB*), as well as prokaryotic two-component nitrogen regulatory system genes

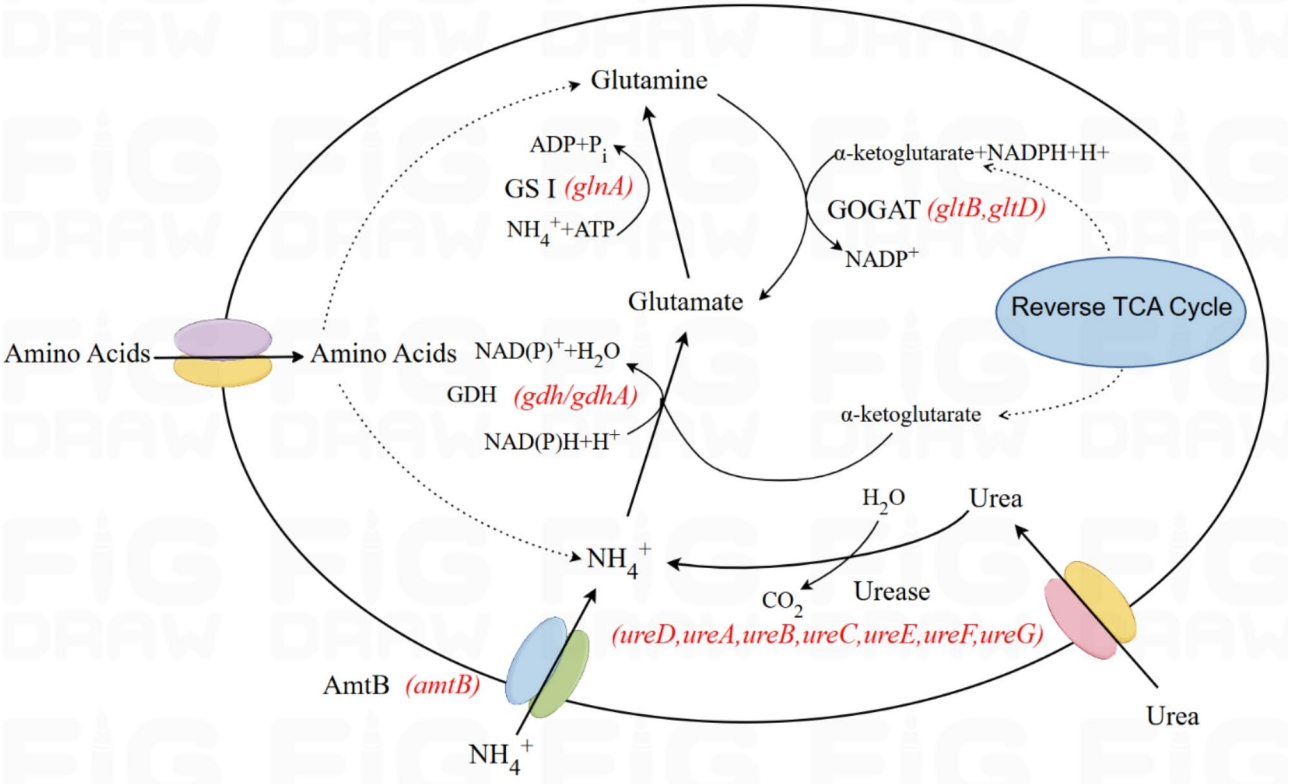

Fig. 2 Nitrogen metabolism pathways identified in *P. penneri* S99 through bioinformatic analyses

(*glnL* and *glnG*). Furthermore, significant differences were noted for *glnL* and *glnG* expressions levels ($P < 0.05$). (Fig. 6)

The expression levels of GDH (*gdhA*, *gdh*), Urease (*ureD*, *ureA*, *ureB*, *ureC*, *ureE*, *ureF*, and *ureG*) and prokaryotic two-component nitrogen regulatory system (*glnL*, *glnG*) were found to be upregulated in the presence of urea compared to amino acids. Furthermore, significant difference was noted for *glnL*, *glnG*, and *ureC* expressions levels ($P < 0.05$). Conversely, GS (*glnA*), GOGAT genes (*gltB*, *gltD*), adenylate transferase gene (*glnE*), high-affinity ammonium transporter gene (*amtB*), and nitrogen regulatory proteins (*GlnK*, *GlnB*) showed downregulation with statistically significant differences observed among *glnB* ($P < 0.05$). (Fig. 6)

The expression levels of adenylate transferase (*glnE*), nitrogen regulatory protein (*glnK*), GOGAT (*gltB*, *gltD*), GS (*glnA*), and prokaryotic two-component nitrogen regulatory system (*glnL*, *glnG*) were found to be upregulated in the presence of ammonia compared to amino acids. Notably, significant differences were observed for *glnL* and *glnG* expression levels under these conditions ($P < 0.05$). Conversely, the genes encoding GDH (*gdhA*, *gdh*), Urease (*ureD*, *ureA*, *ureB*, *ureC*, *ureE*, *ureF*, and *ureG*), high-affinity ammonium transporter (*amtB*) and nitrogen regulatory protein (*glnB*) exhibited downregulation with statistically significant differences observed among *ureD* and *glnB* ($P < 0.05$). (Fig. 6)

Discussion

This study compared the growth and substrate utilization of *P. penneri* S99, a highly active rumen-dominant ureolytic bacterium, when it was provided with urea, ammonia, and amino acids as nitrogen sources. The findings indicated that *P. penneri* S99 could thrive solely on ammonia and urea as nitrogen sources, exhibiting comparable growth patterns on both substrates. However, it exhibited a higher maximum biomass and growth rate when amino acids were the sole source of nitrogen. This is consistent with the phenotype of *S. dextrinosolvens* Z6 [13], which, unlike R. albus 8 [4], is unable to utilize amino acids as a nitrogen source. Based on genomic analysis, *P. penneri* S99 was found to have genes encoding ammonia transporters, but no genes encoding urea and amino acid transporters were identified. This may be attributed to the incomplete genome we obtained, which

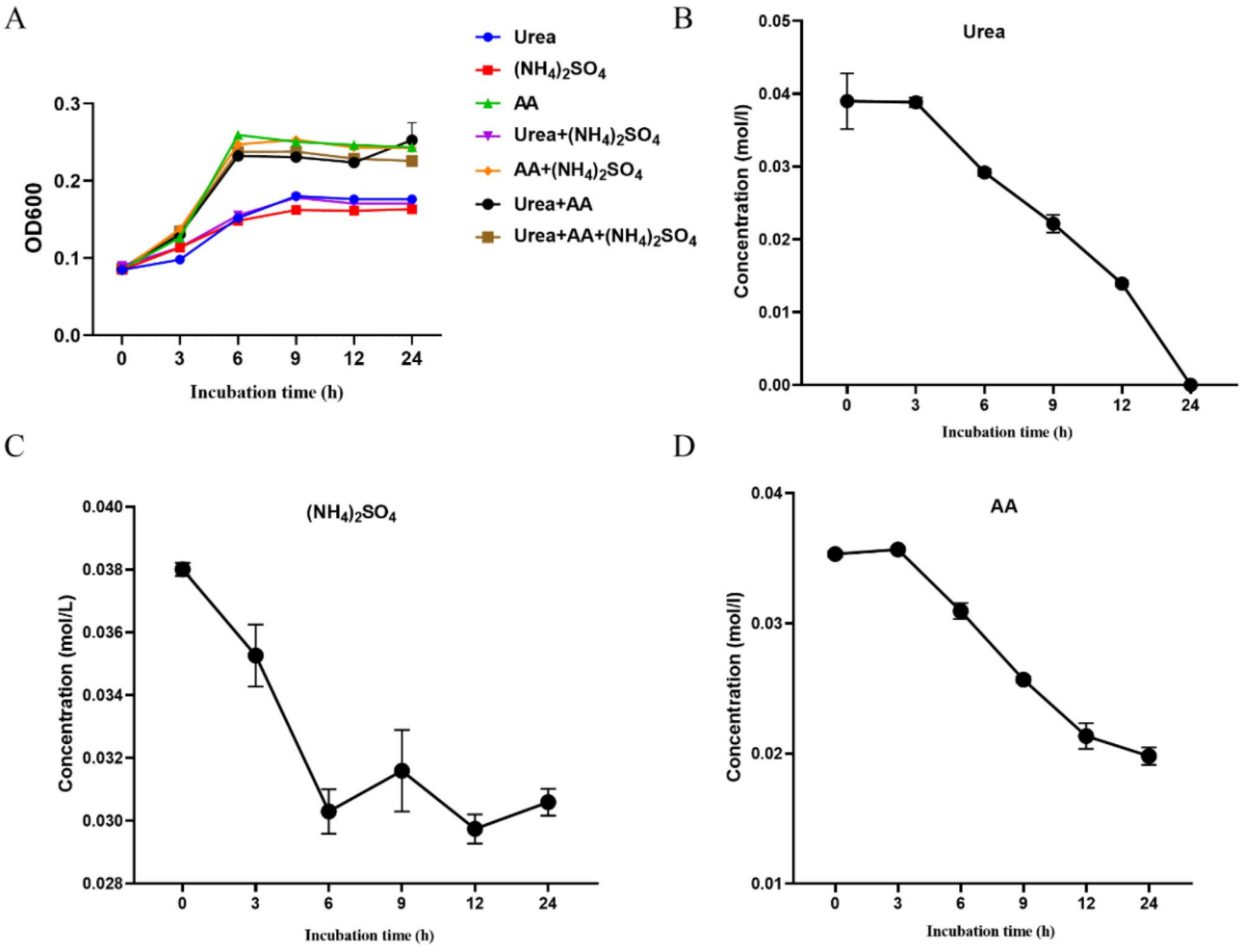

Fig. 3 Growth patterns and substrate content variations of *P. penneri*S99 under different nitrogen sources. (**A**) Growth curve of *P. penneri* S99 under different nitrogen sources; (**B**) Changes in urea content with increasing incubation time of *P. penneri* S99; (**C**) Changes in ammonia content with increasing incubation time of *P. penneri* S99; (**D**) Changes in amino acid content with increasing incubation time of *P. penneri* S99

contains gaps. In future studies utilizing third-generation sequencing technologies, such as nanopore sequencing, a more optimal solution may be provided [28].

The transcriptional abundance of *glnB*, *glnL*, and *glnG* exhibited significant differences as the nitrogen sources were changed. This result showed that, like most bacteria, S99 maintained nitrogen metabolism balance through the prokaryotic two-component nitrogen regulatory system and the nitrogen regulatory protein PII1, ensuring normal cell growth under different nitrogen supply environments [29]. The *glnB* gene was significantly up-regulated in the amino acid medium, while the *glnL* and *glnG* genes were significantly down-regulated in the same medium. Amino acids served not only as nutrients but also as signal molecules that regulated the expression of genes related to nitrogen metabolism [30]. For instance, certain amino acids, such as methionine, provided methyl groups for DNA, RNA, and protein methylation modifications, which subsequently impacted gene expression and regulation [31].

The transcriptional abundance of the urease gene increased in response to urea as the sole nitrogen source. However, urease gene expression was detected in all three different nitrogen sources, and complete hydrolysis of urea was also observed when other nitrogen sources were added to the medium containing urea. The presence or absence of urea in the medium did not affect the transcriptional activity of urease; therefore, we hypothesized that urease expression in *P. penneri* S99 was constitutive [32]. Similarly to *P. penneri* S99, *Corynebacterium glutamicum* [33], *Ruminococcus albus* [4], and *Klebsiella pneumoniae* [34] also demonstrated constitutive urease expression, exhibiting detectable urease activity under nitrogen-limited conditions even in the absence of urea induction. The expression of urease by composition indicated that the expression of the urease gene was not influenced by environmental factors [35]. The abundance of *P.*

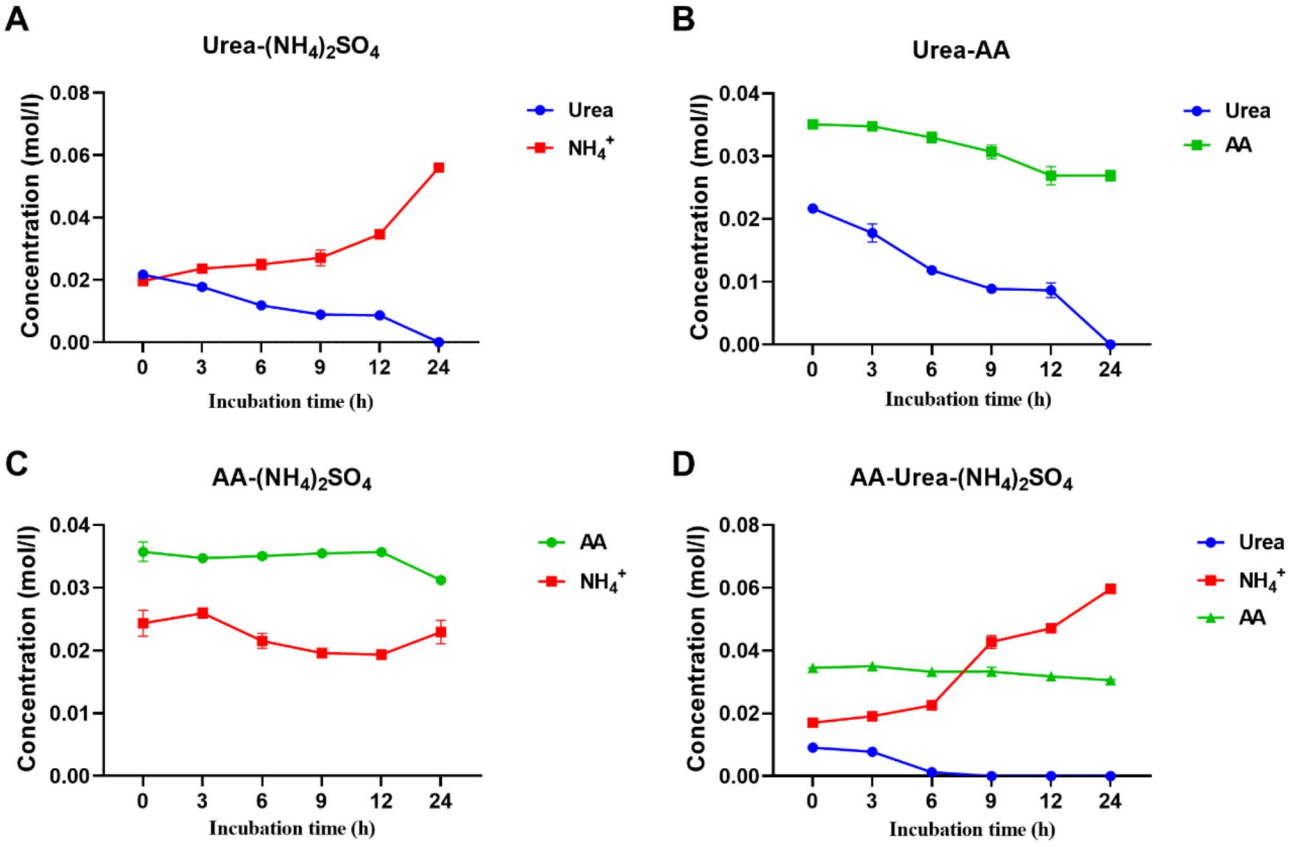

Fig. 4 Growth patterns and substrate content variations of *P. penneri*S99 under different nitrogen sources. (**A**) The changes in urea and ammonia content vary with incubation time as *P. penneri* S99 grows in a mixture of nitrogen sources, including urea and ammonia; (**B**) During the growth of *P. penneri* S99 in a mixed nitrogen source of urea and amino acids, the changes in urea and amino acid content were observed with increasing incubation time; (**C**) As *P. penneri* S99 grows in a mixture of amino acids and ammonia as nitrogen sources, the levels of amino acids and ammonia change over time during incubation; (**D**) During growth in mixed nitrogen sources comprising urea, amino acids, and ammonia, *P. penneri* S99 exhibits changes in levels of urea, amino acids, and ammonia as a function of incubation time

penneri S99 transcripts in both urea and ammonia was essentially similar, except for the presence of the urease gene. This suggested that the ammonia produced within cells from urea was enough to sustain cellular growth.

GDH hydrolyzes glutamic acid to produce α-ketoglutaric acid and ammonia, and the transcriptional abundance patterns of *gdh* and *gdhA* genes were correlated with GDH activity [36]. In this study, it was found that the transcriptional abundance of the GDH gene increased relatively during the exponential growth phase when urea and amino acids were the only nitrogen sources, which was consistent with the findings in Wen et al. [37]. Under nitrogen limitation, *Prevotella bryantii* exhibited an increase in NADH-GDH activity. Similarly, the activity of NADP+-GDH was found to significantly increase in response to nitrogen starvation in *Corynebacterium glutamicum* [38]. However, Harper's study [39] showed that the activity of NADP+-GDH aminating reaction did not change significantly in response to prolonged exposure to nitrogen limitation, implying that post-transcriptional modification serves as a regulatory mechanism in response to nitrogen availability.

GS catalyzes the synthesis of glutamine from ammonium ions and glutamate, whereas GOGAT facilitates the conversion of glutamine and α-ketoglutaric acid into glutamate. Together with GS, they constituted the GS-GOGAT cycle, which played a crucial role in glutamate synthesis under conditions of limited ammonium availability [40]. When ammonia was the sole nitrogen source for exponential growth, the concentration of ammonia decreased and the transcriptional abundance of glnA and gltB/D increased relative to each other. This finding was consistent with previous Magasanik's research, which showed that GS activity was highly upregulated in gut bacteria when exposed to a low ammonia environment [41]. In Escherichia coli, the corresponding activity of GS showed an increase under N limitation [42]. The Type III GS gene was not found in the strain *P. penneri* S99 in this study, which was also an important pathway for ammonia assimilation in a low nitrogen environment [43].

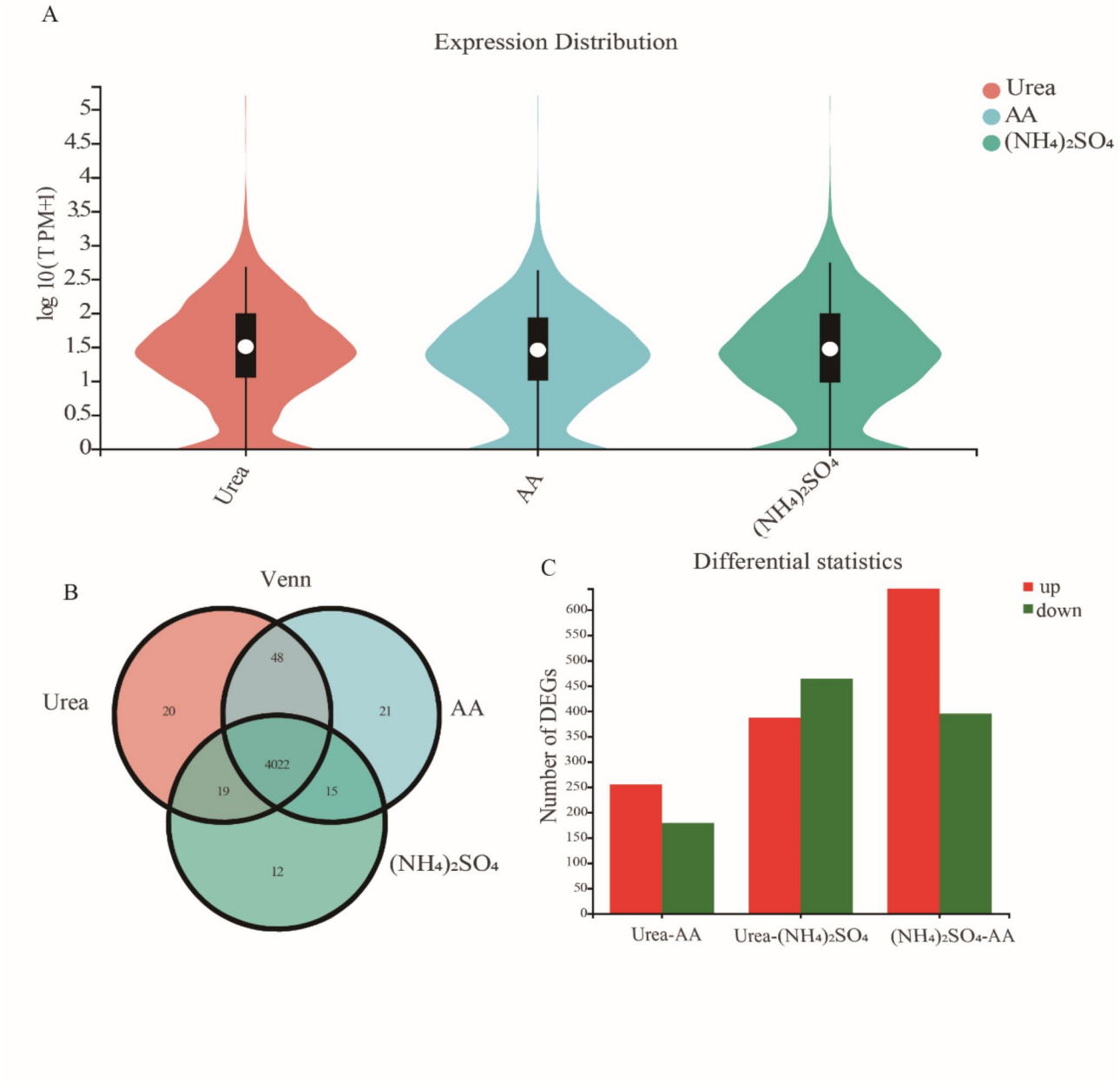

Fig. 5 Gene expression levels of*P. penneri* S99 in different nitrogen sources. (**A**) The violin plot displays the distribution of expression levels, with each color representing a different sample. The inflated portions indicate the most concentrated range of gene expression across all samples. (**B**) A Venn diagram is used to illustrate gene expression among different groups of samples, with each colored circle representing a group and the numbers indicating the shared and unique genes between two or three groups. (**C**) A bar chart is used for statistical representation of differential gene expression, where the x-axis represents differentially expressed groups and the y-axis represents the number of differentially expressed genes. Red denotes upregulated genes, while green denotes downregulated genes

The diverse ureolytic bacteria in the rumen may lead to different research outcomes when based on a single strain, due to the complexity of the rumen ecosystem. This method enables a comprehensive analysis of nitrogen metabolism mechanisms in ureolytic bacteria and provides guidance for practical applications.

Conclusions

In this study, we found that *P. penneri* S99 maintained intracellular ammonia levels and normal growth through nitrogen-regulating genes in different nitrogen sources. *P. penneri* S99 can grow normally in three different nitrogen sources: amino acids, ammonia, and urea, with no significant difference in the expression of ammonia assimilation genes among these sources. The expression of the urease

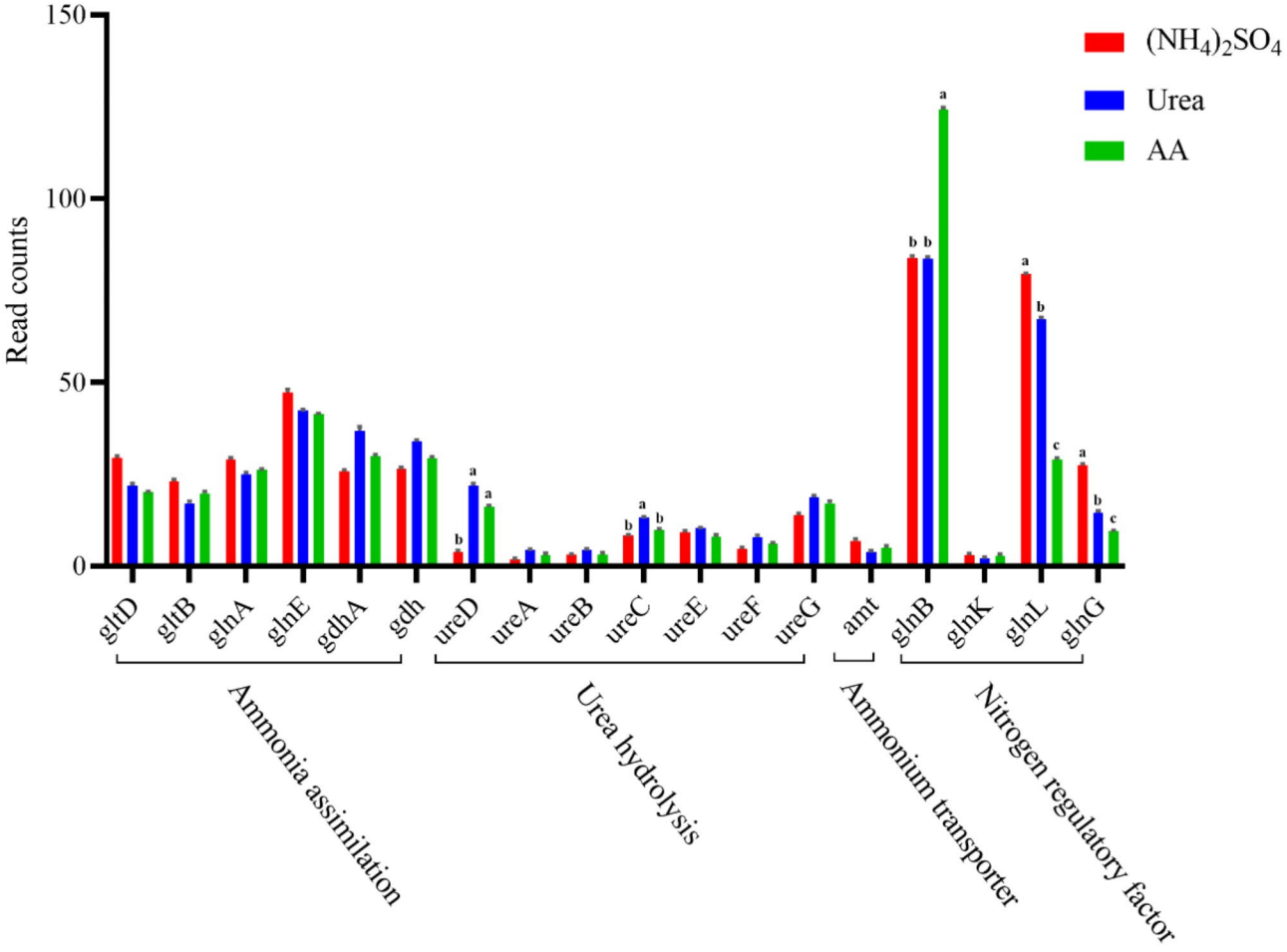

Fig. 6 Comparative analysis of differentially expressed genes associated with nitrogen metabolism in cells cultured in media supplemented with amino acids, ammonium ions, or urea

gene is constitutive, and urea is preferred. Further studies on *P. penneri* S99, including constructing a co-expression network based on gene expression correlation, identified potential transcription factors co-expressed with urease genes. This was verified by gene knockout experiments and provided regulatory targets for modulating rumen metabolism. In addition, expanding the resource base of ureolytic bacteria and comprehensively analyzing the mechanisms of action of different bacteria in rumen nitrogen metabolism will help provide a theoretical basis for improving the utilization rate of rumen nitrogen, particularly urea.

Acknowledgements
Thanks to the projects that have provided funding for this study.

Author contributions
SL designed and conducted the experiments, analyzed the data, and drafted the manuscript. NZ helped revise the manuscript. SZ analyzed the data and revised the manuscript. JW acquired financial support and revised the manuscript.

Funding
This study was funded by National Key R&D Program of China (2022YFD1301000), the Agricultural Science and Technology Innovation Program (CAAS-ZDRW202304) and State Key Laboratory of Animal Nutrition and Feeding (2004DA125184G2108).

Data availability
The transcriptome sequences of Proteus penneri S99 have been deposited in the Genome Sequence Archive under the primary accession code CRA018531 (BIG Sub - GSA).

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

Received: 13 August 2024 / Accepted: 5 February 2025
Published online: 28 February 2025

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