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CHANGES IN GENE TRANSCRIPTION AND PROTEIN EXPRESSION INVOLVED IN THE RESPONSE OF *Agrobacterium* sp. ATCC 31749 TO NITROGEN AVAILABILITY DURING CURDLAN PRODUCTION

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The changes in transcription of genes involved in nitrogen metabolism and curdlan biosynthesis, and total protein expression were firstly analyzed to define the responses of *Agrobacterium* sp. ATCC 31749 to nitrogen source availability during curdlan fermentation. The transcription of all nitrogen metabolism and regulation genes increased significantly under nitrogen limitation. The genes of carbon (*exoC*) and nitrogen (*ntrB*, *ntrC*, and *nifR*) metabolism showed distinctive transcriptional responses to nitrogen limitation. Their relative expression level was increased by 14, 9, 7 and 7-fold, respectively. Two-dimentional electrophoresis (2-DE) revealed that the expression of 14 proteins were elevated and 6 proteins were down-regulated significantly under nitrogen starvation. Furthermore, 4 proteins (GroEL, ABC transporter, Atu1730 and enoyl-acyl carrier protein reductase) in which the expression level changed significantly were identified. The results showed that *Agrobacterium* sp. regulates its carbon flux and nitrogen assimilation effectively for better survival.

Nutrient limitation is the prerequisite for the accumulation of many microbial metabolites in which curdlan production is a distinct case. Curdlan is a water insoluble β-(1,3)-exopolysaccharide produced by *Agrobacterium* species under nitrogen limitation [1]. Similar phenomenon was also observed in many other biopolymer-producing bacteria, such as *Azospirillum brasilense* for the production of polyhydroxyalkanoates (PHA) and poly-3-hydroxybutyrate (PHB) [2]. However, hitherto the mechanism of the nitrogen regulation on biopolymers synthesis in bacteria has not been fully understood. Reports showed that the *ntrBC* and *ntrC* mutants of strain *A. brasilense Sp*7 could accumulate a large amount PHB in the presence of a high concentration of ammonia in the medium. The classical bacterial nitrogen metabolic regulation system is the NtrB-NtrC two-component system (Ntr family) [3]. The protein NtrB – histidine protein kinase (EC 2.7.13.3) catalyzes the phosphorylation and activation of its partner response regulator NtrC- response regulator (EC 2.7.13.3) under nitrogen limitation. The activated NtrC then turns on many other genes and proteins responsible for the initiation of the nitrogen starvation response. It was found that the Ntr family positively regulated biosynthesis other macromolecules, such as alginate, lipase and other biopolymers [3–5].

In this study we investigated the transcription and proteomic profiles of *Agrobacterium* sp. ATCC 31749 in response to nitrogen limitation in order to understand the mechanism of nitrogen regulation on curdlan biosynthesis. In *Agrobacterium* sp., 4 genes coding for enzymes of nitrogen regulation components (*ntrB*, *ntrC*, *ntrX*, *ntrY*), 3 genes for nitrogen assimilation and fixation (*nifR*, *glnA*, *gltB*), 5 genes for carbon metabolism and curdlan biosynthesis (*glmU*, *Crd*, *exoC*, *musC*, *pssA*) were selected for evaluating their transcription response (Table 1). The roles of these genes in *Agrobacterium* sp. ATCC 31749 metabolic pathway and the rationale for the tests are summarized in Fig. 1 [6]. The two-dimensional electrophoresis method was applied to study changes of total protein expression in response to nitrogen starvation of *Agrobacterium* sp. The results showed that the chosen Ntr family of genes and carbon metabolism genes apparently altered their relative expression level in response to nitrogen limitation as well as many accompanying protein expression modulating systems.

MATERIALS AND METHODS

Microorganism, culture medium and conditions of cultivation. The strain of *Agrobacterium* sp. ATCC 31749 was used in this study. The batch fermentation medium contained (g l^{-1}): glucose – 50, NH_4Cl – 1.6, KH_2PO_4 – 2.7, $MgSO_4$ – 0.5 and 10 ml of a trace element solution [7]. The batch fermentation was carried out in a 7 l jar fermenter (BioFlo 110, NBS, USA). Culture temperature was controlled at 30°C. Agitation speed and aeration rate were maintained at 400 rpm and 1 v/v min (volume of air per volume of medium per min), respectively during cultivation.

Estimation of curdlan yield, cell dry weight and ammonia concentration. The biomass and curdlan production were determined by the dry weight method. The cells were centrifuged at 6000 g for 30 min at 4°C. The supernatant was collected for the analysis of ammonium. The pellet containing cells and curdlan was

Table 1. Genes function and primer sequences for RT-PCR in this study

Gene	Protein	Primer F	Primer R	Length
Crd	Putative curdlan synthase	TGTGACGACACACGTCGCGG	GCGTCTGGACGACAGCCACC	247
glmU	Bifunctional N-acetylglucosamine-1-phosphate uridyltransferase	CGCGAAGCGATCAAACGCG	TTGCGTTCCTCGTCGGTGGC	230
mucS	Exopolysaccharide II synthesis transcriptional activator ExpG	CGTTGTTGCCGACGCACACG	GACACGCGAACGCTGGTGGT	289
pssA	Phosphatidylserine synthase	GCCGGACAAGCCCTTCGCTT	CGTAGCGGCGATGCCAGAGG	254
nifR	Nitrogen regulation protein	GGCTGTGCTCGCCGGACATT	TCATGCGGCCTTCCTTGCGG	277
ntrB	Two component sensor kinase	CACAGCCCGGCAGATGCGAA	CCGAGGCGTGGCGAACTCAG	269
ntrC	Two component response regulator	TCGAGACGGAGGCGCTGGA	CCGGTGCGAACCGCCATCTT	187
ntrX	Two component response regulator	TGACAGGGTTGCCCCCACCA	TCCGTGCCGAACAGCGCAAT	177
ntrY	Two component sensor kinase	GCGGGAATGGTGGTTGCCGA	CCGCCGCCCTGCCCTTTTTA	247
gltB	Glutamate synthase	CGCTCCCCGATCCTGACCCA	CGAAACGTGCCGGTCGGTGA	216
exoC	Phosphoglucomutase	CGCGGCGGCGGATGATTTTG	CGCATCCGGCTCGTAACGCT	172
glnA	Glutamine synthetase	TCAACCACGTGCCGGATGCC	CCGACGGACCGTTTTCCCCG	207

resuspended in NaOH solution (0.5 M) for 1 h and the centrifugation was repeated. The resulting curdlan in the supernatant was neutralized and precipitated by 2.0 M HCl. The cells and curdlan were washed and dried to a constant weight in an oven at 80°C. Ammonium (NH_4^+) concentration was determined by the sodium salicylate method [8].

RNA extraction. *Agrobacterium* sp. ATCC 31749 cells were harvested by centrifugation at 4°C, 6000 g for 10 min and cells were disrupted with glass beads at 4°C for and centrifuged for 6 × 30 s at 6000 g. Thereafter one fifth volume of chloroform was added into the supernatants. The solution was mixed thoroughly and left to stand at room temperature for 5 min. The mixture was subsequently centrifuged at 4°C, 13000 g for 15 min, and supernatant was transferred to a new 1.5 ml centrifuge tube. Propanol of the same volume was added to the tubes and the centrifugation was repeated. The precipitate was washed with 0.7 ml 70% ethanol and collected by centrifugation again. Purified RNAs were suspended in 30 μl of (dimethylpyrocarbonate)-treated water (DMPC) 0.1%.

Reverse transcription and real-time PCR. The cDNA was synthesized using the Fermentas RevertAid™ First Strand cDNA Synthesis Kit (Fermentas, Lithuania). Primers were designed in order to have a length of about 20 bases at Tm of about 60°C and synthesized by SBS (SBS Genetech, Shanghai, China). Details of primers were listed in Table 1. The accession ID (Identification) of tested genes in sequence database (NCBI) were: 40556679 (*Crd*), 1133482 (*nifR*), 1133483 (*ntrB*), 1133484 (*ntrC*), 1133485 (*ntrY*), 1133486 (*ntrX*), 1132231(*glnA*), 1132183 (*gltB*), 1135948 (*exoC*), 1133100 (*pssA*), 1133201 (*mucS*), 1133825 (*glmU*).

Real-time PCR was carried out with an ABI 7300 quantitative PCR instrument (ABI, USA) with RT-PCR Master Mix (SYBR Green, TOYOBO, Japan). Thermal cycling conditions were designated as follows: initial denaturizing at 95°C for 5 min, followed by 45 cycles of 94°C for 10 s and 60°C for 30 s. An additional step starting from 90 to 60°C was performed to establish a melting curve to verify the specificity of the real-time PCR reaction for each primer pair. For each measurement, a threshold cycle value (C_T) was determined. Results were analyzed using the comparative critical threshold (C_T) method in which the amount of target RNA is adjusted to a reference relative to an internal calibrated target RNA as described [9].

Sample preparation for two-dimensional electrophoresis. *Agrobacterium* sp. cells were harvested by centrifugation at room temperature at 6000 g for 15 min. The pellets were washed 3 times with a Tris-HCl buffer (40 mM, pH 8.0) and 50 μl cell pellets were resuspended in 1 ml of a lysis buffer containing 7 M urea, 2 M thiourea, 1% w/v amidosulfobetaine-14, 40 mM Tris, 0.001% bromophenol blue, 1 mM EDTA, 10 μl tributyl phosphate and 10 μl carrier ampholyte. After sonication (JY92, XinZhi Biotechnology Company, Ningbo, China) for 3 min in ice bath (ultrasonic-15 s, spacing interval-4 s, cycle-20 times) the cell debris was removed by centrifugation at 12000 g for 60 min [10]. The supernatants were stored at –80°C until analyzed by two-dimensional electrophoresis (2-DE).

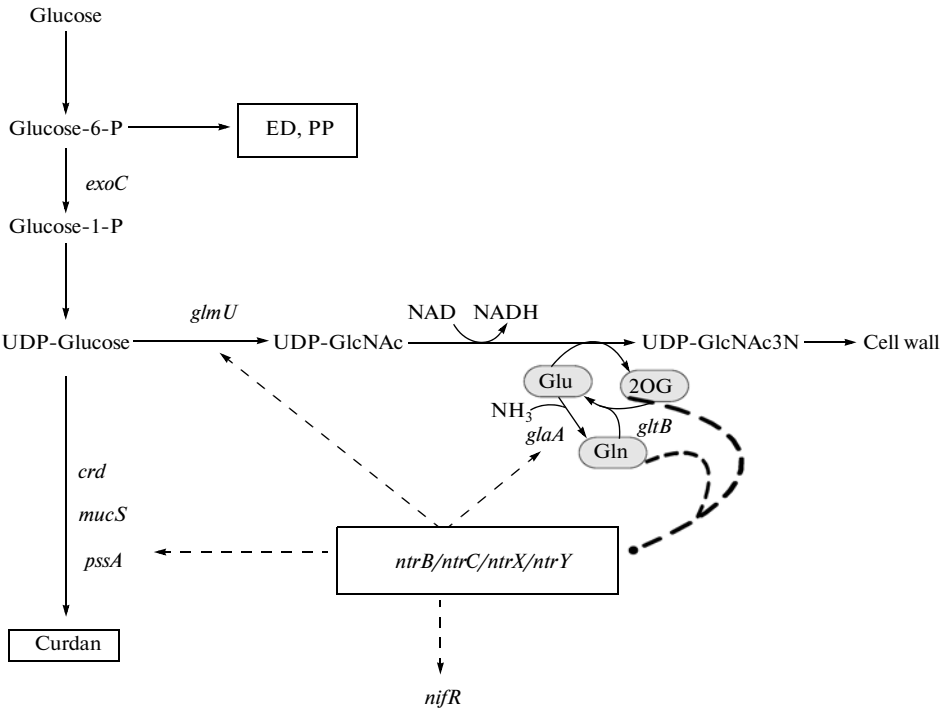

Fig. 1. Metabolic network of the test genes involved in curdlan production by *Agrobacterium* sp. ATCC 31749. UDP-Glucose – uridine diphosphoglucose; UDP-GlcNAc – beta-1,3-N-acetylglucosaminyltransferase; ED – Entner-Doudoroff pathway.

Two-dimensional electrophoresis and MALDI-TOF mass spectrometric analysis. The first dimension of 2-DE was carried out on a GE IPGphor™ Electrophoresis System at 20°C. Linear pH 4–7 immobilized pH gradient (IPG) gel strips (Bio-Rad Laboratories, CA, USA) were rehydrated overnight by placing the strips gel-side-down in the sample containing rehydration solution in a strip holder and covering them with mineral oil (Bio-Rad Laboratories, USA). The first dimension was performed for 30 min at 250 V, for 1 h at 1000 V, for 5 h at 10000 V, and until 70000 V h at 10000 V for the analytical and preparative gels that had been prepared for image analysis. The IPG gel strips were then equilibrated for 15 min in an equilibration solution of 0.375 M Tris-HCl buffer pH 8.8 containing 6 M urea, 20% glycerol (v/v) and 2% sodium dodecyl sulfate (SDS) with 2% dithiothreitol (DTT) for 15 min and then 2.5% iodoacetamide for 15 min. The equilibrated gel strips were placed on a 12.5% polyacrylamide gel and the second dimensional separation was carried out using an Ettan Dalt six Electrophoresis system (GE Healthcare, USA) at 12°C. SDS-PAGE was carried out at 2 W/gel for 30 min and 16 W/gel for 6 h until the bromophenol blue reached to the bottom of the gel. Silver-staining of proteins was performed using a modified method, as described elsewhere [11]. Triplicate gels used to visualize proteins by silver-staining were scanned with GE Image Scanner. The images were analyzed by using PDQuest 8.0.1 (Bio-Rad Laboratories, USA).

The samples for the matrix-assisted laser desorption-ionization-time of ight (MALDI-TOF) mass spectrometry analysis were extracted from the Comassie-stained spots [12]. Protein analysis was performed using a MALDI-TOF mass spectrometry system (Voyager DE-STR, USA). The web-based search engine MASCOT 2.2 and the most recent Swiss-Prot database (http://www.matrixscience.com/search form select.html) were used to identify protein spots by querying the trypsin digested peptide fragment data.

Statistic analysis. All data were entered into the SPSS 15.0 program for standard deviation and significance analysis. The significant P value was set as 0.05.

RESULTS

The batch growth profile of *Agrobacterium* sp. ATCC 31749. A typical growth profile of *Agrobacterium* sp. ATCC 31749 was shown in Fig. 2. Apparently, the biomass accumulated in the nitrogen-sufficient period and no curdlan was produced at this phase. At the end of nitrogen exhaustion, the biomass reached the maximum level and curdlan synthesis was initiated. Samples analyzed were taken from the cultures with cultivation times of 8, 12, 24 and 36 h (labeled in Fig. 2 as C, A, D, B, respectively).

Changes in relative expression level of genes related to nitrogen metabolism of *Agrobacterium* sp. in response to nitrogen limitation. Two samples were taken for relative expression (RE) level analysis: culture in growth phase

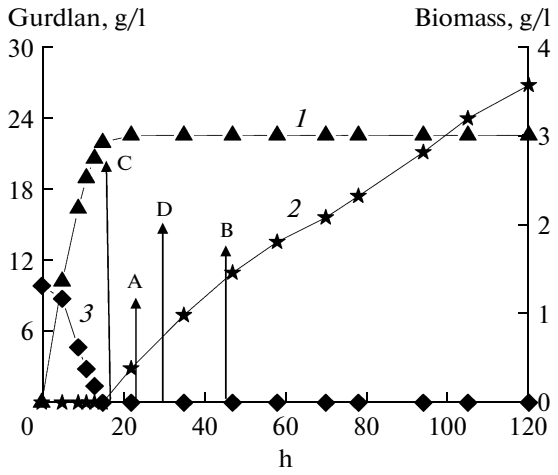

Fig. 2. The kinetic of cell growth and curdlan production in batch fermentation. A, B – sampling time for RT-PCR at 12 and 36 h; C, D – sampling time for 2-DE at 8 h and 20 h: *1* – biomass; *2* – curdlan production; *3* – recidual NH_4Cl.

with sufficient nitrogen at 12 h (sample A), in curdlan synthesis phase with exhausted nitrogen at 36 h (sample B), as shown in Fig. 2. Sample A was defined as the calibration point using 16sRNA as internal reference, the RE level elevated higher than 2-fold means that the genes were increased their expression significantly [13]. The results are shown in Fig. 3.

When the availability of nitrogen in the environment was changed from sufficient to limited, an apparent transcriptional increase of Ntr family of genes was observed and their RE levels all exceeded 2-fold, resulting in a significant elevation. As shown in Fig. 3a, the highest increase in gene expression level was *ntrB* and its RE level increased nearly 9-fold. The RE level of *ntrC* increased 7-fold, 5-fold for *ntrX* and 3-fold for *ntrY*. Activated NtrC can turn on many genes expression in the cell, but its gene expression was also controlled by nitrogen availability.

Glutamate (Glu) and glutamine (Gln) serve as the key nitrogen donors for the biosynthesis of many building blocks and some small molecules in all bacteria. The most important pathway which facilitates the incorporation of nitrogen into Glu and Gln is the Gln synthetase (EC 6.3.1.2)/Glu synthetase (EC 6.3.1.2) (GS/GOGAT, *glnA* and *gltB*) pathway, which is ubiquitous in bacteria. In addition, NifR is the main enzyme responsible for nitrogen fixation regulation for some soil bacteria such as *Agrobacterium* sp. [14]. The RE level of *gltB* and *glnA* genes showed a moderate increase of 3 and 5-fold respectively under nitrogen starvation, and the RE level of *nifR* was also elevated significantly.

Changes in RE level related to curdlan biosynthesis and carbon metabolism genes under nitrogen limitation. The bifunctional N-acetylglucosamine-1-phosphate uridyltransferase (EC 2.7.7.23; 2.3.1.157, *glmU*) is a cytoplasmic bifunctional enzyme involved in the biosynthesis of the nucleotide-activated UDP-GlcNAc (EC 2.4.1.149) [15]. As shown in Fig. 3 b, the expression of *glmU* is quite sensitive to changes in the available nitrogen level of the culture medium, and it's RE level increased 3-fold. Phosphoglucomutase (EC 5.4.2.2, *exoC*) is central in carbon metabolism and catalyzes the conversion of G-1-P to G-6-P. The RE level of *exoC* increased to the highest level of 15-fold during the transition from sufficient nitrogen source to limited level in the culture medium. *Crd* gene encodes the key enzyme of curdlan synthase (EC 2.4.1.12) for curdlan biosynthesis [16]. Under nitrogen starvation *Crd* gene was activated and its expression level was elevated 5-fold. Similar result was observed in exopolysaccharide II synthesis, in which transcriptional activator ExpG (*mucS*) is a regulator controlling EPS II synthesis in response to nitrogen availability [17]. Phosphatidylserine synthase (EC 2.7.8.8, *pssA*) is a membrane-associated enzyme involving in curdlan transport [18]. When nitrogen source was used up and curdlan bio-

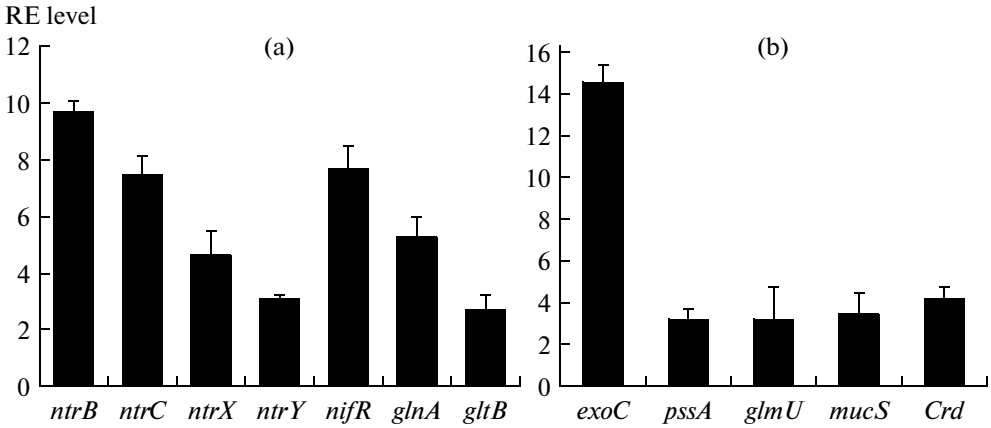

Fig. 3. Changes in the RE level of the curdlan biosynthesis, nitrogen (a) and carbon (b) metabolism related genes under nitrogen limitation in *Agrobacterium* sp. ATCC 31749.

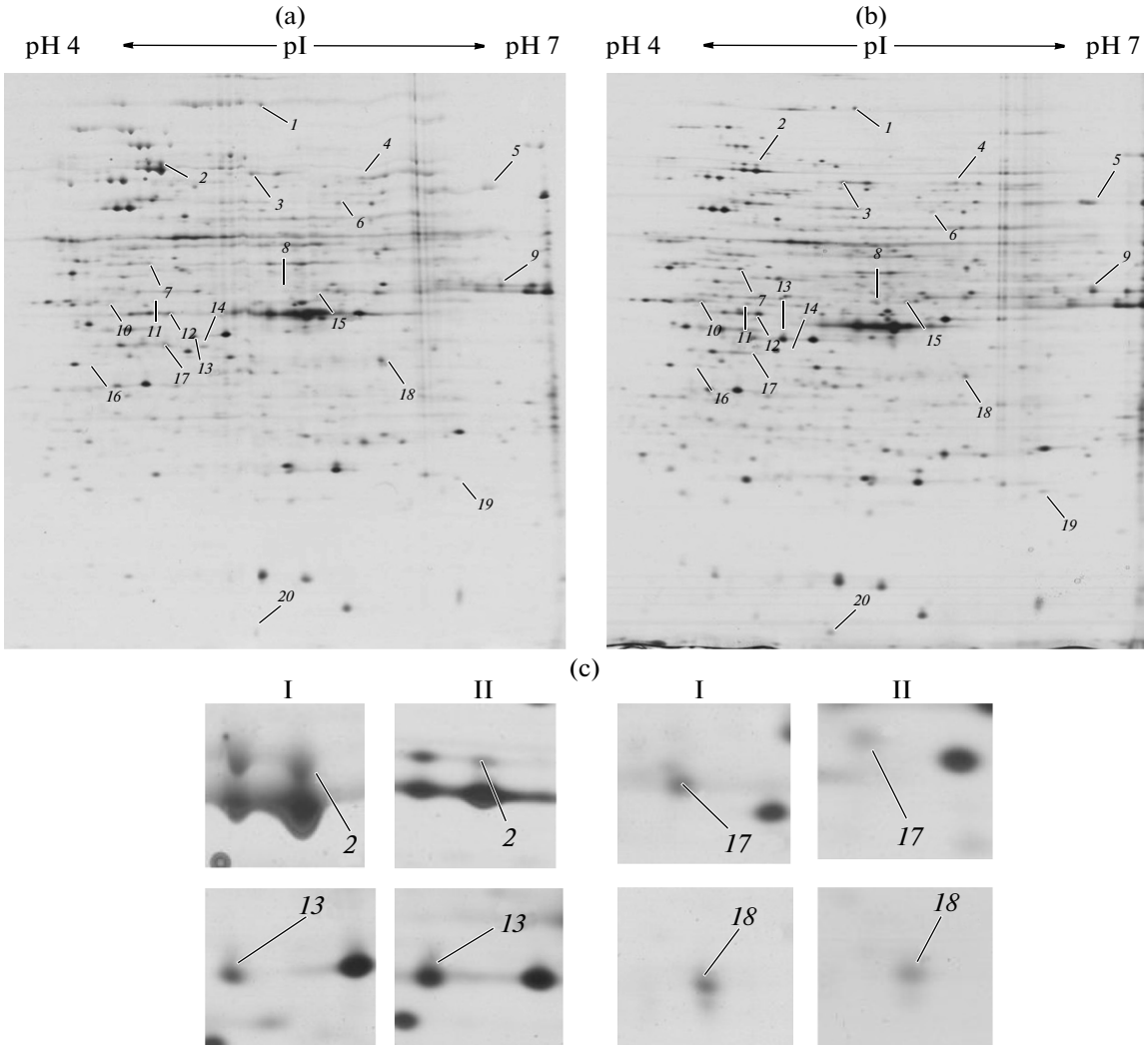

Fig. 4. Silver-stained 2-DE SDS-PAGE at the 8 h (a) and 20 h (b) of the curdlan batch-fermentation process, and close-up views of the 4 identified proteins on 2-DE maps: a – 2-DE map of sample at 8 h fermentation; b – 2-DE map of sample at 20 h fermentation; c – close-up view of identified proteins spots, and among them I represents protein 2-DE map at 8 h, II represents protein 2-DE map at 20 h.

synthesis was initiated and the RE level of *pssA* gene also increased 3-fold.

Proteomic analysis of *Agrobacterium* sp. ATCC 31749 in response to nitrogen limitation. The samples for 2-DE analysis were: culture in growth phase of 8 h (sample C) and in curdlan synthesis phase of 20 h (sample D), as shown in Fig. 2. Triplicate 2-DE were run for 2 samples with good reproducibility. The 2-DE maps of 2 samples are shown in Fig. 4 a and b. Further analysis of the 2-DE map was carried out using the PDQuest8.0.1 software. As a result, nearly 750 protein spots were found on the two maps and the protein spots distribution position were generally identical and the matching rate was about 78%. When comparing 2 maps 20 protein expressions changed significantly and 14 of them were elevated (labeled with numbers as 1, 3, 5, 7, 8, 9, 10, 11, 12, 13, 15, 16, 19, 20). Six of them (labeled with numbers as 2, 4, 6, 14, 17, 18) decreased significantly.

Identification of changed proteins by MALDI-TOF mass spectroscopy. Subsequently, 5 proteins were chosen from the 20 proteins exhibited significant changes for identifying. Four proteins were successfully identified – 3 proteins with known functions and the other was unknown protein as shown in Table 2. Close-up views of 2-DE maps of the tested 4 proteins spots were shown in Fig. 4c, and spots on I maps represented protein expression under nitrogen sufficient conditions, spots on II maps represented protein expression under nitrogen limitation. The No. 2 protein was a molecular chaperone of GroEL. The main function of this protein is to combine and to stabilize other protein which has unstable conformation that promotes new peptide folding and transmembrane transport of protein associated with cell or-

Table 2. Identification of differential proteins of *Agrobacterium* sp. ATCC 31749 in different phases by MALDI-TOF/TOF-MS/MS and protein database searching

Spot no.	Protein name*	Accession no.	Mr, kDa/pI	Protein score	Matched peptides	Protein function
2	Chaperonin GroEL	gi|15888025	57.63/5.08	234	15	Helps new protein folding exactly
13	ABC transporter	gi|159185771	36.14/5.70	620	18	Transports salt ions from vivo to vitro
17	Hypothetical protein Atu1730	gi|15889035	30.10/5.13	302	12	–
18	Enoyl-acyl carrier protein reductase	gi|15888100	29.29/6.51	263	13	Catalyzes the last step of fatty acids extension

* The proteins were searching from the protein database of *A. tumefaciens* str. C58 whose genome is highly homologous to *Agrobacterium* sp. ATCC 31749.

ganelle [19]. The protein expression decreased apparently under nitrogen source exhaustion. The No. 13 protein was an ABC transmembrane transport protein capable of transporting different molecules such as carbohydrates, amino acids, peptides and cell metabolites into the cell [20]. The expression of the No. 13 protein was elevated. When nitrogen source was absent in the environment, toxic metabolites accumulated and cell growth creased. Conceivably, this protein may remove harmful metabolites out of the cells and the osmotic pressure balance was maintained. The No. 18 protein was an enoyl-acyl carrier protein reductase (EC 1.3.1.9), one of the key enzymes in fatty acid biosynthesis. Fatty acid is the precursor for the biosynthesis of phosphatide, lipopolysaccharide and other lipid components which are the important components for cell wall and biofilm formation in bacteria.

DISCUSSION

In this work we investigated the response of *Agrobacterium* sp. ATCC 31749 to nitrogen limitation and the relationship between nitrogen and carbon metabolism in unbalanced nutrient situation by the appropriate transcriptional and proteomic data. One of the most interesting observations is that with regard to the target genes, *Agrobacterium* sp. reacts immediately to environmental changes of nitrogen availability.

The Ntr family of genes showed profound sensitivity to nitrogen availability in the culture medium. Similar phenomena were also found in other bacteria in which the Ntr family of genes was responsible for switch on nitrogen assimilation. In *E. coli* the intracellular NtrB and NtrC increased upon nitrogen limitation. The activated NtrC would elevate the transcription level of *glnA* and *gltB* which were controlled by nitrogen availability [21]. Kumar and Shimizu [22] also reported that the transcription level of *gltB* and *glnA* was enhanced under high C/N condition in the same bacterium. The *gltB* mutant of *Pseudomonas putida* KT2442, was found to be impaired in the capacity to survive under prolonged nitrogen starvation [23]. Thus, the Ntr family of genes and nitrogen assimilation proteins were responsible for the survival of the bacterial cells in nitrogen starvation environment by elevating their expression.

As a result, genes related to curdlan biosynthesis were rapidly activated to produce curdlan in the bacterial surface to protect the bacteria under adverse environment of nitrogen exhaustion [16]. The transcription of the *exoC* gene was highly elevated under nitrogen starvation, and more carbon flux of G-1-P was channeled into curdlan biosynthetic pathway. The phenomenon was similar to previous reports that *exoC* mutants of *A. tumefaciens* could not convert G-1-P into G-6-P, an intermediate for the biosynthesis of UDP-glucose which is the precursor for exopolysaccharide biosynthesis [24]. Thus, *Agrobacterium* sp. elevates *exoC* gene expression in order to supply more G-6-P for the curdlan biosynthetic pathway. Janczarek and Skorupska [25] reported that the gene *pssA* in *Rhizobium leguminosarum* was involved in exopolysaccharide biosynthesis and its production was enhanced when the specific gene transcription was increased under nitrogen limitation. Analyzing the changes in the RE level of genes examined under nitrogen starvation we could postulate that the Ntr family as a regulator of nitrogen metabolism would increase their transcription level in order to activate some genes transcription related to nitrogen assimilation and exopolysaccharide biosynthesis in response to nitrogen availability.

Consequently, it is conceivable that down-expression of enoyl-acyl carrier protein reductase, the important enzyme for cell wall and lipopolysaccharide formation, inhibits cell growth under nitrogen limitation since the cell wall synthesis would be blocked. Accordingly, the reduced lipopolysaccharide biosynthesis enabled the UDP-glucose to be channeled to curdlan exopolysaccharide synthesis.

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