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NtrC-Sensed Nitrogen Availability Is Important for Oxidative Stress Defense in *Pseudomonas putida* KT2440[§]

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The *zwf*, which encodes glucose-6-phosphate dehydrogenase, is repressed by NtrC under nitrogen-limited condition. Previously, we demonstrated that induction of *zwf-1* is required for protecting *Pseudomonas putida* cells under oxidative stress, which could be possible probably because of derepression of HexR on the *zwf-1* gene under oxidative stress. These findings led us investigate that NtrC still represses the *zwf-1* under nitrogen-limited oxidative stress condition, which makes cells more sensitive under such condition. Interestingly, deletion of the *ntrC* gene significantly reduces growth rate, but renders cells more resistant to oxidative stress, under nitrogen limited condition in *P. putida*. More vitality of the *ntrC* mutant under oxidative stress condition was also confirmed by the fluorogenic redox dye using flow cytometry. The results of transcriptome analysis demonstrated that the derepression of several oxidative stress genes along with the *zwf-1* gene might confer high resistance to oxidative stress in the *ntrC* mutant. Here, we presented the data for the first time, showing that different sets of genes are involved in nitrogen-rich and nitrogen-limited oxidative stress conditions and NtrC-sensed nitrogen availability is one of the most important prerequisite for full cellular defense against oxidative stress in *P. putida*.

***Keywords*:** *zwf-1*, SoxR, HexR, nitrogen, soil model bacterium, NADPH

Reactive oxygen species (ROS) are generated either during normal aerobic metabolisms or during exposure to exogenous pollutants. The ROS can then damage a host of macromolecules, including proteins, lipids, and DNA inside cells (Stadtman, 1992; Cabiscol *et al*., 2000; Imlay, 2003). Many bacteria utilize the *zwf-1* gene product, glucose-6-phosphate dehydrogenase (G6PDH), as a defensive response to oxidative damage (Lundberg *et al*., 1999; Pomposiello and Demple, 2001). The disruption of G6PDH production increases bacterial sensitivity to oxidative stresses (Lundberg *et al*., 1999; Girò *et al*., 2006). In *Escherichia coli*, SoxS controls the G6PDH production in response to superoxide or nitric oxide stresses (Demple, 1996; Pomposiello and Demple, 2001). Unlike *E. coli*, we reported that the SoxRS regulon does not exist within the genome of *Pseudomonas putida* KT2440, and that G6PDH production could be regulated by HexR (Lee *et al.*, 2006; Park *et al*., 2006; Kim *et al*., 2008). In *P. putida*, the *zwf-1* gene product belongs to the glucose metabolism operon and is controlled negatively by HexR. The HexR protein binds directly to the *zwf-1* promoter region and regulates *zwf-1* induction as a repressor under a variety of carbon source conditions. We suggested previously that HexR might directly sense oxidative stress and derepress the *zwf-1* expression under oxidative stress conditions (Kim *et al*., 2008). Interestingly, the deletion of the *hexR* gene increases resistance to oxidative stress. This effect may be attributable to constitutive overexpression of the *zwf-1* gene product, which is essential for the regeneration of the NADPH pool inside cells (del Castillo *et al*., 2008; Kim *et al*., 2008; Daddaoua *et al*., 2009).

When bacteria grow in a soil environment, nitrogen is frequently limited and thus, bacterial regulatory response promotes the nitrogen assimilation system. Nitrogen regulation has been extensively studied in the enterobacteria (Merrick and Edwards, 1995; Leigh and Dodsworth, 2007). Under nitrogen-limited conditions, the NtrC functions as a global activator of nitrogen assimilation and allows cells to uptake ammonia (Ninfa *et al*., 2000; Zimmer *et al*., 2000; Gyaneshwar *et al*., 2005). The NtrC belong to the two-component signal transduction system, in which NtrB modulates NtrC via phosphorylation (Stock *et al*., 2000; Arcondeguy *et al*., 2001; Verhamme *et al*., 2002; Ninfa and Jiang, 2005). The transcriptomic analysis data demonstrate that there are as many as 58 NtrC-activated open reading frames in *P. putida*. Many genes involved in ammonia and amino acid transport function in an NtrC-dependent manner. Interestingly, *zwf-1*, along with other genes for glucose metabolism, is repressed by NtrC under nitrogen-limited conditions (Hervás *et al.*, 2008). The putative NtrC-binding site in the promoter regions of the *zwf-1*, gene were also determined and appear to be located upstream (~70 bp) of the HexR binding site in *P. putida* KT2440 (Fig. 1A). It appears that the NtrC balances the speed of glucose metabolism and nitrogen assimilation. We previously demonstrated that that the production of G6PDH is required for the protection of *P. putida* cells under oxidative stress conditions (Kim *et al.*, 2008). We hypothesize that any condition that represses the production of G6PDH will render cells more sensitive to oxidative stress. Nitrogen limitation appears to be a possible mechanism triggering the repression

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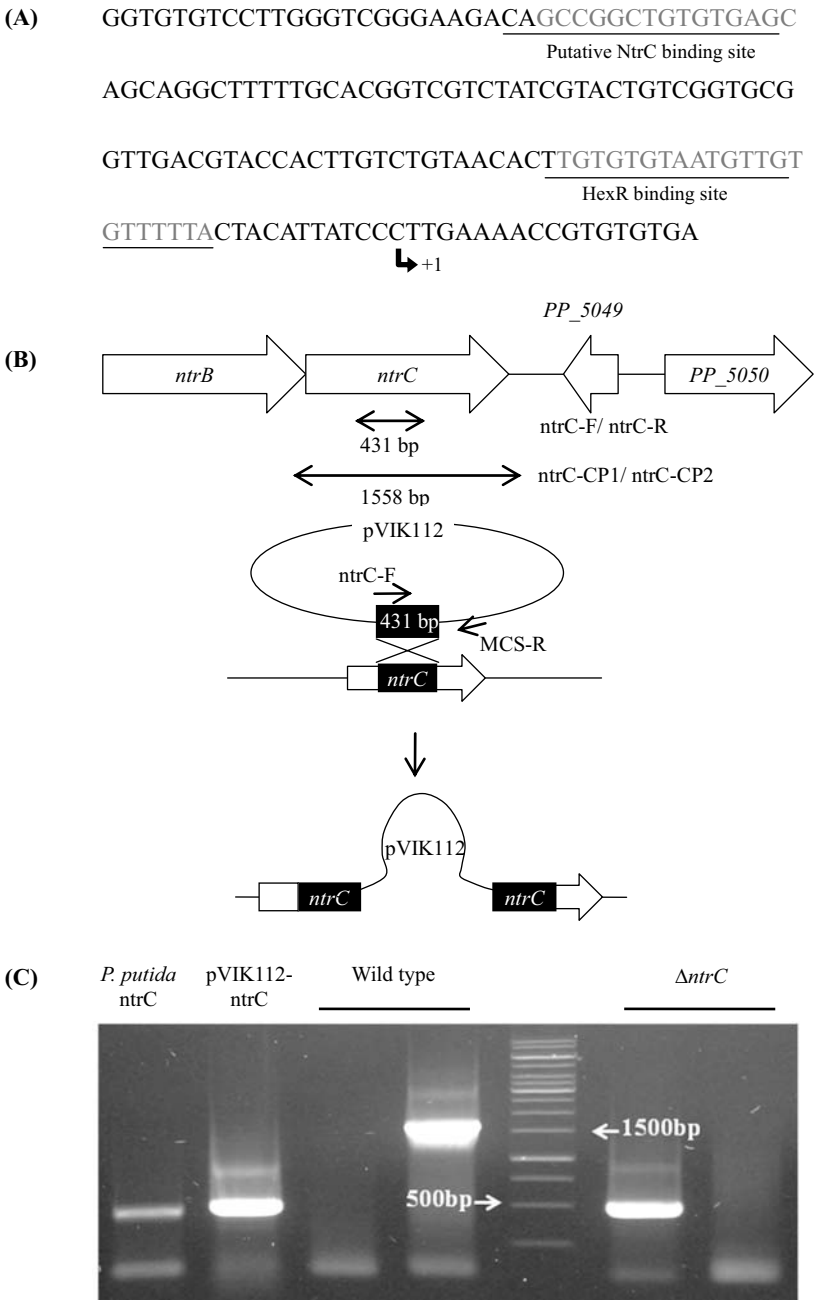

Fig. 1. Chromosomal disruption of the *ntrC* and *zwf-1* promoter region in *P. putida* KT2440. (A) Location of HexR and NtrC binding sites in *zwf-1* promoter region. +1 is transcription start site of *zwf-1*. (B) The simple *ntrC* gene disruption strategy and the region PCR-amplified with primer pairs used in this study. (C) PCR verification of the *ntrC* mutant construction [Lanes: 1, *P. pitida* WT (Primer: ntrC-F, ntrC-R); 2, *E. coli* S17-1 λ pir (pVIK112 ntrC) (Primer: ntrC-F, MCS-R); 3, *P. putida* WT (Primer: ntrC-F, MCS-R); 4, *P. putida* WT (Primer: ntrC-CP1, ntrC-CP2); 5, Fermentas 1 kb ladder; 6, Δ*ntrC* (Primer: ntrC-F, MCS-R); 7, Δ*ntrC* (Primer: ntr-CP1, ntrC-CP2)]

of *zwf-1* expression, even under oxidative stress conditions. In order to gain insight into the regulation of *zwf-1* by NtrC under oxidative stress conditions, we assessed the above case with the *ntrC* mutant.

Materials and Methods

Bacterial strains, culture conditions, and DNA manipulation

Bacteria were cultivated at 37°C (*E. coli*) or 30°C (*P. putida*) in LB medium or mineral salts medium (M9) containing 10 mM glucose as a carbon source, 10 mM ammonium chloride, and 10 mM L-serine for nitrogen-excess conditions, and 10 mM L-serine alone without ammonium chloride for nitrogen-limited conditions, with vigorous aeration. Whenever required, antibiotics were added at the following concentrations: rifampicin, 200 μg/ml; and kanamycin, 50 μg/ml. The wild-type and *zwf-1* mutant were cultivated overnight in LB liquid medium; from this, 10^9 cells were inoculated into fresh M9 medium containing different nitrogen conditions.

Cloning procedures and mutant construction

In order to delete the *ntrC*, a 431 bp fragment of its internal region was amplified via PCR using the ntrC-F (5′-CGCGAATTCGCGCCT GCCGGTCATCATCA-3′) and ntrC-R (5′-CGCGGTACCGGCGCA ACAGCTCCGACCC-3′) primers (Fig. 1B). The fragment was then cloned into the *Eco*RI and *Kpn*I cloning sites of the pVIK112 vector (Kalogeraki and Winans, 1997), generating pVIK-ntrC. The constructed plasmid was then introduced into *E. coli* S17-1 λ pir (Simon *et al*., 1983). Conjugation was conducted using *E. coli* S17-1 λ pir (pVIK-ntrC) and *P. putida* KT2440-R, a rifampin-resistant derivative, as the donor and recipient. Primers used in this study: MCS-R primer (ACC ATG GTC ATA GCT GTT TCC TG), ntrC-CP1 (CGC AAG CTT CAT ACC GCC TTC TCG ATT TAC), ntrC-CP2 (CGC GAA TTC CCT GTG GGA GCG GCC TTG TG).

Oxidative stress sensitivity assay

The wild type and the *ntrC* mutant strain were grown overnight in liquid LB medium and subsequently diluted 100-fold under M9 medium with a variety of nitrogen conditions. Bacteria were cultivated at 30°C in M9 medium containing 10 mM glucose as a carbon source, 10 mM ammonium chloride, and 10 mM L-serine for nitrogen-excess conditions, and 10 mM L-serine alone without ammonium chloride for nitrogen-limited conditions, with vigorous aeration. After further incubation until the cells had reached exponential phase (OD_{600} ~0.5), serially diluted cells were spotted on LB agar with or without cumen hydroperoxide (CHP) and menadione (MD).

LIVE/DEAD BacLight™ bacterial viability assay in FACS

The cells were grown to the mid-log phase (OD_{600} of approximately 0.7) at 30°C, with aeration. Then, cells were treated by adding 3 mM CHP during 30 min at 30°C. Treated cells washed in PBS buffer and aliquot ~1 ml to flow cytometry tubes. Cells stained with the LIVE/DEAD BacLight RedoxSensor Green Viability kit (Molecular Probes, Invitrogen, USA) for 10 min at room temperature in the dark, according to the manufacturer's instructions. Fluorescence intensity was measured on a FACS Calibur (Becton Dickinson, USA) and the mean fluorescence channel (MFC) value of 20,000 cells was determined upon analysis of the live or dead cell population which was defined by forward and side scatter using BD CellQuest Pro software (BD Biosciences, USA). The data were analyzed in FSC/FL-1, FSC/FL-3, and FL1/FL-3 dot plot.

Northern blot analysis

Total RNA was isolated from 5-10 ml of exponentially growing cells using an RNeasy kit (QIAGEN, USA) in accordance with the manufacturer's instructions. RNA concentrations were estimated via absorbance at 260 nm. Samples of total RNA (10 μg) were loaded on denaturing agarose gels containing 0.25 M formaldehyde, separated, and then stained with ethidium bromide to visualize 23S and 16S rRNA. The fractionated RNA was transferred to nylon membranes (Schleicher and Schuell) using a Turboblotter (Schleicher and Schuell). The amount of *zwf-1* mRNA was determined by hybridizing the membrane with a specific ^{32}P-labelled probe (Invitrogen, USA) prepared via PCR amplification from *P. putida* strain KT2440 with the kt zwf-1 Pp-1 (ATC CGC GAC GAG AAG GTG AAA GTG)/ kt zwf-1 Pp-2 (GGC CCT GCT CCT TGG TCA TCAC) primer pair.

cDNA microarray

The cells were grown overnight in liquid LB medium and subsequently diluted 100-fold under M9 medium with a variety of nitrogen conditions. After further incubation until the cells had reached exponential phase (OD_{600} ~0.5), 10 mM CHP is treated for 15 min. Total RNA was isolated from 10 ml of exponentially growing cells using an RNeasy kit (QIAGEN) in accordance with the manufacturer's instructions. The integrity of bacterial total RNA was assessed by capillary electrophoresis with an Agilent 2100 Bioanalyzer (Agilent, USA) and purified further with an RNeasy Mini kit (QIAGEN). cDNA probes for cDNA microarray analysis were prepared by the reverse-transcription of total RNA (50 μg) in the presence of aminoallyl-dUTP and 6 μg of random primers (Invitrogen) for 3 h. The cDNA probes were cleaned up using Microcon YM-30 column (Millipore, USA) and then followed by coupling of Cy3 dye (for reference) or Cy5 dye (for test sample) (Amersham Pharmacia, Sweden). The Cy3 or Cy5-labeled cDNA probes were purified with QIAquick PCR Purification kit (QIAGEN). Dried Cy3 or Cy5-labeled cDNA probes were resuspended in hybridization buffer containing 30% formamide, 5× SSC, 0.1% SDS, 0.1 mg/ml salmon sperm DNA. The Cy3 or Cy5-labeled cDNA probes were mixed together and hybridized to a microarray slide. After overnight at 42°C, the slide was washed twice with washing solution 1 containing 2× SSC, 0.1% SDS for 5 min at 42°C, and once with washing solution 2 containing 0.1× SSC, 0.1% SDS for 10 min at room temperature, and finally four times with 0.1× SSC for 1 min at room temperature. The slide was dried by centrifugation at 650 rpm for 5 min. Hybridization image on the slide was scanned by Axon 4000B (Axon Instrument, USA).

Microarray data analysis

Hybridization image was analyzed by GenePix Pro 3.0 software (Axon Instrument) to obtain gene expression ratios (reference vs test sample). Gene expression ratios were normalized by GenePix Pro 3.0 software. Clustering image was obtained from hierarchical clustering, which involves computing 'distances' between data elements. All data are sorted by 2-fold change of ratio of gene expression between CHP treatment and control in the wild-type.

Results and Discussion

Sequence analysis of the *zwf-1* region and construction of the *ntrC* mutant

Previously, we demonstrated that HexR might directly sense oxidative stress and derepresses the expression of the *zwf-1* gene in *P. putida* KT2440 under oxidative stress condition. It has been shown that HexR can bind the promoter region of the *zwf-1* (Hervás *et al*., 2008; Kim *et al*., 2008). The putative NtrC binding site (red color in Fig. 1A) in the promoter regions of the *zwf-1* gene was also determined and appears to be located upstream (~70 bp) of the HexR binding site (blue color in Fig. 1A) in *P. putida* KT2440. If binding of NtrC to the promoter region of the *zwf-1* is not released under nitrogen-limited condition, the *zwf-1* expression will be repressed even in the presence of either oxidative stress or glucose. Thus, cells become more sensitive to oxidative stress. We tested this scenario with the *ntrC* mutant. Disruption of the *ntrC* gene was achieved using a single crossover recombination method with the pVIK112 (Fig. 1B). The PCR with ntrC-F/-R primer pairs was used to amplify the internal region of the *ntrC*. Detailed procedures for the mutant construction were described in 'Materials and Methods' section. To make sure that the homologous recombination occurred in the recipient strain, PCR verification was conducted using the ntrC-F and

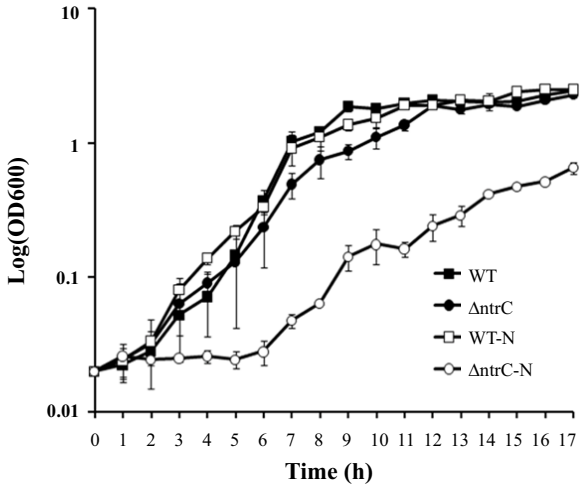

Fig. 2. Growth curves of the wild-type (■) and the *ntrC* mutant (●) under nitrogen- rich conditions, the wild-type (□) and the *ntrC* mutant (○) under nitrogen-limitation condition.

MCS-R primer pairs. The MCS-R primer was designed based on the sequence of the pVIK112 plasmid. When the ntrC-CP1/-CP2 primer pair was used, a 1,558 bp PCR product was observed only in wild-type cells (Fig. 1C). Because of the orientation of the *ntrC* gene, the polar mutation caused by insertion of the vector into the genome doesn't occur.

Impaired growth of the *ntrC* mutant under nitrogen-limited condition

We monitored the growth of the wild-type and *ntrC* mutant in M9 medium containing different nitrogen sources (Fig. 2). The growth pattern of the *ntrC* mutant was shown to be similar to the wild-type strain under normal conditions. In the nitrogen-limited medium containing serine as a nitrogen source, the *ntrC* mutant strain evidenced a longer lag phase than the wild-type (Fig. 2). When proline was supplied as a nitrogen source, these tendencies were also detected (data not shown). This growth defect might be attributable to the lack of an efficient nitrogen assimilation pathway. Under excess nitrogen conditions, cells do not need to consume less-than-optimal nitrogen sources. However, when nitrogen is limited, serine can be utilized as a sole nitrogen source. Consistent with other reports (Zimmer *et al.*, 2000; Hervás *et al.*, 2008; Hervás *et al.*, 2009), our data also showed that NtrC plays critical roles in cellular growth under nitrogen-limitation conditions.

Tolerance of the *ntrC* mutant against oxidative stress

The *ntrC* mutant appeared to be more resistant than the wild-type in the presence of menadione (MD) or cumene hydroperoxide (CHP) (Fig. 3A). The greater vitality of the *ntrC* mutant under oxidative stress conditions was also verified using fluorogenic redox dye via flow cytometry (Fig. 3). Greater shifts to the right indicate the death of the cells in FACS analysis. The analytic data were displayed by dot and histogram plot. The y-axis of the histogram shows dead cells by coloring them with FL-3 red; thus, a right direction shift means that the cells are in a dead state with propidium iodide. Under both nitrogen-rich (Fig. 3B) and nitrogen-limited (Fig. 3C) conditions, the wild-type cells were shown to be more sensitive to oxidative stress. However, more severe reductions in cell vitality were noted in the wild-type cells with CHP under nitrogen-limited conditions (Fig. 3C). The deletion of NtrC increases oxidative stress resistance. This is probably due

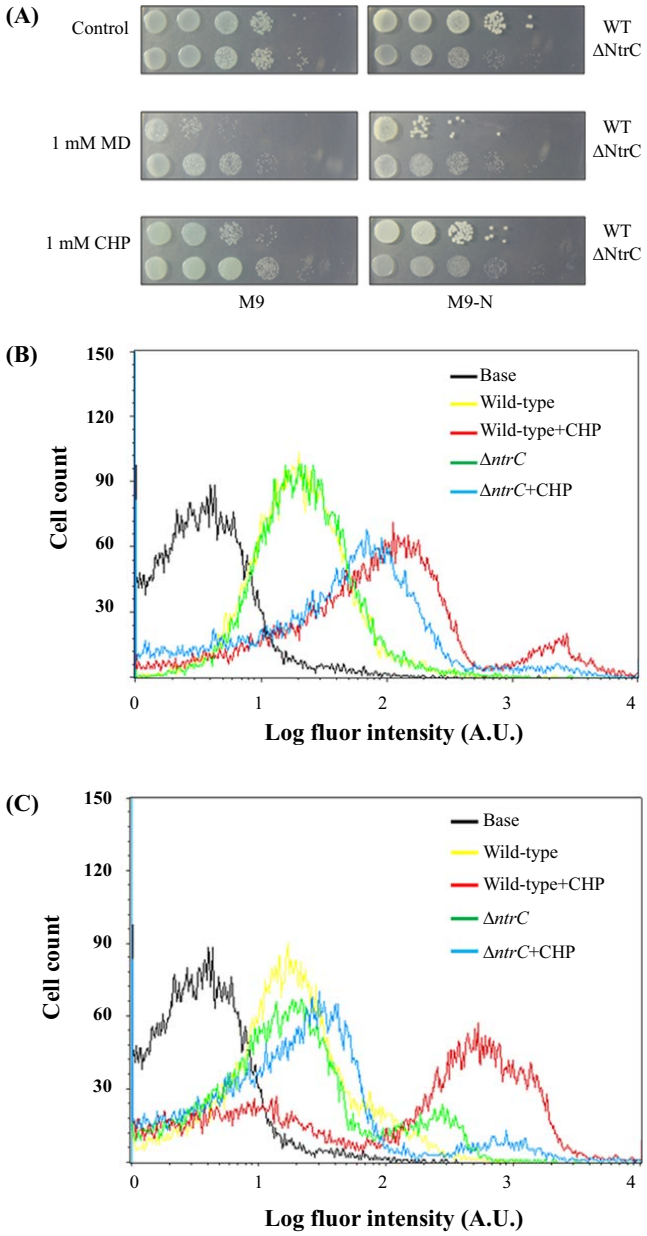

Fig. 3. Sensitivity assay of *P. putida* and *ntrC* mutant strains. (A) Figures of agar plates. M9 is nitrogen excess condition and incubated during an 1 day. M9-N is nitrogen limitation condition and incubated during 2 days. There is not different to cell number in M9-N between an 1 day and 2 days. (B, C) Cell viability test using FACS. Representative measurements are shown and were taken 30 min following addition of 3 mM CHP. Black and green represent time-zero baseline measurements in wild-type and Δ*ntrC* cells. Nitrogen rich M9 medium (B) and nitrogen limited M9 medium (C) used for cell viability test with proline 10 mM. The X-axis represents FL-3 (PerCP) fluorescent channel.

to the constitutive production of NADPH from G6PDH, which could be used for the repair of oxidative stress-damaged proteins, as NADPH is consumed by many oxidative stress defense enzymes--including glutathione reductase, thioredoxin reductase, and ferredoxin reductase (Girò *et al*., 2006). Additionally, we also speculated that *ntrC* deletion could induce other enzymes that might prove helpful for the survival of the *ntrC* mutant under oxidative stress conditions.

Expression analysis of the *zwf-1* under oxidative stress condition

In order to understand the regulation of *zwf-1* under nitrogen limitation conditions, we measured the *zwf-1* expression. G6PDH has been known to be an antioxidant regulating $NADP^+$/NADPH level. Previously, we reported that the *zwf-1* expression increased when cells were exposed to oxidative stress such as menadione, arsenic, and cumene hydroperoxide (Kim *et al*., 2008). We confirmed that the *zwf-1* gene was highly expressed in the presence of menadione (MD) in the wild-type (Fig. 4). The *zwf-1* expression level under nitrogen limited MD-amended condition (Fig. 4, lane 4) is similar to that of control (Fig. 4, lane 3) probably because NtrC still repressed the expression of the *zwf-1*. Because nitrogen-rich and nitrogen-limited condition have different nutritional sources and different growth rates, direct comparison of the *zwf-1* gene expression between control lanes (lanes 1 and 3) could be difficult. However, the *zwf-1* transcription was affected by the *ntrC* deletion. The expression of the *zwf-1* gene was derepressed in the *ntrC* mutant, as compared to the wild-type strain. This high expression level was maintained even though oxidative stresses were exposed to the *ntrC* mutant (Fig. 4). Under nitrogen-limited condition the *zwf-1* expression in the *ntrC* mutant was still higher than wild-type control, but slight induction of the *zwf-1* gene was observed in the presence of MD (Fig. 4, lane 8), suggesting that HexR (or other regulator) could be responsible for that induction. Slight lower induction of the *zwf-1* gene under nitrogen limited condition (lane 7) compared to the control of the mutant (lane 5) was probably due to the growth defect of the mutant under nitrogen limited condition.

Transcriptomes of wild-type and the *ntrC* mutant under nitrogen-limited oxidative stress condition

In an effort to obtain more information regarding the global gene expression pattern of the *P. putida ntrC* mutant under nitrogen-limited oxidative stress conditions, the transcriptional profiles of *P. putida* strains KT2440 with the *ntrC* mutant under various conditions were compared via cDNA microarrays. Transcriptional profiling under nitrogen-limited oxidative stress differs significantly from that noted under nitrogen-rich oxidative stress conditions (Supplementary data Tables 1 and 2). Our data is for the first time showing microarray data for oxidative stress response in *P. putida* (Supplementary data Table 2). The *zwf-1* gene is one of the most induced genes under oxidative stress condition (~4.02 fold induction in Supplementary data Table 2). Genes that might possibly be involved in oxidative stress defense were selected on the basis of up-regulation under nitrogen-limited oxidative stress conditions, a list of which is provided in Fig. 5A and Supplementary data Table 1. Both cases share a few genes in common for up-regulation, suggesting that different sets of genes are involved in nitrogen-rich and nitrogen-limited conditions. The putative type I pili subunit CsuA/B protein (PP2359) is up-regulated profoundly in both cases, and its expression is not dependent on NtrC. Enhanced pili production might promote biofilm and aggregate formation, which protect cells under oxidative stress conditions. Many genes associated with oxidative stress (particularly *sodA*, *radA*, PP4768, PP4856, and PP4924) are not abundantly expressed in the *ntrC* mutant with CHP (Fig. 5A and Supplementary data Table 3). The increased expression of *zwf-1* was not noted under nitrogen-limited oxidative stress conditions in the wild-type cells, probably because the NtrC represses the expression of the *zwf-1* gene (Fig. 5B, lane 2). However, the level of *zwf-1* expression increased dramatically under nitrogen-rich oxidative stress conditions in wild-type cells (Fig. 5B, lane 3). Under nitrogen-limited conditions, the *ntrC* mutant evidenced a high level of *zwf-1* expression (Fig. 5B, lanes 4 and 5), regardless of the nitrogen availability conditions. We identified all the genes whose expression patterns were similar to those of *zwf-1* expression under each condition, as they might confer resistance against the *ntrC* mutant along with the *zwf-1* gene (Fig. 5B). Interestingly, some oxidative stress genes (*katE*, catalase; *radC*, DNA repair protein), cell wall biosynthesis genes (*murI*, glutamate racemase), and regulatory genes (PP0270, sensor histidine kinase; PP1307, AsnC family transcriptional factor; PP3603 and PP4734, GntR family transcriptional factors) have been identified as examples of such cases. Surprisingly, putative NtrC binding sites have been detected in the promoter regions of many genes, which are the *zwf-1*, *katE*, *murI*, *uxpA*, and *radC* genes (Fig. 5B, red color-marked). It is worth investigating the direct binding of NtrC to these promoter regions, which have not previously been shown to be controlled by the NtrC. The derepression of these genes with the *zwf-1* gene might confer high resistance to oxidative stress in the *ntrC* mutant. Other genes [*glpF* (PP1076), *glpD* (PP1073)] whose expression appears to be repressed by the NtrC do not follow the expression pattern of the *zwf-1* gene (Supplementary data Table 3, Hervás *et al.*, 2008). NtrC activates a variety of nitrogen assimilatory genes (*glgX*, *glgA*, *amtB*, *ureE*, etc). Consistent with the results of a previous study (Hervás *et al.*, 2009), our transcriptional profiling also showed that those genes are abundantly expressed under nitrogen-limited conditions (Fig. 5C, lane 1 and Supplementary data Table 4). However, the *ntrC* mutant, as well as CHP

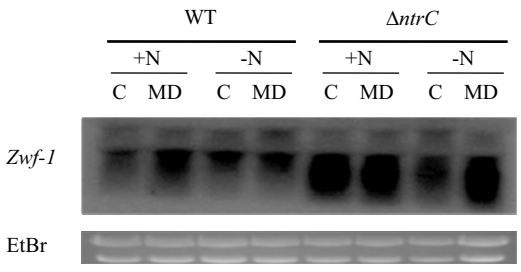

Fig. 4. Northern blot analysis of the *zwf-1* transcriptional level. C, control; -N, nitrogen limitation condition; unindicted lane is nitrogen excess conditions. Poor nitrogen source is proline and cells were treated by 1 mM MD in 10 min.

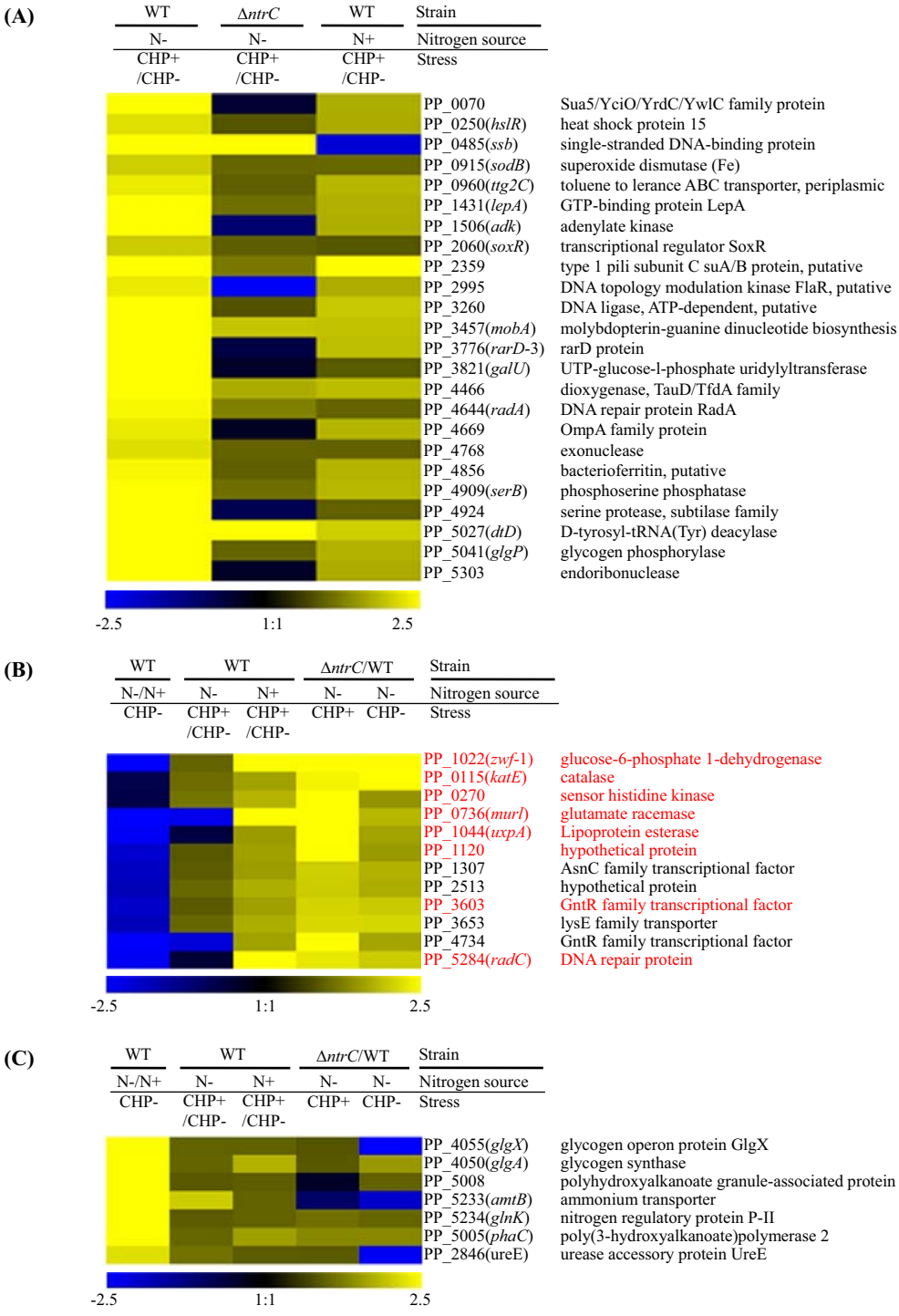

Fig. 5. The first line indicates strains and the second line means nitrogen conditions (N+, nitrogen rich; N-, nitrogen limitation). The third line suggests that cells are treated with CHP (CHP+) or without CHP (CHP-). The backslash mark (/) indicates ratio of gene expression, which is a numerator per a denominator. (A) Global transcriptome analysis reveals a relationship between nitrogen assimilation and oxidative stress in *P. putida*. The ratio of gene expression in the microarrays is shown in the Figure [as a heat map using a log2-based color scale (yellow, induced; blue, repressed)]. Genes were sorted by the first column, which indicates the ratio of gene expression between CHP treatment and control in the wild-type. (B) Global transcriptome analysis for derepressed genes in the *ntrC* mutant in the presence and absence of CHP. Genes were sorted by the fourth column, which indicates the ratio of gene expression between the *ntrC* mutant and the wild-type in the presence of CHP. The ratio of gene expression in the microarrays is shown in the Figure [as a heat map using a log2-based color scale (yellow, induced; blue, repressed)]. (C) Global transcriptome analysis for activated genes under nitrogen-limited conditions in the absence of CHP. Genes were sorted by the first column, which indicates the ratio of gene expression between nitrogen-limited conditions and nitrogen-rich conditions in the wild-type without CHP treatment.

treatment, represses the expression of such genes (Fig. 5C, lanes 2-4), although a slight induction of the *amtB* gene was noted in the presence of CHP under nitrogen-limited conditions (Fig. 5C, lane 2). Here, our data showed that the deletion of the *ntrC* gene renders cells more resistant to oxidative stress under nitrogen-limited conditions. This report is, to the best of our knowledge, the first study to suggest that NtrC-sensed nitrogen availability is one of the most important prerequisite for full cellular defense against oxidative stress in *P. putida*. This observation might be more relevant to a typical soil system, where nitrogen availability is frequently limited.

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