NtrC, The Master Regulator Controls the Utilization of Alternative Nitrogen Sources in Pseudomonas Stutzeri A1501

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Research Article

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Abstract

*Pseudomonas stutzeri* A1501 is the model strain for studying associative nitrogen fixation and possesses the nitrogen regulatory NtrC protein in the core genome. Nitrogen source is one of the important factors affecting the efficiency of biological nitrogen fixation in the natural environment. However, the regulation of NtrC in nitrogen metabolism of *P. stutzeri* A1501 is not clear. In this work, phenotypic analysis of the ntrC mutant characterized the roles of NtrC for the nitrogen metabolism and oxidative stress response of *P. stutzeri* A1501. To systematically identify NtrC-controlled gene expression, RNA-seq was performed to further analyze the gene expression differences between the wild type strain and the ΔntrC mutant under nitrogen fixation conditions. A total of 1431 genes were found to be significantly altered by the ntrC deletion, among which 147 associative genes had NtrC-binding sites, and pathways for nitrogen fixation regulation, the acquisition and catabolism of nitrogenous compounds and nitrate assimilation were particularly discussed. Furthermore, the oxidative stress-related gene (katB), upregulated by the ntrC deletion, was suggested to be the potential target gene of NtrC, underlining the importance of NtrC to nitrogenase protection against oxygen damage. Based on these findings, we propose that NtrC is a high-ranked element in the regulatory network of *P. stutzeri* A1501 that controls a variety of nitrogen metabolic and oxidative stress responsive traits required for adaptation to complex rhizosphere environment.

Introduction

Nitrogen is one of the most important limiting elements in the environment. Bacteria have evolved many systems to adjust the cell metabolic system according to the environmental nitrogen supply (Shimizu 2016). NtrC, a regulator protein of nitrogen metabolism, is ubiquitous in bacteria and is the global regulator involved in the regulation of expression of genes in response to nitrogen limitation (Jiang and Ninfa 1999, 2009; Schumacher et al. 2013). In bacteria, NtrC and NtrB constitute a two-component regulatory system of nitrogen metabolism, which is mediated by protein phosphorylation signal conduction (Arcondéguy et al. 2001; Ninfa and Jiang 2005). Once phosphorylated, NtrC binds DNA at specific promoters and activates transcription of target genes (Weiss et al. 1992; Chen and Reitzer 1995).

Homologs of the ntrC genes have been found in many nitrogen-fixing bacteria, but the role of NtrC in nitrogen fixation is not essential. NtrC activates the transcription of nifLA in *Klebsiella pneumoniae* (Merrick 1983; Minchin et al. 1989), however, in *Azotobacter vinelandii, Bradyrhizobium japonicum*, and *Azospirillum brasilense*, NtrC is not involved in the expression of nif gene expression, but plays a role in some other aspects of nitrogen assimilation, such as nitrate utilization and glutamine synthase (GS) activity (Toukdarian and Kennedy 1986; Martin et al. 1988; Liang et al. 1993). In addition, the nitrogen assimilation control gene, nac, of *K. pneumoniae* is also regulated by NtrC (Collins et al. 1993). In *Rhodobacter capsulatus*, NtrC is necessary for urea utilization (Masepohl et al. 2001). The NtrC protein was also found to regulate the biosynthesis of alginate, lipase, and biofilm (Leech et al. 2008; Krzeslak et al. 2008; Kim et al. 2009; Cheng et al. 2018). In both *Pseudomonas aeruginosa* and *Pseudomonas*
fluorescens, NtrBC and CbrAB form a network to control the C/N balance (Li and Lu 2007; Zhang and Rainey 2008; Sánchez et al. 2017).

Pseudomonas stutzeri A1501 was isolated from the rice rhizosphere in China and is a model strain for studying associative nitrogen fixation (Desnoues et al. 2003; Rediers et al. 2003; He et al. 2008). This strain has specific nitrogen metabolic properties, including nitrogen fixation under microaerophilic conditions, denitrification under anaerobic conditions and nitrification under aerobic conditions (Lalucat et al. 2006). The regulation of nitrogen fixation (nif genes) depends on the general nitrogen regulatory system in the core genome (NtrBC and related genes) and the nif-specific regulatory system (NifLA) acquired by horizontal transfer in P. stutzeri A1501 (Yan et al. 2008). In addition, due to the associated nitrogen-fixing bacteria and root system could not form root nodules and other special tissue structures, the nif gene expression is also greatly affected by environmental factors. It is conceivable that the protein NtrC may participate in the expression of the regulator (NifA) in P. stutzeri (Xie et al. 2006). However, its role and mechanism of NtrC involved in the nitrogen metabolism of P. stutzeri A1501 have not been clarified clearly. In this study, we have provided evidences that NtrC is required for regulation of nitrogen metabolism and environmental adaption of P. stutzeri A1501, including nitrogen compounds utilization, nitrate assimilation, denitrification and nitrogen fixation, as well as oxidative stress response. Furthermore, we utilized RNA-seq to compare the global expression profile of ntrC deletion mutant to wild type under nitrogen fixation conditions and identified some potential targets regulated by NtrC, such as genes involved in nitrogen fixation, assimilatory nitrate reduction, urea metabolism, electron transport and ammonium transport, demonstrating that NtrC is a global regulator controlled the nitrogen availability of P. stutzeri A1501 under nitrogen fixation conditions. Given the global regulatory role of NtrC in nitrogen fixing bacteria, the A1501 NtrC will be a promising element for studying the synthetic biology of nitrogen fixation systems and enhancing the nitrogen-fixing efficiency of root-associated diazotrophs.

Materials And Methods

Bacterial strains and culture conditions

The bacterial strains and plasmids used in this study are listed in Table 1. Bacteria were cultivated at 30 °C (Pseudomonas stutzeri) or 37 °C (Escherichia coli) in Luria-Bertani (LB) or mineral lactate medium (medium K). The mineral lactate medium (g L^{-1}) contained KH_2PO_4 0.4, K_2HPO_4 0.1, NaCl 0.1, MgSO_4.7H_2O 0.2, MnSO_4. H_2O 0.01, Fe_2(SO_4)_3. H_2O 0.01, Na_2MoO_4.H_2O 0.01, C_3H_6NaO_3 6 mL, (NH_4)_2SO_4 0.4. When required, nitrogen sources were supplemented at the following final concentrations: 10 mmol L^{-1} (NH_4)_2SO_4, 10 mmol L^{-1} serine, 10 mmol L^{-1} urea or 10 mmol l^{-1} KNO_3. Cultivation of the strain under anaerobic conditions was carried out by using the LB medium supplemented with 10 mM KNO_3 as a terminal electron acceptor of denitrification. The cultivation vessel, which was 60 mL in volume and contained 20 mL of medium, was sealed with butyl rubber, and the remaining oxygen was removed by flushing the tubes with argon for 10 min. The cultivation vessels were inoculated in triplicate with equal amounts of A1501/A1511 cells and shaken at 200 rpm at 30 °C. At regular time intervals.
OD$_{600}$ of 200 µL samples was analyzed. Antibiotics were used at the following concentrations (in µg mL$^{-1}$): ampicillin (Amp) 100; kanamycin (Km) 50; tetracycline (Tc) 10; chloramphenicol (Cm) 40.

Table 1
Strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain/plasmid</th>
<th>Relevant characteristics</th>
<th>Source</th>
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<tr>
<td>P. stutzeri strains</td>
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<tr>
<td>A1501</td>
<td>Wild-type, Culture Collection: CGMCC 0351</td>
<td>Lab collection</td>
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<tr>
<td>A1511</td>
<td>ntrC deletion mutant ,Cm$^r$</td>
<td>This study</td>
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<tr>
<td>A1512</td>
<td>A1511 containing pLAntrC, Tc$^r$ and Cm$^r$</td>
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<td>A1513</td>
<td>A1511 containing pLAcbrB, Tc$^r$ and Cm$^r$</td>
<td>This study</td>
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<td>E. coli strains</td>
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<tr>
<td>JM109</td>
<td>Competent cell for cloning</td>
<td>Takara</td>
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<td>Plasmids</td>
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<tr>
<td>pLAFR3</td>
<td>Mobilizable vector, Tc$^r$</td>
<td>(Staskawicz et al. 1987)</td>
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<tr>
<td>pKatCAT5</td>
<td>Source of chloramphenicol resistance cassette, Cm$^r$</td>
<td>Lab collection</td>
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<tr>
<td>pLAntrC</td>
<td>pLAFR3 derivative carrying the A1501 WT ntrC gene under the control of its endogenous promoter, Tc$^r$</td>
<td>This study</td>
</tr>
<tr>
<td>pLAcbrB</td>
<td>pLAFR3 derivative carrying the A1501 WT cbrB gene under the control of its endogenous promoter, Tc$^r$</td>
<td>This study</td>
</tr>
<tr>
<td>pK18mobSacB</td>
<td>Allelic exchange vector, Km$^r$</td>
<td>(Schäfer et al. 1994)</td>
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<td>pk18/delntrC</td>
<td>pK18mobsacB derivative carrying a BamH I/Hind III fragment for homologous recombination, Cm$^r$, Km$^r$</td>
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</tr>
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<td>pRK2013</td>
<td>Helper plasmid for conjugation into P. stutzeri A1501, Km$^r$</td>
<td>(Figurski and Helinski 1979)</td>
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<td>pMD18-T</td>
<td>2.96 kb cloning vector, Amp$^r$</td>
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Construction of the ntrC deletion mutant and complementation plasmids

For ntrC gene replacement, a sacB-based strategy was employed (Schäfer et al. 1994). To construct the ntrC-null mutant (A1511), amplification of a 772 bp DNA fragment located upstream of ntrC was performed using the primer set upF/upR, and amplification of an 806 bp DNA fragment located
downstream of ntrC was performed using the set downF/downR (Table S1). Restriction enzyme sites (BamHI and HindIII) incorporated into the oligonucleotide primers to facilitate vector construction are underlined in the oligonucleotide sequences shown in Table S1. An 882 bp DNA fragment containing the Cm resistance cassette was amplified from the plasmid pKatCAT5 by PCR using the primers CmF and CmR. The three amplicons were fused into a 2.46 kb fragment, in which the Cm gene is located between the other two amplicons by overlap extension PCR according to the strategy of PCR-based fusions (Shevchuk et al. 2004). The fusion PCR product was then cloned into the multiple cloning sites of the pMD18-T vector (TaKaRa, Japan). The resulting plasmid DNA was double digested with BamHI/HindIII and then cloned into the BamHI/HindIII sites of pK18mobsacB (Schäfer et al. 1994). The resulting plasmid, pK18/delntrC, was mobilized from E. coli into P. stutzeri A1501 by conjugation using pRK2013 (Figurski and Helinski 1979) as the helper plasmid. After mating, cells were spread on LB plates containing 50 µg/mL Km and 40 µg/mL Cm to screen for clones in which pK18/delntrC had integrated into the A1501 genome via a single recombination event. Another recombination event was then induced to replace ntrC with cat and for removal of the Km^r and sacB genes from the genome. A colony of a single recombinant was then grown in a nonselective LB medium at 30°C. Cultures were diluted and spread onto LB agar supplemented with 10% (wt/vol) sucrose and 40 µg/mL Cm. The ntrC mutant strain was selected for kanamycin-sensitive and sacB-negative colonies. Correct recombination was checked using the primers testF and testR, followed by nucleotide sequencing of the amplicon obtained. The resulting ntrC deletion mutant, named A1511, was used for further study.

DNA fragments containing WT genes for ntrC or cbrB with their promoter and terminator regions were amplified by PCR to construct complementation plasmids. Two complementation DNA fragments containing ntrC or cbrB was doubly digested with HindIII/BamHI and then ligated into the broad host plasmid pLAFR3 (Staskawicz et al. 1987). The resulting two complemented plasmids, named pLANtrC and pLAcbrB, were used for further studies.

**RNA isolation for qRT-PCR**

Total RNA was isolated from bacteria cultured under the described conditions using the SV total RNA isolation system (Promega, Madison, WI) according to the manufacturer’s instructions. Total RNA was quantified using microspectrophotometry (NanoDrop Technologies, Inc.). RNA integrity was measured using an Agilent 2100 Bioanalyzer (Agilent Technologies, Inc.). RNA samples with RNA integrity numbers (RINs) above 7.0 and threshold cycle (CT) values above 32 were used for qRT-PCR.

**Quantitative real-time PCR**

Expression levels of selected genes were determined by qRT-PCR with a Power SYBR green PCR master mix using an ABI Prism 7500 sequence detection system (Applied Biosystems, USA) according to the manufacturer’s instructions. Primers were designed based on sequences of selected genes, which were imported into OligoPerfect (Invitrogen, USA), a primer design software program designed to generate primer pairs suitable for real-time PCR. Primers used for qRT-PCR are listed in Table S1. All qRT-PCRs were performed in triplicate using a 25-ml mixture containing cDNA (5 ml of a one-fifth dilution), 1×brilliant
SYBR green quantitative PCR master mixture (Stratagene, USA), and approximately 5 pmol of each primer. Amplification and detection of specific products were performed using the following procedure: 95 °C for 10 min, followed by 40 cycles of 95 °C for 30 s, 55 °C for 1 min, and 72 °C for 30 s and then a dissociation curve analysis. The 16S rRNA gene was used as the endogenous reference control, and relative gene expression was determined using the $2^{-\Delta\Delta CT}$ relative quantification method. To obtain a standard curve for the real-time PCR (RT-PCR), PCR was performed with each primer set by using calibrated amounts of chromosomal DNA; these reactions were performed at the same time as the qRT-PCR.

**Nitrogenase activity assays**

Nitrogenase activity was determined according to the derepression protocol previously described (Desnoues et al. 2003). Bacterial suspensions were incubated at an OD$_{600}$ of 0.1 in N-free minimal lactate medium (0.5% oxygen and 10% acetylene) at 30°C. Protein concentrations were determined using a standard protein assay (Bio-Rad, Hercules, CA) with bovine serum albumin as a standard. The specific activity of nitrogenase was expressed as nmol ethylene per hour per milligram of protein. Each experiment was repeated at least three times.

**RNA-seq**

Strains A1501 and A1511 were cultured for 5 h under nitrogen fixation conditions. RNA was extracted using TRIzol LS reagent (Invitrogen, USA) following the manufacturer’s instructions. Host-cell RNA was depleted using a MICROBEnrich kit (Ambion, USA), and bacterial 23S and 16S rRNAs were subsequently depleted with a MICROBExpress bacterial mRNA enrichment kit (Ambion, USA). Total RNA-seq libraries were then constructed and sequenced using an Illumina HiSeq 2500 instrument with the paired-end method by Tianjin Biochip Corporation (Tianjin, China). The raw tag sequence data were analyzed for gene annotation, genome annotation, and functional annotation. The quality of all steps was controlled in accordance with the recommendations of Illumina.

**Transcriptome data analysis**

In order to analyze gene expressed variation of different samples, the fragments per kb of CDS per million mapped reads (FPKM) value were used to normalize the data and represent the overall gene expression. The differently expressed genes between the two samples were selected according to their significance in Chi-square tests ($p < 0.05$, with Bonferroni correction) and at least 2-fold differences. Each transcriptome experiment was repeated independently at three times (biological replicates).

**Bioinformatics analysis**

For the phylogenetic analysis, the amino acid sequences of NtrC proteins from different organisms were obtained from the NCBI. Multiple sequence alignments of full-length proteins were performed using ClustalX (Crooks et al. 2004). The pairwise deletion option was used to circumvent the gaps and missing data. We used the neighbor-joining tree generated by the MEGA (Molecular Evolutionary Genetics Analysis) program with 1000 replicates of bootstrap analysis (Datsenko and Wanner 2000).
For the WebLogo analysis, MEME (http://meme.sdsc.edu/) (Bailey and Elkan 1994) and BioProspector (http://robotics.stanford.edu/~xsliu/BioProspector/) (Liu et al. 2001) were used for sequence analysis of upstream regions of significantly changed genes. The sequence logo was created with WebLogo (http://weblogo.berkeley.edu/) (Crooks et al. 2004).

**Phenotype microarrays (PMs) analysis**

Growth phenotype of the ntrC mutant was assessed using the PM3 plates (Biolog, Hayward CA). They are 96-well microtiter plates with each well containing defined medium with a unique nitrogen (PM3) compound plus an indicator dye for cell respiration. Excluding a carbon-free well (negative control) for each plate, the PM3 assay assesses the ability of a bacterium to utilize 95 nitrogen compounds as the sole nitrogen source (succinate is the carbon source). Experiments were performed following the manufacturer's instructions. A total of 100 mL of this cell suspension inoculated into the Biolog inoculating fluid was transferred into each well and incubated at 30 °C for 24 h. The colour intensity was measured every 15 min using the OmniLog reader.

**Abiotic stress-resistance assays**

Wild type A1501, ntrC mutant A1511 and complemented strain A1512 were grown in LB medium at 30°C to an OD<sub>600</sub> of 0.6 and were then transferred into fresh LB medium in the presence or absence of 0.5 mM CHP. At the time indicated (oxidative stress, 10 min), 10-fold serial dilutions were made, and 8 µL of each dilution was spotted onto solid LB plates. These plates were incubated at 30°C for 24 h before colony growth was observed and enumerated.

**Results**

**Growth analysis of a P. stutzeri A1501 ntrC mutant and a complemented derivative under different nitrogen sources**

The genomic sequence analysis showed that the *P. stutzeri* A1501 contains a single copy of a putative ntrC-like gene (*PST0349*), which has an open reading frame (ORF) of 1436 bp (Yan et al. 2008). In other bacteria, the *ntrC* gene is located in an operon downstream of the gene coding for its potential sensor kinase NtrB and the gene *glnA* which encodes a glutamine synthetase (Liu et al. 2017). Similar to other bacteria, in *P. stutzeri* A1501, the *ntrC*-like gene is located in the downstream of NtrB, however, it is distant from the gene *glnA*. *P. stutzeri* has only one gene coding for a PII homologue, in contrast to enterobacteria, which have two paralogs coding for GlnK and GlnB (van Heeswijk et al. 1996). Further phylogenetic analysis indicated that the product of *ntrC* was highly conserved in Pseudomonas species, and compared with the nitrogen-fixing bacteria, the deduced amino acid sequence of NtrC of A1501 shows the highest similarity (86% identity) to *A. vinelandii* DJ NtrC protein, however, only had 45% similarity to the homologous protein of *A. brasilense* (Fig. S1).

In bacteria, NtrC was verified as the regulatory player in nitrogen metabolism (Yeom et al. 2010; Kukolj et al. 2020). To study the role of NtrC in A1501, a mutant strain carrying a deletion of *ntrC* gene, designated
A1511, and the functional complement strain, termed A1512, were constructed (see methods). While the \textit{ntrC} mutant grew at a similar rate and to the same final optical density as the wild type in minimal medium containing ammonium sulphate or serine as a sole nitrogen source and sodium lactate as the carbon source, the mutant lost the utilization capacity for nitrate and urea. The complemented \textit{ntrC} mutant (A1512) was able to reach a similar final optical density (OD$_{600}$) as the wild type (Fig. 1). Our qRT-PCR results showed that when A1511 cells were grown in minimal medium containing nitrate or urea as a sole nitrogen source, the transcription of nitrate assimilatory gene (\textit{nasB}, \textit{nasC} and \textit{nasG}) was significantly reduced compared to that of wild type strain. The transcriptional levels of urease accessory protein encoding gene \textit{ureE} and urease encoding gene \textit{ureC} were also strongly repressed in \textit{ntrC} mutant (Fig. 2). Meantime, the NtrC-putative binding site was found in the promoter region of \textit{nasB} or \textit{ureE} by bioinformatical analysis (Table S2), suggesting that NtrC might positively regulate nitrate assimilation and urea metabolism of A1501 in a direct manner.

**NtrC affects the metabolic activity of alternative nitrogen sources**

To further understand the physiological function of NtrC in nitrogen catabolism, the ability of the wild-type strain and the \textit{ntrC} mutant to utilize 95 different nitrogen sources was examined using the Biolog Phenotype Micro Array (PMs) assays. The utilization of 24 N sources was found to be affected by \textit{ntrC} deletion. In 11 cases (nitrate, nitrite, urea, L-cysteine, L-isoleucine, L-leucine, cytosine, thymine, N-acetyl-L-glutamic acid, uracil and uric acid), the \textit{ntrC} mutant was compromised significantly in substrate utilization. In contrast, with 10 various amines (D-glucosamine, formamide, acetamide, phenylethylamine, ethylamine, N-butylamine, methylamine, putrescine, L-alanine and ammonia) the \textit{ntrC} mutant showed enhanced metabolic activity compared to the wild-type (Fig. 3). The inability of A1511 to utilize nitrate as a sole nitrogen source suggests that NtrC controls the expression of genes essential for the assimilation of nitrate. Denitrification is one of the most important processes in bacterial nitrogen cycle. A1501 can take nitrate as electron acceptor and show nitrogen fixation activity under anaerobic conditions (Lin 1987). To study the role of NtrC in nitrate respiration, we investigated the denitrification ability of wild type A1501 and \textit{ntrC} mutant A1511 under anoxic condition. Results shown in Figure S2 confirmed that the ability of A1511 to use nitrate as terminal electron acceptor was decreased 70% compared with the WT under anoxic condition. These results suggest that NtrC is essential for the nitrogen sources utilization under aerobiotic or anoxic conditions.

**NtrC is required for the positive regulation of nitrogen fixation**

NtrC regulates the function of nitrogenase specific regulator NifA in \textit{K. pneumoniae}, but it has no effect on the expression of nitrogenase complex in \textit{A. brasilense} and \textit{A. vinelandii} (Zhang et al. 1997; Wang et al. 2012). To evaluate the role of NtrC on nitrogenase system of A1501, the nitrogen-fixing activity was detected under nitrogen fixation (nitrogen-free and microaerobic) conditions. The \textit{ntrC} deletion resulted in
about 90% reduction in nitrogen-fixing activity, and the defect was restored by the introduction of a single copy of \textit{ntrC} (Fig. 4a). The results of quantitative real-time PCR (qRT-PCR) showed that the expression levels of the encoding genes for nitrogenase reductase NifH, nitrogenase specific regulator NifA, nitrogen regulatory PII protein GlnK, ammonium transporter AmtB and glutamine synthetase GlnA were decreased to various extents in the \textit{ntrC} mutant compared with the wild type, whereas these inductions were fully or partially restored to wild-type levels by the complementation plasmid with a wild-type \textit{ntrC} gene (Fig. 4b). Furthermore, the conserved putative NtrC-binding site sequence was found in the promoter region of \textit{nifA}, \textit{glnK} or \textit{glnA}, suggesting that their expression might be transcriptionally activated by NtrC and NtrC positively regulated the nitrogen fixation of \textit{P. stutzeri} A1501 (Table S2).

The two-component system CbrAB and NtrBC form a network to control the C/N balance in \textit{P. aeruginosa} (Li and Lu 2007). We found that the complementary strain A1513 (A1511 containing pLAcbrB) could recover the defect of nitrogenase activity caused by \textit{ntrC} deletion (Fig. 4a). The results of quantitative real-time PCR (qRT-PCR) showed that the expression levels of nitrogen fixation-related genes were fully or partially restored to wild-type levels by the complementation plasmid with \textit{cbrB} gene (Fig. 4b), strongly indicating that CbrB and NtrC regulate the nitrogen fixation in a cooperative manner.

**Genome-wide analysis of the NtrC regulatory network in \textit{P. stutzeri} A1501 under nitrogen fixation conditions**

In order to further identify genes responding to the nitrogen fixation conditions in an NtrC-dependent manner, a global transcriptional profiling analysis was conducted with wild type A1501 and the null-\textit{ntrC} mutant A1511 under nitrogen fixation conditions. Compared to the wild type, the expression levels of a total of 1431 genes exhibited more than a two-fold change in A1511 under nitrogen fixation conditions. Among these genes, the transcription of 1253 genes was enhanced and the expression of 178 genes was repressed in the \textit{ntrC} mutant (DESeq analysis p-value < 0.05, Fold Change > 2.0 or < 0.5). In particular, among these downregulated genes, the 49 kb expression island containing \textit{nif} and other associated genes was markedly downregulated by \textit{ntrC} inactivation, indicating the dominant role of NtrC in the nitrogen fixation regulation of \textit{P. stutzeri} A1501, and these findings are consistent with the phenotypic and expressional analysis described above, indicating the reliability of RNA-Seq.

The \textit{ntrC} mutant resulted in changes in genes expression for several functional categories under nitrogen fixation conditions. These altered genes were further classified according to the COG functional classification system, and the relative occurrence of genes belonging to each category is shown in Fig. 5. Most interesting is the strong downregulation of genes involved in transport and metabolism enzyme functions (43%), indicating that the deletion of \textit{ntrC} alter the composition of proteins related to the transport and catabolism of nitrogenous compounds. Furthermore, genes related to energy production and conversion (5%) are upregulated, suggesting that the \textit{ntrC} mutant might affect the biosynthetic capabilities of the cell under the nitrogen fixation conditions.

Next, to investigate the potential targets of NtrC involved in nitrogen metabolism, the promoter regions of the 1431 changed genes were analyzed and 147 NtrC-dependent genes exhibited putative NtrC-binding
sites, which contain the highly conserved GC and GC elements with an 11-nucleotide spacing by WebLogo analysis (Fig. S3). Among the 756 top ranked differentially expressed genes ($P < 10^{-2}$, Fold Change $> 2.0$ or $< 0.5$), 141 genes showed dramatically repressed in the ntrC mutant. The ntrC null mutant resulted in the genes involved in nitrogen assimilation and nitrogen fixation, such as the glutamine synthetase ($glnA$, PST0353), the PII sensor proteins ($glnK$, PST0502) and nitrogen fixation regulatory protein ($nifA$, PST1313; $nifL$, PST1314) showed 0.06, 0.14, 0.16 and 0.13 folds) reduced transcription, respectively (Table S2). In line with the inability of the ntrC mutant to grow with urea or nitrate as the sole nitrogen source, the genes required for urea ($ureD-2$, $ureE$, $ureF-2$, $ureG$ and $ureA$) and nitrate ($nasS$, $nasT$, $nasA$, $nasF$, $nasD$ and $nasB$) transport and utilization displayed strongly downregulated transcription. Additionally, the genes coding for electron transport ($mfABCDGEH$) and ammonium transporter ($amtB1$ and $amtB2$) were significantly repressed in the ntrC mutant. Since $amtB1$ and $amtB2$ are co-transcribed with $glnK$, which had the NtrC-binding site in the promoter region, we inferred that the transcription of $amtB1$ and $amtB2$ is NtrC-dependent. We also found the transcription of the genes $ureE$ encoding urease, $nasB$ encoding nitrite reductase, $nasF$ encoding nitrate transporter and $mfA$ encoding electron transporter is NtrC-dependent, which have the putative NtrC-binding site in the promoter region, indicating that these genes may be the key gene under NtrC control for nitrate assimilation and urea catabolism. Additionally, the transcription of several genes ($PST2280$, $PST2508$ and $PST4035$) involved in chemotaxis was decreased in the ntrC mutant, in particular, the putative NtrC-binding stie was found in the promoter of $PST2280$ coding for methyl-accepting chemotaxis receptor protein (MCPs) and $PST2508$ coding for methyl-accepting chemotaxis transducer. Chemotaxis is the directed motility by means of which microbes sense chemical cues and relocate towards more favorable environments. Since MCPs are the most common receptors in bacteria, we inferred that NtrC might contribute to the interaction of A1501 with plant hosts. Among the 615 top ranked genes with dramatically increased transcription, the expression levels of several genes involved in the glycolytic pathway were enhanced significantly in the ntrC mutant, including ($PST0991$ coding for glucose dehydrogenase, $sucD$ coding for succinyl-CoA synthetase, $PST3494$ coding for probable glyceraldehyde-3-phosphate dehydrogenase, $eda-1$ coding for 4-hydroxy-2-oxoglutarate aldolase, $glk-1$ and $glk-2$ coding for glucokinase, $PST3496$ coding for 6-phosphogluconolactonase, $PST3497$ coding for glucose-6-phosphate 1-dehydrogenase and $PST3500$ coding for 6-phosphogluconate dehydratase) showed 3.1, 2.9, 6.2, 9.7, 3.0, 5.3, 4.8, 6.1 and 11.2 folds (Table S2). Based on these data, we define NtrC as the master nitrogen regulator and infer that it not only activates pathways for nitrogen fixation but also represses carbon catabolism under nitrogen fixation conditions, possibly to prevent excessive carbon and energy flow in the cell.

The ntrC mutant shows altered oxidative stress response

Oxygen concentration is one of the main environmental factors affecting biological nitrogen fixation due to the extreme oxygen sensitivity of nitrogenase. To test directly whether the ntrC mutant displayed altered resistance to oxidative stress, we compared the growth of wild-type strain A1501, the ntrC mutant A1511 and the complementary strain A1512 under oxidative stress condition by the addition of the oxidizing agent cumene hydroperoxide (CHP). As shown in Fig. S4a, both A1511 and A1512 displayed
growth rates similar to that of the wild-type strain in LB medium, indicating that deletion of the ntrC gene had no effect on bacterial survival under normal growth conditions. But we found that the deletion of ntrC resulted in a significant increase growth in the presence of 0.5 mM CHP, and the complementary strain recovered the growth capacity to the wild-type level under the same treatment (Fig. S4a). Consistent with observations mentioned above, the oxidative stress-related genes were increased to various extents in the ntrC mutant compared with the wild type (Fig. S4b), especially catalase encoding gene katB, whose expression was increased by 11 folds. Bioinformatic analysis revealed one NtrC-binding site in the katB promoter region, and we inferred that katB is the target gene of NtrC involved in directly regulating optimal oxidative stress resistance.

Discussion

In this study, we used global gene expression and phenotypic analyses to characterize the role of NtrC in nitrogen metabolism of P. stutzeri A1501 and found 1431 genes were significantly differentially expressed altered by ntrC mutant. This large number of differentially expressed genes (33.95% of the genome) shows that a major NtrC-dependent transcriptomic response is initiated by P. stutzeri A501 under nitrogen fixation conditions. As expected, genes that known or predicted to be involved in nitrogen metabolism form the majority in the NtrC regulon. In particular, nifA, which codes for the transcriptional activator of all nif operons (Chengtao et al. 2004; Demtröder et al. 2019), showed a 0.16 fold decrease, and glnK, which codes for a PII family protein (Xu et al. 1998; Blauwkamp and Ninfa 2002), showed a 0.14 fold decrease in ntrC mutant. In P. stutzeri, GlnK is required for both NifA synthesis and activity, in particular by preventing the inhibitory effect of NifL on NifA activity (Xie et al. 2006; He et al. 2008). These data were in agreement with the observation that inactivation of NtrC affected nitrogenase activity, suggesting a role in positive regulation of the nif genes. Homologs of the ntrC genes have been found in many nitrogen-fixing bacteria, and their roles in nitrogen fixation have been best characterized in K. pneumoniae. In this organism, NtrC plays an important role in the transcription of nifLA regulatory genes, with NifA activating the transcription of other nif operons (Merrick 1983). Whereas, in some diazotrophs, such as A. vinelandii, B. japonicum, and A. brasilense, NtrC are not necessary for nif gene expression. In this study, although the NtrC acts as transcriptional activator of nifA, the mutant was Nif+, its nitrogen fixing activity was just far lower than that in wild type. Conceivably, NtrC has positive regulation on nitrogen fixing and possibly CbrB protein can substitute NtrC to keep some nitrogenase activity.

The largest category of genes in the NtrC regulon is nitrogen scavenging. This is logical from an evolutionary perspective, as the soil dwelling P. stutzeri A1501 encounters various nitrogen sources in the environment and must compete with other soil microbes for nutrients. The genes encoding nitrogen transporter and binding proteins, ammonium transporters, uptake systems for nitrate/nitrite, urea, and amino acids/ peptides are all upregulated by NtrC in P. stutzeri A1501 under nitrogen fixation conditions. A similar situation is observed for nitrate/nitrite uptake and assimilation in that P. stutzeri A1501 contains two nitrate transporters, binding and response protein (PST2003, NasA, NasF, NasE and NasD) and nitrite reductase (NasB and NasC) are all upregulated by NtrC in P. stutzeri A1501. The assimilatory nitrate
reduction to ammonium is a two-step process: reduction of nitrate to nitrite by nitrate reductase followed by reduction of nitrite to ammonium by nitrite reductase. As confirmed in this study, the assimilatory nitrite reductase NasBC (PST2409 and PST2410) is upregulated by NtrC under nitrogen fixation conditions, but the nitrate reductase enzyme NasG (PST2411) is not. Therefore, the uptake and assimilation of nitrite, not nitrate, appears to be an important nitrogen stress response in *P. stutzeri* A501. Our study also identified a NtrC regulated response regulator NasT (PST2401). In our previous study, *nasT* mutate was unchanged the nitrate uptake capacity of *P. stutzeri* A1501, but could not grow using nitrate as the nitrogen source (unpublished data). However, the precise role of this regulator and nitrate/nitrite respiration in the nitrogen stress response in *P. stutzeri* A1501 requires further investigation.

The phenotype of the *ntrC* mutant indicated that NtrC was not only absolutely required for nitrogen metabolism in *P. stutzeri*, but also related to optimal resistance to oxidative stress. The *ntrC* mutant showed significant upregulation of oxidative stress response genes, especially *katB*, which is the most pivotal enzyme for oxidizing agent CHP (Manso et al. 2020). Bioinformatics analysis revealed one NtrC binding site to the promoter region of *katB*, indicating that KatB is the potential target regulated by NtrC. Due to oxidative stress is the crucial problem of the survival of nitrogen fixing bacteria, we inferred that NtrC might regulate oxidative stress resistance via the direct transcriptional activation of *katB*. In this work, NtrC was shown to be involved in regulating the consumption of some nitrogenous compounds. When it was inactivated and consequently lost function, the ability for utilizing some nitrogenous compounds by *P. stutzeri* was impaired. These results further confirmed that *ntrC* gene was a regulator for the metabolism and assimilation of some nitrogen sources in *P. stutzeri*. However, not all metabolisms of nitrogenous compounds were related to NtrC. For example, the *ntrC* mutant could grow well with (NH$_4$)$_2$SO$_4$ and some amino acids as the nitrogen source (Glu and Gln). However, the two amino acids were the key signaling molecules in nitrogen metabolism pathway and switches for nitrogen assimilation in bacteria (Manso et al. 2020). Further studies are required to clarify the NtrC-based mechanisms underlying the response of this bacterium to nitrogen signal and oxidative stress at the cellular and molecular levels.

Taken together, the results of this study provide a framework for understanding the transcriptional changes of numerous key genes related to various nitrogen metabolism processes controlled by NtrC in *P. stutzeri* A1501 under nitrogen fixation conditions. Chief among these differentially expressed genes are those involved in nitrogen fixation, amino acid catabolism, assimilatory nitrate and ammonium transport. By combining the transcriptome data with bioinformatics analyses, some potential new targets genes responsible for electron transport and oxidative stress response regulated by NtrC were discovered, which would help enhance the knowledge of NtrC-based mechanisms underlying both nitrogen metabolism and environmental adaptation network in *P. stutzeri* A1501.

**Declarations**

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**Conflicts of interest** The authors declare that they have no conflict of interest.

**References**


30. Minchin SD, Austin S, Dixon RA (1989) Transcriptional activation of the *Klebsiella pneumoniae nifLA* promoter by NtrC is face-of-the-helix dependent and the activator stabilizes the interaction of sigma 54-RNA polymerase with the promoter. EMBO J 8:3491–3499


33. Sánchez DG, Primo ED, Damiani MT, Lisa AT (2017) *Pseudomonas aeruginosa gbdR* gene is transcribed from a σ54-dependent promoter under the control of NtrC/CbrB, IHF and BetI. Microbiology (Reading) 163:1343–1354. https://doi.org/10.1099/mic.0.000502

Figure 1

Growth of wild type P. stutzeri A1501 (●), ntrC mutant strain A1511 (▲) and the functional complement strain A1512 (■) on ammonium sulphate (a), L-serine (b), nitrate (c) or urea (d) as the sole source of nitrogen. Growth was measured in K medium supplemented with ammonium sulphate, L-serine, nitrate or urea (10 mmol·L⁻¹) as the sole nitrogen source. Results are means and standard errors of three independent cultures.
Figure 2

The effect of ntrC deletion on the expression of nitrate assimilation related genes (a) and urea catabolism related genes (b). Relative levels of transcripts are presented as mean values ± standard deviations (SDs) calculated from three sets of independent experiments and normalized to levels in the wild-type strain.
Figure 3

Role of ntrC gene in utilization of nitrogen substrates as determined by Biolog phenotype microarray (PM3) analysis. Signal intensities were measured using an OmniLog detection system and expressed as relative OmniLog units (OLU). Data are the means ± the standard error from at least three independent cultures, assayed in triplicate.
NtrC is required for the positive regulation of nitrogen fixation. (a) Nitrogenase activity in wild-type A1501 (circles), ΔntrC A1511 (triangles) and the complemented strains A1512 (squares), A1513 (diamond). (b) The effect of ntrC deletion on the expression of nif genes and their regulators. Relative levels of transcripts are presented as mean values ± standard deviations (SDs) calculated from three sets of independent experiments and normalized to levels in the wild-type strain.
Figure 5

Overview of expression profiling analysis. (a) Functional categories of core subset of up-regulated genes (P < 0.05 and fold change >2) in ntrC mutant versus wild type under nitrogen fixation conditions. (b) Functional categories of core subset of down-regulated genes (P < 0.05 and fold change <0.5) in ntrC mutant versus wild type under nitrogen fixation conditions. The percentage of genes in each section is depicted.

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