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A comparative proteomic analysis of *Gluconacetobacter diazotrophicus* PAL5 at exponential and stationary phases of cultures in the presence of high and low levels of inorganic nitrogen compound

L.M.S. Lery [a,c], W.M.A. von Krüger [a,c,*], F.C. Viana [b,c], K.R.S. Teixeira [b,c], P.M. Bisch [a,c]

[a] *Unidade Multidisciplinar de Genômica – Instituto de Biofísica Carlos Chagas Filho – Universidade Federal do Rio de Janeiro, Brazil*
[b] *EMBRAPA Agrobiologia – Seropédica, Brazil*
[c] *Rede de Proteômica do Estado do Rio de Janeiro, Brazil*

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ABSTRACT

A proteomic view of *G. diazotrophicus* PAL5 at the exponential (E) and stationary phases (S) of cultures in the presence of low (L) and high levels (H) of combined nitrogen is presented. The proteomes analyzed on 2D-gels showed 131 proteins (42E+32S+29H+28L) differentially expressed by *G. diazotrophicus*, from which 46 were identified by combining mass spectrometry and bioinformatics tools. Proteins related to cofactor, energy and DNA metabolisms and cytoplasmic pH homeostasis were differentially expressed in E growth phase, under L and H conditions, in line with the high metabolic rate of the cells and the low pH of the media. Proteins most abundant in S-phase cells were stress associated and transporters plus transferases in agreement with the general phenomenon that binding protein-dependent systems are induced under nutrient limitation as part of hunger response. Cells grown in L condition produced nitrogen-fixation accessory proteins with roles in biosynthesis and stabilization of the nitrogenase complex plus proteins for protection of the nitrogenases from O_2-induced inactivation. Proteins of the cell wall biogenesis apparatus were also expressed under nitrogen limitation and might function in the reshaping of the nitrogen-fixing *G. diazotrophicus* cells previously described. Genes whose protein products were detected in our analysis were mapped onto the chromosome and, based on the tendency of functionally related bacterial genes to cluster, we identified genes of particular pathways that could be organized in operons and are co-regulated. These results showed the great potential of proteomics to describe events in *G. diazotrophicus* cells by looking at proteins expressed under distinct growth conditions.

1. Introduction

Although abundant in the terrestrial atmosphere, gaseous nitrogen (N_2) is chemically inert at ambient temperature, the nitrogenous mineral and organic reserves in the ground are limited and quickly depleted by successive cropping [1]. In tropical regions, conditions of temperature and humidity speed up the processes of decomposition of the organic substances, resulting in highly acid soils with still lower content of nitrogenous compounds [2,3].

As much as 30% of the total fertilizers needed for agricultural crops are nitrogen-derived chemicals produced under high costs of energy [4]. Consequently, decreasing dependence on chemical insumes for agricultural production is a need to reduce cost and the negative impact to the economy and environment. Biological processes, mediated by microbial action, are crucial for cycling of nutrients in the planet and have been used with success to control plagues, domestic illnesses of plants and animals [5]. Therefore, a biological approach, such as the use of N_2-fixing bacteria to provide plants reduced N, remains as one of the best solutions for the problem.

Gluconacetobacter diazotrophicus was first isolated from sugar cane [6], where it has been found as an endophyte in roots, stems and leaves [7–9]. It has also been isolated from coffee plant, sweet-potato, pineapple, cameroon grass, tea, banana, ragi, rice [4,10–14] and, even from insects that infest sugar cane [15]. *G. diazotrophicus* is a N_2-fixing bacterium [6,16], well adapted to the high sucrose environment of the sugarcane plant [6,10], where it fixes nitrogen under microaerophilic conditions [6,17]. Its optimum pH for growth and nitrogen-fixation is 5.5, but in vitro it can grow and fix nitrogen at pH as low as 2.5, however, it does not grow at pH higher than 7.0 [17]. It produces the plant growth promoting hormones indole-3-acetic acid (IAA) and gibberellins A1 and A3 [9,18] and bacteriocins, and more, it is able to solubilize in vitro two essential micronutrient for plant growth, zinc and phosphorous compounds [19,20], therefore, *G. diazotrophicus* provides a good model to study symbiosis with non-leguminous plants [10].

* Corresponding author. Avenida Carlos Chagas Filho, CCS, bloco G, Instituto de Biofísica Carlos Chagas Filho, sala G1-043, Ilha do Fundão, CEP 21941-902, Rio de Janeiro, RJ, Brazil. Tel.: +55 21 25626531; fax: +55 21 22808193.
E-mail address: wmavk@biof.ufrj.br (W.M.A. von Krüger).

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G. diazotrophicus has been the focus of several studies aiming to understand the mechanisms behind diazotrophy. The bacterium contains a major cluster of *nif* and *fix* genes [21–25] and in the presence of low levels of the ammonium it expresses both, the Mo-nitrogenase and NifA, the transcriptional regulator of all *nif* genes [23]. *G. diazotrophicus* Mo-nitrogenase activity is inhibited under high intracellular oxygen concentrations at the level of its synthesis and by irreversible damage to the iron protein [26]. However, it can also be protected due to the formation of a complex with a FeSII protein (Shethna protein) [27]. In this complex the enzyme remains inactive, but can be reactivated in the presence of an efficient respiratory substrate to lower intracellular levels of O_2 [27–29]. Interestingly, *G. diazotrophicus* nitrogenase activity is more tolerant to oxygen than that of most diazotrophs [17], what explains the ability of its colonies to fix nitrogen at a wide range of atmospheric pO_2 [30]. *G. diazotrophicus* Mo-nitrogenase purified components, Gd1(MoFe) and Gd2 (Fe), were found to be quite similar to Av1 and Av2, the components of the *Azotobacter vinelandii* nitrogenase, in terms of size, composition, interactions and catalytic properties, however, they showed narrower pH range for efficient catalysis and higher sensitivity to pH and salt [29].

G. diazotrophicus properties studied so far suggest it is a diazotroph quite distinct from other root-associated organisms [17], therefore, efforts have been done to better understand its physiology, what might contribute to the development of biotechnological approaches for a more sustainable and environmentally safe crop production [31].

One way to identify metabolic pathways involved in a specific biological process is the differential proteome analysis by two-dimensional gel electrophoresis (2D-GE) followed by mass spectrometry (MS) and bioinformatics to characterize proteins of interest. This approach, largely used to provide broad pictures of the physiological processes associated with a given condition in many bacterial species, including *G. diazotrophicus* [32–38], has been used in the present study to identify proteins specifically expressed at exponential (E) and stationary (S) phases of liquid cultures under low (L) and high (H) levels of a combined nitrogen compound. Many novel proteins of *G. diazotrophicus* with roles in specific and in the general metabolic pathways have been detected under the growth conditions used. The present data set together with others recently published [38] constitute a major advance in the molecular microbiology of this species and will contribute to a better understanding of the physiology of the bacterium.

2. Materials and methods

2.1. Bacterial strain and culture conditions

G. diazotrophicus, strain PAL5 (BR11281), was precultured in DYGS medium [39] to exponential phase. The cells were harvested by centrifugation (18,000 ×g, 10 min, 4 °C), washed under the same conditions and resuspended at a final concentration of 2×10^6 cells/ml in 2 l of the modified defined minimal medium LGI (LGIM) at initial pH 5.5. For growth under N-limitation or N-abundance media were supplemented with $(NH_4)_2SO_4$ at 1 mM (low combined nitrogen level, L) or 10 mM (high combined nitrogen level, H) respectively, as previously described [17]. Bacterial growth was carried out in a fermentor (Bioengineering, GAC of 2.5 l), at 30 °C and 400 rpm, under controlled air or air/N_2 flow [17]. To avoid contamination during growth, filters were used at inlets of gases and at openings. During growth levels of dissolved oxygen (dO) and pH of the cultures were monitored continuously and concentrations of $(NH_4)_2SO_4$ were measured as previously described [17]. Culture with H level of $(NH_4)_2SO_4$ was initially saturated with O_2 atmospheric to dO 80% and, during growth, air influx assured nitrogenase complex inactivity [17]. For growth with L level of $(NH_4)_2SO_4$, the chamber was initially fluxed with a mixture of air and N_2 in a proportion of 1:1 to give an initial dO of 50%. During growth, as levels of dO decreased due respiration, gases were injected into the chamber to keep dO at levels compatible with cell growth and nitrogen-fixation [17]. Growth was followed by cell number counts (Newbauer-counting chamber), optical density at 600 nm (spectrophotometer PE — Model Lambda 11) and total protein content [40]. Samples for further analysis were collected during exponential (E, 25–50 h) and stationary (S, 55–70 h) phases of growth.

2.2. Sample preparation and 2D gel electrophoresis (2D-GE)

Cells were harvested by centrifugation (18,000 ×*g*, 10 min, 4 °C) and the cellular pellet was resuspended in a lysis buffer (2% Chaps, 8 M urea, 0.13 M DTT, 0.02% (v/v) Pharmalyte 3–10, 1 mM PMSF). Cell suspension was submitted to 20 cycles of freezing [$N_{2\ (l)}$] and thawing (37 °C) and lysis was monitored by optical microscopy. A clear lysate was obtained by centrifugation at 50,000 ×*g* for 1 h at 4 °C and protein content was estimated by 2D Quant Kit (GE Healthcare).

For the first dimension approximately 500 µg of proteins in 320 µl of solution (2% Chaps, 8 M urea, 0.13 M DTT, 0.02% (v/v) Pharmalyte 3–10, 8 mM PMSF, traces of bromophenol blue) was used to rehydrate an 18 cm strip pH 3–10 (Immobiline DryStrip; GE Healthcare) for 16 h at room temperature. Proteins were focused according to the manufacturer's instructions (GE Healthcare) [41]. The gel strip was equilibrated and then loaded onto a 12–14% gradient gel (SDS-ExcelGel XL) and electrophoresed as recommended [41]. First and second dimension runs were carried out in the Multiphor II unit (GE Healthcare).

Gels were stained with Coomassie Blue R-250 [42] scanned with the ImageScanner LabScan v5.0 and analyzed with the ImageMaster 2D Platinum v5.0 software (GE Healthcare).

2.3. Comparative analysis of gels

Proteomic analysis of *G. diazotrophicus* lysates of cells at E or S-phase of cultures in LGIM with starter doses of 1 or 10 mM of $(NH_4)_2SO_4$, were carried out by 2D-GE. For each condition (exponential/low, EL; exponential/high, EH; stationary/low, SL and stationary/high, SH) triplicate gels were run (Fig. 1). Two gels of the triplicate run for each phase/condition derived from one culture and the third from a distinct culture. Comparison of 2D-GE protein patterns of cells collected at the same phase of growth under similar conditions, in distinct experiments, permitted us to evaluate variability at biological level (biological replicate). Analysis of the same sample in two gels, on the other hand, allowed us to check technical variability (experimental replicate).

Protein spots on all gels were automatically detected with the ImageMaster 2D Platinum v5.0 software which also performs automate matching using filtering, querying and the statistical applications required (IMageMaster™ 2D Platinum software version 5.0, User Manual, [43]). All the spots were also manually confirmed. Images of three replicate gels (member gels) were superposed using the same software and a master gel containing all spots representative of each culture phase and condition (E, S, L and H) was obtained. In each master gel protein spot position, shape and optical density were averaged and unmatched spots or those whose relative volumes differed more than 20% among member gels were not considered (Fig. 1).

Relative values of isoelectric point (pIr) and molecular mass (MWr) of the proteins of interest were determined considering linear distributions of pH (3–10) and the protein molecular weight markers (GE Healthcare), respectively.

In order to identify proteins specific to the different culture phases and conditions under study, intersections of the master gels were obtained and compared. Spots common to two gels were considered differentially expressed if their relative volumes differed more than 3 fold (Fig. 1). Relative volumes (%Vol) were normalized volumes that remain relatively independent of irrelevant variations between gels,

particularly caused by varying experimental conditions [43]. %Vol is expressed as:

$$\%\mathrm{Vol} = \frac{\mathrm{Vol}}{\sum_{S=1}^{n} \mathrm{Vol}_S} \times 100$$

where, Vol is the volume of the spot of interest and Vol_s is the volume of each spot *S* in a gel containing *n* spots.

2.4. Mass spectrometry analysis and protein identification

Protein spots were cut from gels and stored at −20 °C or processed immediately. Dried gel fragments were treated with porcine trypsin (Promega, Madison, WI, USA) and peptides were extracted with 50% acetonitrile (ACN), 5% trifluoroacetic acid (TFA). Sample volume was reduced to 10 µl (Speed Vac) and 1 µl was mixed with equal volume of a saturated solution of α-cyano-4-hydroxycinnamic acid matrix in 50% ACN, 0.1% TFA. The mix was spotted onto a MALDI-TOF sample plate and allowed to crystallize at room temperature. Analysis was performed in a Voyager DE PRO BioSpectrometer Workstation (Applied Biosystem, Foster City, CA, USA). Spectra were obtained in reflectron-delayed extraction mode with high resolution for 800–4000 Da range.

MALDI-TOF-TOF analysis was performed in cases where identification by MALDI-TOF was not successful. Precursor ion fragmentation, using N_2 as collision-induced dissociation gas and collision cell pressure kept at 2.8×10^{-6} Torr, was carried out in a 4700 Explorer Proteomics Analyzer (Applied Biosystem).

Trypsin autolysis peptide masses 842.5 and 2211.1 and calibration mixture 1 or 2 (Sequazyme Peptide Mass Standard kit, PerSeptive Biosystems, Foster City, CA, USA) were used, respectively, as internal and external standards in both MS procedures.

Peptide mass fingerprints were analyzed by two independent approaches: online Protein Prospector MS-Fit interface (http://prospector.ucsf.edu) that matched the mass spectrometry data to protein sequences in NCBI database (*G. diazotrophicus* database version NC_010125.1, GI:162145846, with 3.778 protein sequences on 04/28/2008; Bacterial protein sequence database with 8.022.379 protein sequences on 04/28/2008). Only proteins with hit in both NCBI databases were considered identified.

For protein identification a first MOWSE score above 10^4 and at least a 10-fold difference in MOWSE score from the second possible hit was required. Additionally, a minimum of 20% of protein coverage and 4 peptides of mass spectra should match the sequence of the protein in the databank. Congruence in the results of both approaches was an essential requirement and meant that the protein hit in NCBI database was homologous or similar to the ORF hit in the *G. diazotrophicus* database.

The MS/MS spectra data showing the daughter ions, generated by the collision-induced dissociation and internal fragmentations (b or y ions), were used to search for an ORF in the *G. diazotrophicus* database using local MASCOT interface [44]. The criteria of identification were a Mascot score above 70, a minimum of 20% of protein coverage and 4 peptides from the mass spectra with hits in the database.

2.5. Bioinformatics analysis of proteins

A series of computational tools were used to assign or validate potential functions to the differentially expressed proteins. The BLASTp program [45,46] and PFAM database [47] were used, respectively, to search for sequence similarities, protein domains or conserved protein regions.

3. Results and discussion

3.1. Characterization of member and master gels

The high similarity among the protein patterns on the three 2D gels of a set (member gels) run for each phase/condition, i.e., exponential/low, EL; exponential/high, EH; stationary/low, SL and stationary/high, SH, showed us that the technical approach used was correct and the results are reliable.

The three member gels were compared and a master gel, containing only the spots whose relative volumes differed less than

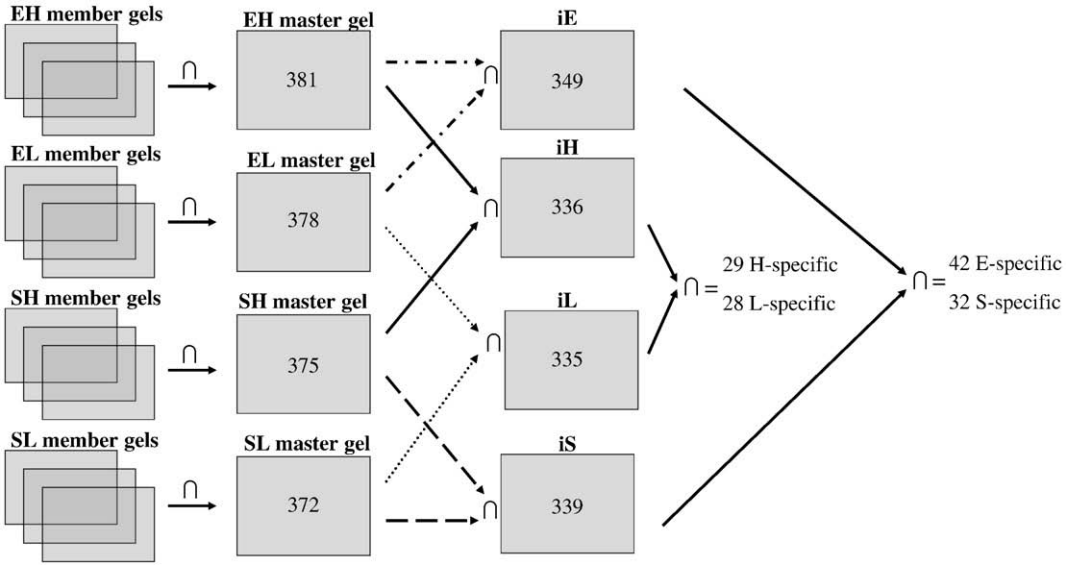

Fig. 1. Experimental design for comparative two-dimensional gel analysis. Three member gels obtained for each culture phase [exponential (E) or stationary (S)] and condition of growth [low (L) or high (H) starter concentration of $(NH_4)_2SO_4$] were used to obtain a master gel that contained only the spots whose relative volumes differed less than 20% among the replicates. The four master gels were: EH, EL, SH and SL. Master gels EH and EL were compared and an iE (intersection exponential) virtual gel with 349 spots was generated. Comparison of the master gels SH and SL generated an iS (intersection stationary) virtual gel with 339 spots. iL and iS virtual gels were compared and 42 spots specific of the E-phase and 32 of the S-phase of growth were identified. EH and SH master gels were also compared and 336 spots common to both originated an iH virtual gel (intersection high). Similarly, comparison of EL and SL master gels generated iL (intersection low) gel with 335 spots. Comparison of iH and iL virtual gels revealed 29 spots specific to the H and 28 to the L condition of growth.

20% among the replicates, was obtained for EH, EL, SH and SL status with 381, 378, 375 and 372 reproducible spots, respectively (Fig. 1). In total, 307 spots common to all gels plus phase or/and condition-specific spots (42 E, 32 S, 29 H, 28 L; 3 EH, 7 SH, 1 EL, 5 SL, Fig. 1) could be visualized over a pH range 3–10, but largely (75%) between pH 4–7 and molecular weights from 22 kDa to 97 kDa (for *ca.* 80% of the proteins, Fig. 2).

A remarkable feature of all *G. diazotrophicus* 2D profiles was a huge protein spot with p*I* around 4 and molecular weight of 45 kDa. Another distinctive characteristic was clusters of spots, possibly, isoforms of proteins. Many of these presented little or no observable mass alteration, but differed in charge (Fig. 2). Protein isoforms have been identified in a variety of organisms suggesting that they might be common and could represent a new aspect of protein function regulation [32,38,48,49].

3.2. Differential protein expression analysis

Morphological and physiological changes in *G. diazotrophicus* during E and S-phases of growth are largely unknown. Similarly, molecular components and cellular mechanisms of adaptation to nitrogen compounds starvation have not been extensively studied in this bacterium. In an attempt to better understand these processes a comparative proteomic approach was used to detect changes in expression, abundance and post-translational modifications of proteins. In this paper, the protein expression of *G. diazotrophicus* at E and S culture phases, under L and H growth conditions was analyzed.

Comparisons of master gels EL×EH and SL×SH, resulted in intersection gels called iE and iS, respectively (Fig. 1). Protein spots not common to iE and iS, but present only on iE gel were characteristic of E-phase, similarly, those unique to iS gel were proper of the stationary phase, independently of the initial inorganic nitrogen concentrations in the culture media. Accordingly, 42 spots were expressed preferentially in cells at E and 32 at S-phases of growth (Fig. 1).

Some proteins were only observed on gels from cells of EL and SL or EH and SH phase/conditions (Fig. 1). The intersection of the master gels EL×SL, the iL virtual gel, was compared to the intersection of the master gels EH×SH, the iH virtual gel (Fig. 1). Some protein spots of the 131 proteins (42E+32S+29H+28L=131) expressed differentially by *G. diazotrophicus* 46 were identified by mass spectrometry. Twenty-nine were identified by MALDI-TOF analysis of tryptic digests. Those not identified by peptide mass fingerprint (PMFs), were submitted to tandem MS/MS fragmentation to generate information on their amino acid sequences.

In all, the amount of differentially expressed proteins confidently identified was: 31% (13 spots in 42) from E-phase grown cells, 34% (11 in 32 spots) from S-phase grown cells, 68% (19 in 28 spots) of those expressed under L condition and 10% (3 in 29 spots) of those specific to cells grown under H condition (Tables 1–4). The remaining proteins (86) could not be identified by MS or MS/MS, in some cases due to poor quality of mass spectra derived from low amount and/or mixture of proteins in the spots. However, in cases where good quality spectra were obtained data might have not matched sequence entries, most probably, due to poor representation in the protein sequence databases [50].

The 46 proteins identified in the present were only present on iL (28) or on iH virtual gel (29) and were considered specific of cells grown under L or H levels of combined nitrogen, respectively (Fig. 1).

3.3. Protein identification

Of the 131 proteins (42E+32S+29H+28L=131) expressed differentially by *G. diazotrophicus* 46 were identified by mass spectrometry. Twenty-nine were identified by MALDI-TOF analysis of tryptic digests.

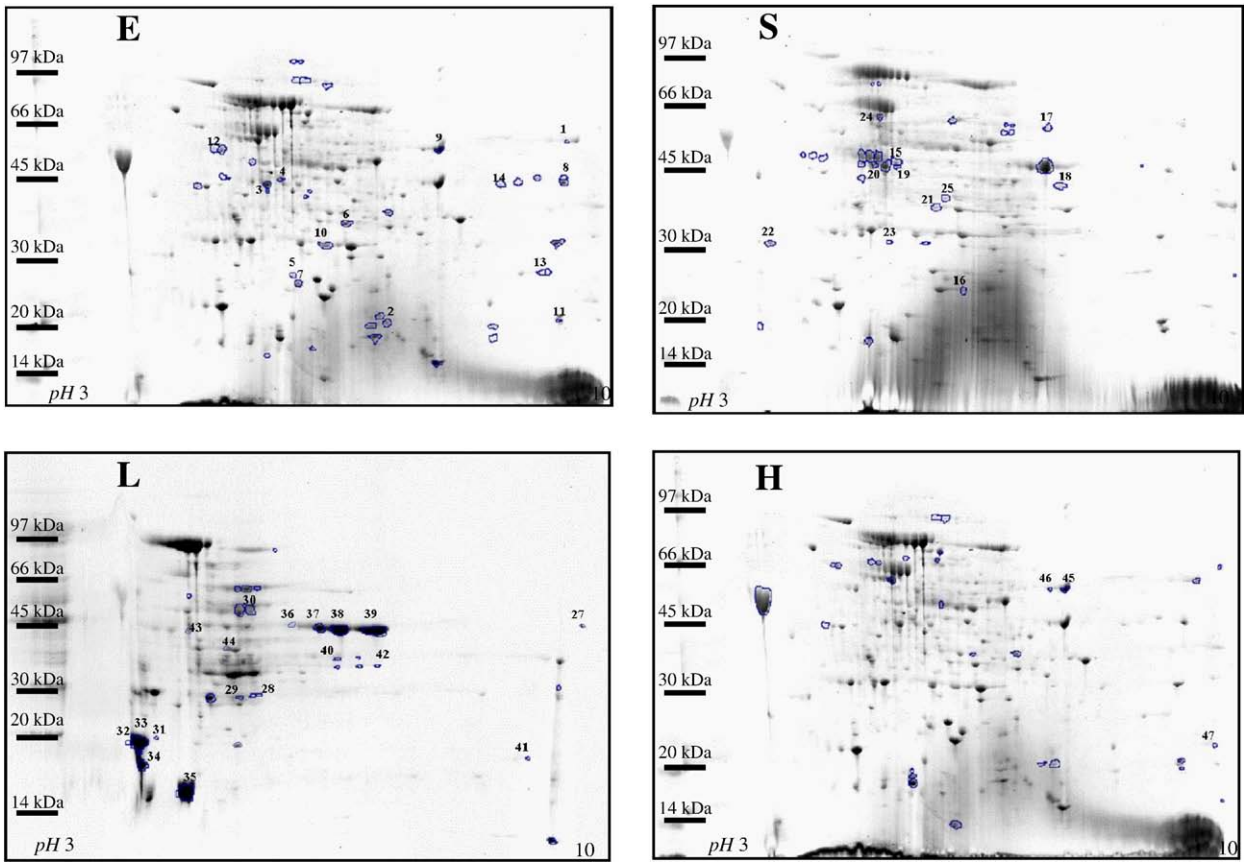

Fig. 2. Protein expression patterns of *G. diazotrophicus* showing spots (outlined in blue) specific to the growth phase (E or S) and culture condition (L or H), detected as described in the legend of Fig. 1. Spots identified by mass spectrometry were numbered according to Tables 1–4.

Those not identified by peptide mass fingerprint (PMFs), were submitted to tandem MS/MS fragmentation to generate information on their amino acid sequences.

In all, the amount of differentially expressed proteins confidently identified was: 31% (13 spots in 42) from E-phase grown cells, 34% (11 in 32 spots) from S-phase grown cells, 68% (19 in 28 spots) of those expressed under L condition and 10% (3 in 29 spots) of those specific to cells grown under H condition (Tables 1–4). The remaining proteins (86) could not be identified by MS or MS/MS, (possibly 28 spots), in some cases due to poor quality of mass spectra derived from low amount and/or mixture of proteins in the spots. However, in cases where good quality spectra were obtained data might have not matched sequence entries, most probably, due to poor representation in the protein sequence databases [50].

The 46 proteins identified in the present work are products of 38 different genes; therefore, many of them represent products expressed from a single gene. In fact, clusters of spots constitute a distinctive characteristic of *G. diazotrophicus* 2D profiles (Section 3.3, [38]). Some proteins presented MWr and pIr slightly distinct from the theoretical values (MWt and pIt) due to cleavage of signal peptides and/or to charge alterations resulting from post-translational modifications of the proteins.

3.4. Functional categorization of proteins identified

Tables 1–4 present the 46 proteins identified in the present work, grouped according to their putative functions in the cells.

3.4.1. Proteins induced during the exponential (E) and stationary (S) growth phases

The major group (4 proteins — 31%) differentially induced in *G. diazotrophicus* during the E growth phase was formed by those of DNA replication, recombination and repair processes (Table 1), whereas transporters and transferases grouped the majority of the proteins (8 proteins — 72%) expressed during the S-phase of the culture (Table 2). Other E-phase cell specific categories include proteins with roles in cell signaling, secretion and motility, nucleotide metabolism, lipid metabolism, and others. Proteins involved in energy and coenzyme metabolisms, on the other hand, were detected in both, E and S-phase cells (Tables 1–2). S-phase cells expressed additionally a protein of translation processes (Table 2).

Table 1
G. diazotrophicus proteins differentially expressed at least three fold during exponential (E) phase in comparison to stationary (S) growth phase

Spot	Locus	Protein	Method	Cob	PM	Score
Intracellular pH (pHi) homeostasis						
12	1566	Sensor protein KdpD	MS	41	16	3.17E+07
2	693	ATP synthase delta chain	MS	77	12	7.19E+04
Synthesis of carbon and energy storage compounds						
3	1240	Acetoacetyl-CoA thiolase	MSMS	44	18	3.84E+07
Cofactor metabolism						
8	1901	Molybdenum cofactor biosynthesis protein A	MSMS	35	11	1.79E+07
4	2953	Biotin synthase	MS	34	14	2.00E+07
6	3616	Conserved hypothetical	MS	60	16	1.63E+07
Nucleic acid metabolism						
9	3581	Phage integrase	MSMS	65	25	1.33E+07
10	29	Transposase	MSMS	22	12	1.56E+07
11	3613	Resolvase	MS	42	9	4.10E+04
1	3776	Cytosine-specific methyltransferase DdeI	MS	21	5	1.46E+07
5	2014	Nucleoside diphosphate kinase	MS	64	20	1.84E+07
Cell envelope biogenesis						
7	2522	UDP-glucuronate 5′-epimerase	MSMS	46	25	1.96E+07
Secretion and motility						
13	2154	Signal peptidase I	MS	38	11	1.33E+07

Cob: % of identified protein covered by matched peptides; PM: total number of peptides that matched the sequence in the database.

Table 2
G. diazotrophicus proteins differentially expressed at least three fold during stationary (S) phase in comparison to exponential (E) growth phase

Spot	Locus	Protein	Method	Cob	PM	Score
Transferases						
16	1778	Glucosamine-fructose-6-phosphate aminotransferase	MS	46	16	1.02E+07
18	2536	Glycosyl transferase	MSMS	54	10	3.76E+07
20	1186	Phosphoenolpyruvate-protein phosphotransferase	MS	37	18	1.49E+07
Transporters						
15	463	Extracellular solute-binding protein of ABC-type dipeptide transport system	MS	36	19	1.07E+07
24	2287	ABC transporter, ATP-binding protein	MSMS	31	14	1.89E+07
17	2628	Ribose transport ATP-binding protein RbsA	MS	40	18	3.50E+07
Nutritional stress						
25	2393	Leucyl-tRNA synthetase	MS	27	20	4.94E+07
19	809	tRNA(Ile)-lysidine synthase	MS	50	20	7.49E+07
21	3036	NADH-quinone oxidoreductase chain E	MS	32	10	4.79E+07
General metabolism						
22	1995	*N*-carbamoyl-l-amino acid hydrolase	MS	56	24	3.35E+07
Other						
23	1917	HemK-homolog protein	MS	34	9	2.48E+07

Cob: % of identified protein covered by matched peptides; PM: total number of peptides that matched the sequence in the database.

3.4.2. Proteins induced under low (L) and high (H) levels of combined nitrogen

The vast majority (7 proteins — 37%) of proteins induced differentially in *G. diazotrophicus* under L growth condition were those of regulatory pathways, followed by proteins of cell envelope biogenesis, nitrogen-fixation and coenzyme metabolism (Table 3). Minor groups comprehended proteins with roles in symbiosis, energy metabolism, secondary metabolism and translation.

Only three proteins were induced specifically in cells grown under H condition, with functions in amino acid and DNA metabolism, motility and secretion (Table 4).

3.5. Putative roles for the proteins induced during the exponential (E) growth phase

The majority of proteins expressed by *G. diazotrophicus* cells during the E growth phase were involved in DNA metabolism and energy production and conversion, in agreement with the high energy requirement of this phase of growth. Putative roles for some proteins expressed by the cells during E-growth phase are discussed below.

3.5.1. Intracellular pH (pHi) homeostasis

The initial pH of the medium (pHo) used to grow *G. diazotrophicus* in this study was 5.5, but during growth pH dropped to 2.7 and only recovered slightly at the stationary phase, under both, L and H culture conditions (results not shown). Therefore, many proteins expressed during the E growth phase might be involved in the maintenance of pHi.

The osmosensitive K^+ channel sensor histidine kinase KdpD (spot 12, Table 1, GDI1566), for instance, is a signal transduction protein, whose increased expression in E-phase cells, under the culture

Table 3
G. diazotrophicus proteins differentially expressed at least three fold under low (L) in comparison to high (H) concentration of $(NH_4)_2SO_4$

Spot	Locus	Protein	Method	Cob	PM	Score
Nitrogen-fixation						
35	450	Nitrogenase-stabilizing/protective protein NifW	MSMS	37	6	1.96E+04
41	430	FeMo cofactor biosynthesis protein NifB	MSMS	45	10	1.87E+04
44	2267	NifR3-like protein	MSMS	39	10	2.01E+09
Cell wall metabolism						
30	1624	Glycosyl transferase	MS	30	21	1.39E+07
43	1778	Glucosamine-fructose-6-phosphate aminotransferase	MSMS	30	14	1.10E+04
27	3185	Peptidoglycan synthetase FtsI	MS	41	16	2.92E+07
36	2185	Outer membrane lipoprotein	MSMS	46	7	1.28E+04
37	2185	Outer membrane lipoprotein	MSMS	41	5	1.02E+04
38	2185	Outer membrane lipoprotein	MSMS	37	4	1.99E+04
39	2185	Outer membrane lipoprotein	MSMS	46	6	1.79E+04
General metabolism						
29	2054	Dienelactone hydrolase	MS	42	13	4.38E+07
Regulators						
31	3301	Polyhydroxyalkanoate synthesis regulators	MS	44	4	6.45E+04
32	3301	Polyhydroxyalkanoate synthesis regulators	MS	52	7	9.95E+04
33	3301	Polyhydroxyalkanoate synthesis regulators	MS	48	5	7.11E+04
26	1273	Transcriptional activatory	MS	65	18	1.28E+07
28	682	Transcriptional regulator, GntR family	MS	46	9	1.07E+07
Others						
42	935	50S ribosomal protein L25	MSMS	39	11	2.56E+04
34	1720	Dihydrolipoyllysine-residue acetyltransferase	MS	51	7	3.55E+04
40	2955	Putative succinoglycan	MSMS	33	5	1.10E+07

Cob: % of identified protein covered by matched peptides; PM: total number of peptides that matched the sequence in the database.

condition used, could help pHi homeostasis [51]. In *Escherichia coli* and other bacteria, the two component system KdpD/KdpE regulates the expression of the *kdpFABC*, a system for high affinity K^+ transport, induced in response to K^+ limitation, osmotic upshock and low pHo. The influx of K^+ via Kdp-ATPase is accompanied by extrusion of H^+ with a subsequent increase in pHi [51,52]. The *E. coli* subunits KdpA, KdpB and KdpC are essential for Kdp-ATPase function *in vivo*, KdpF, on the other hand, is important for the stability of the complex [53]. *G. diazotrophicus* genome also encodes *kdpE* (GDI1567) and the essential Kdp-ATPase genes, *kdpABC* (GDI1563, GDI1564, GDI1565) therefore, we suggest that *G. diazotrophicus* Kdp-ATPase could contribute to pHi homeostasis, under the conditions studied.

The ATP synthase delta chain (*atpD*, spot 2, Table 1, GDI0693) is a subunit of the multisubunit enzyme (ATP synthase) that catalyzes the respiratory synthesis of ATP coupled to the influx of protons in mitochondria and chloroplasts. But, in bacteria, both, ATP synthesis and hydrolysis are catalyzed by the ATP synthase, depending on the cell's metabolic requirements [54]. In many acid-tolerant bacterial species it has been observed an increased activity of the enzyme in response to reduced pHo [55–58]. Higher ATP synthase activity under low pHo would cause ATP hydrolysis coupled to outward proton pumping, in order to keep constant pHi in these acid-tolerant species. We hypothesized that an increased expression of AtpD, under low pHo, would similarly contribute, at least in part, for the high acid tolerance of *G. diazotrophicus* exponential-phase cells.

3.5.2. Synthesis of carbon and energy storage compounds

The acetoacetyl-CoA thiolase (spot 3, Table 1, GDI1240) is a ubiquitous enzyme that catalyzes the reversible thiolytic cleavage of 3-ketoacyl-CoA into acyl-CoA and acetyl-CoA and can be found as multiple isozymes in prokaryotes [59]. In many bacterial species acetoacetyl-CoA thiolases (*phaA*, EC: 2.3.1.9) are involved in the biosynthesis of polyhydroxyalkanoic acids (PHAs), storage polyester compounds deposited as inclusions in the cytoplasm [60]. Genes coding for the enzymes involved in the biosynthesis of PHA have been studied in a broad range of bacteria, including endophytic nitrogen-fixing species such as *Herbaspirillum seropedicae* [61], *A. vinelandii* [62], *Bradyrhizobium japonicum* and *Rhizobium* sp. [63] and *Sinorhizobium meliloti* [64] that use PHA as a main carbon store. However, a relationship between PHA metabolism and the nitrogen-fixation process in these bacteria has not been established. The most widely distributed PHA biosynthetic pathway in bacteria includes a b-ketothiolase (*phaA*), which condenses two molecules of acetyl-CoA to give acetoacetyl-CoA, a acetoacetyl-CoA reductase (*phaB*), and a PHA synthase (*phaC*) [60,65,66]. Nothing is found in the literature on the metabolism of PHAs in *G. diazotrophicus*, however, the differential expression of a putative *phaA* by cells at the exponential growth phase, as occur in other bacterial species [66], suggests that the bacterium might be able to store carbon and energy in the form of PHA. Interestingly, a search in the *G. diazotrophicus* genome database for other genes required for PHA synthesis leads us to three other loci: GDI3304, annotated as *phbC* for a putative poly-beta-hydroxybutyrate polymerase, GDI3305 for a putative acetoacetyl-CoA reductase (*phaB*) and GDI3301, for a polyhydroxyalkanoate synthesis regulator (*phaR*). A putative PhaR was, in fact, identified in the present study as one of the proteins differentially expressed under L growth condition (spots 31–33, Table 1, refer to item General metabolism). Therefore, it is well possible that the acetoacetyl-CoA thiolase, identified in this work, could function as a PhaA, which along with a putative PhaB (GDI3305), PhaC (GDI3304) plus the putative regulator PhaR (GDI3301) would enable production of PHAs via acetyl-CoA.

3.5.3. Cofactor metabolism

Molybdenum (Mo) exhibits biological activity when in the form of Mo-cofactor (Moco) that consists of a Mo atom covalently bound to one or two dithiolates attached to a unique tricyclic pterin moiety commonly referred to as molybdopterin (MPT) [67]. Moco-containing enzymes catalyze important redox reactions. Some of them are vital for nitrogen assimilation, phytohormone synthesis, purine catabolism and stress response [68]. With the exception of bacterial nitrogenases, where Mo is part of the FeMo cofactor (FeMoco), MTP or a modified MTP is the active site at the catalytic center of all other Mo-enzymes [68]. Some proteins of the dimethyl sulfoxy (Me_2SO) reductase family in *Rodobacter capsulatus* and *E. coli* require a MTP form of MoCo covalently bound to GMP (molybdopterin guanine dinucleotide, bis-MGD), produced via reactions catalyzed by MobA and MobB [69]. In the present study we managed to identify the MTP biosynthesis protein A (*moaA*, spot 8, Table 1, GDI1901), which, in *E. coli* and other bacterial species catalyzes the first step in molybdenum cofactor biosynthesis, the conversion of 5¢-GTP to an oxygen-sensitive tetrahydropyranopterin. MoaA is a *S*-adenosyl-L-methionine (SAM)-dependent enzyme of the Radical SAM superfamily proteins, that

Table 4
G. diazotrophicus proteins differentially expressed at least three fold under high (H) in comparison to low (L) concentration of $(NH_4)_2SO_4$

Spot	Locus	Protein	Method	Cob	PM	Score
DNA metabolism						
45	3581	Phage integrase	MS	45	25	6.03E+06
Protein metabolism						
46	1990	Peptidase M24	MS	22	11	1.67E+07
47	2154	Signal peptidase I	MS	38	11	1.33E+07

Cob: % of identified protein covered by matched peptides; PM: total number of peptides that matched the sequence in the database.

generate radical species by the reductive cleavage of SAM through an unusual Fe–S center [68]. By searching the *G. diazotrophicus* genome database, we further identified all the genes of the biosynthetic pathway of MTP, *moaC*, *moaE*, *moaD*, *moeA*, *moeB* and *moaB*/*mogA* (respectively loci GDI1938, GDI1822, GDI1821, GDI1924, GDI1293 and GDI0771). Additionally, the *G. diazotrophicus* genome contains *mobB* and *mobA* genes (loci GDI0166 and GD0167, respectively), suggesting that bis-MGD might be required for the activity of some of the bacterium Mo-dependent reductases.

Biotin synthase (*bioB*, spot 4, Table 1, GDI2953), similarly to MoaA (GDI1901), is a member of the emerging Radical SAM superfamily due to the presence of a conserved CX3CX2C motif (C is cysteine and X is any amino acid), a Fe–S cluster [70]. In *E. coli*, BioB catalyzes the last step of the biosynthesis of biotin (vitamin H), which requires, additionally, BioF, BioC, BioD, BioA, BioH [71]. However, in the nitrogen-fixing symbiotic *S. meliloti* and other rhizobium species, BioB might perform a distinct role, since, in these organisms, several key genes of biotin biosynthesis are absent or nonfunctional [72]. Interestingly, *G. diazotrophicus*, similarly to *E. coli* and differently to some nitrogen-fixing symbiotic organisms, possesses, besides *bioB* (GDI2953), a cluster of other genes of the biotin biosynthetic pathway, corresponding to the loci GDI1919 (*bioA*), GDI1920 (*bioF*), GD1922 (*bioCD*), suggesting that it might be able to produce biotin when required.

Another protein identified among the expressed differentially in E-growth phase cells is the product of GDI3616 (spot 6, Table 1) that has been annotated as an uncharacterized conserved protein of unknown function. However, FASTA analysis and searches in the Pfam database of protein domain families showed that GDI3616 product has sequence identity and motifs common to the bacterial glutathione-dependent formaldehyde-activating enzyme, Gfa (data not shown). Gfa catalyzes the condensation of formaldehyde and glutathione to *S*-hydroxymethylglutathione, the first step of the formaldehyde-detoxification pathway, and all known members of the family contain 5 strongly conserved cysteine residues. Gfa from *Paracoccus denitrificans*, one of the best studied, shows 41.9% identity and 74.2% similarity to the product of GDI3616 and shares with it the conserved cysteine cluster [73,74]. Therefore, there are reasons to believe that the product of GDI3616 is indeed a member of the bacterial Gfa family.

3.5.4. Nucleic acids metabolism

Three proteins specific to the E-phase grown cells (Table 1) are known to be involved in non-homologous DNA recombination processes [75]: a member of "phage integrase" family (spot 9, GDI3581), a putative resolvase (spot 11, GDI3613) and a transposase (spots 10, GD0029). These enzymes are generally encoded by genes of chromosomally integrated plasmids, phage, transposons and insertion sequences [76], suggesting they have been acquired by horizontal gene transfer [77].

Cytosine-specific methyltransferase DdeI (spot 1, Table 1, GDI3776), which specifically methylates cytosines in DNA [78], is, most probably, a member of a restriction/modification system of *G. diazotrophicus*, a defense tool against foreign genetic material.

The nucleoside diphosphate kinase (*ndk*, spot 5, Table 1, GDI2014), on the other hand, catalyzes the last reaction of nucleoside triphosphate (NTP) biosynthesis, the reversible transfer of γ-phosphates between nucleoside di- and triphosphate at very high efficiencies through a conserved histidine residue [79]. NDK shows little substrate specificity, utilizing the ribose and deoxyribose forms of both purine and pyrimidine NDPs as substrates, thus functioning as a housekeeping enzyme in the maintenance of nucleotide pools for DNA and RNA synthesis [80]. Bioinformatics analysis of *G. diazotrophicus* NDK molecule shows that it shares 53.1% identity (80.4% similar) in sequence with its counterpart from *E. coli* and has also the His117 at the active site and other residues (Arg87, Thr93, Arg104, Asn114, Ser119 and Glu128) conserved among all known NDKs [81,82]. Therefore, the potential metabolic roles of NDK in *G. diazotrophicus* might be those involved in growth and cell signaling [83], highly active processes in cells at the E-phase of culture.

3.5.5. Other functions

Other proteins expressed differentially during E growth phase included: the UDP-glucuronate 5¢-epimerase (spot 7, GDI2522), an enzyme that plays roles in the cell envelope biogenesis, carbohydrate transport and metabolism, and a putative signal peptidase type I, SPaseI (*lepB*, spot 13, Table 1; spot 47, Table 4, GDI2154). In all the bacterial species analyzed so far, SPaseI has been shown to be essential for cell viability [84].

3.6. Putative roles for the proteins induced in the stationary (S) growth phase

In response to nutrient starvation, in their natural habitat or in culture, bacterial cells enter the S-growth phase, a physiological state characterized by a transcriptional switch, whereby many genes are repressed and others are induced. Two major groups of proteins specifically induced in *G. diazotrophicus* cells during S-phase of growth were the transporters and transferases. At this phase of culture cells are not much involved in neosynthesis, but rather, with the ability to respond to nutrient starvation, due to reduced anabolic activities and the arrest of energy generating systems. Putative roles for the proteins expressed by S-growth phase cells are discussed below.

3.6.1. Transferases

One of such proteins is the glucosamine-fructose-6-phosphate aminotransferase (GlmS, spot 16, Table 2, GDI1778), which catalyzes the formation of glucosamine 6-phosphate from fructose 6-phosphate and glutamine, as discussed below in Section 3.7.

A glycosyl transferase (spot 18, Table 2, GDI2536) and a phosphoenolpyruvate phosphotransferase (EI protein, product of *ptsI*, spot 20, Table 2, GDI1186) detected specifically in cells during the S-growth phase are also sugar metabolism proteins. Phosphoenolpyruvate phosphotransferase/EI protein is a sugar phosphotransferase system (PTS) protein that catalyzes the uptake and concomitant phosphorylation of numerous carbohydrates [85].

3.6.2. Transporters

Three transporters of the ABC (ATP-Binding Cassette) family were found up-regulated in S-phase cells. One of them, DppA, is the extracellular solute-binding protein of the ABC-type dipeptide transport system (*dppA*, spot 15, Table 2, GDI0463), also super expressed by *Bacillus subtillis* cells under ammonium starvation [86] and by *Streptococcus pyogenes* in response to amino acid starvation [87]. In enteric bacteria and in the N_2-fixing *Rhizobium leguminosarum*, DppA uptakes into the cytoplasm exogenous dipeptides and also δ-aminolevulinic acid (ALA), a heme precursor structurally similar to dipeptides [88,89]. In addition to its role in transport, in *Rhodobacter sphaeroides* DppA acts as a molecular chaperone protecting the periplasmic acid-unfolded dimethyl-sulfoxide reductase (DMSOR) from aggregation [90]. These findings suggest that, besides working as a dipeptide transporter when amino acids are limited, *G. diazotrophicus* DppA might also act as a periplasmic chaperone, somehow contributing to the acid resistance of the starved S growth phase cells.

The other two ABC family proteins were putative ATP-binding subunits of transport systems (spots 17 and 24, Table 2, GDI2628 and GDI2287, respectively). GDI2628 is one of the four *rbsA* genes we have found in the *G. diazotrophicus* genome database. Its product is the ATP-binding protein (RbsA) of the ribose affinity ABC transporter system, also expressed by *E. coli* under nutrient deficiency [91] and by *Mycobacterium smegmatis* during the S-growth phase [92]. Therefore, the differential expression of GDI2628 gene product by stationary

phase *G. diazotrophicus* cells might indicate that they are able to transport sugar into the cytoplasm to cope with nutritional stress.

3.6.3. Nutritional stress

G. diazotrophicus overexpression of a leucyl tRNA synthetase (*leuS*, spot 25, Table 2, GDI2393) and of a tRNA (Ile)-lysidine synthase (*tilS*, spot 19, Table 2, GDI0809) involved, respectively, in the synthesis of $tRNA^{Leu}$ and $tRNA^{Ile}$ during the S-growth phase of culture is probably a response to amino acid depletion, as happens in *E. coli* and other bacterial species under limiting supply of these nutrients [93,94].

3.6.4. General metabolism

NADH-quinone oxidoreductase (*nuoE*, spot 21, Table 2, GDI3036), on the other hand, is a cation-pump of the respiratory complex I (NDH I-type) that, in several bacterial species, couples the transfer of electrons from NADH to ubiquinone with the translocation of cations across the membrane [95,96]. In *A. vinelandii* NDH I activity is increased in cells grown under O_2-depleted conditions, the effect being independent of the NH_3 availability [97–99]. Thus, the expression of a subunit of this respiratory complex by stationary *G. diazotrophicus* cells is consistent with the low level of O_2 in the culture media in this phase of growth. In both *S. typhimurium* and *E. coli*, the subunits of NADH I are encoded by the genes of the *nuo* locus, which includes a total of 14 *nuo* genes (*nuo* A–N) [100,101]. Interestingly, in *G. diazotrophicus* a *nuo* gene cluster of 14 genes (GDI2459–2471) is present in the genome, probably as an operon, suggesting that the bacterium has the potential to use the NDH I-type respiratory complex when required.

Other protein identified in S-phase cells was an *N*-carbamoyl-L-amino acid hydrolase also known as L-*N*-carbamoylase (spot 22, Table 2, GDI1995), which catalyzes the hydrolysis of the *N*-carbamoyl-L-α-amino acid to the corresponding free amino acid. Its expression by cells at the stationary phase of culture might also be a response to the amino acid depletion. L-*N*-carbamoylase is specific to L-amino acids, substances used by the food, pharmaceutical and cosmetic industries [102]. Therefore, L-*N*-carbamoylases from different sources have been used to produce optically pure L-amino acids from D, L-5 monosubstituted hydantoin by a process known as hydantoinase [103]. L-*N*-carbamoylases from many bacterial species have been purified and their properties were evaluated for commercial application in the hydantoinase process [104–106]. A multiple alignment including deduced acid sequences of GDI1195 and of well characterized L-*N*-carbamoylases from distinct bacterial sources showed that GDI1195 product shares with them conserved motifs and the five amino acid residues that form the zinc-binding center, the active site of the enzyme [104]. Therefore, the possibility that the L-*N*-carbamoylase of *G. diazotrophicus* (GDI1195) could have a biotechnological application cannot be overlooked.

3.6.5. Other functions

G. diazotrophicus S-phase cells expressed additionally a putative HemK-homolog, called RF MTase (*hemk*-homolog, spot 23, Table 2, GDI1917) that methylates both release factors, RF1 and RF2, in *E. coli* [107]. Analysis of deduced amino acid sequence of the putative RF MTase (GDI1917) shows it shares 37.8% identity (62.6% similar) with its homolog from *E. coli* K12 and presents amino acid sequence motifs conserved in other proteins of the HemK family (data not shown). In many bacterial species *hemk* gene and *prfA* (the gene for RF1) are next to each other in the chromosomes of the cells. Interestingly, in *G. diazotrophicus*, *prfA* (GDI1915) and *hemk* (GDI1917) are very close, in line with the hypothesis that GDI1917 encodes indeed a RF MTase [107]. A recent study on the role of *E. coli* methylation of RF1 and RF2 showed they are required for normal translation termination *in vivo* of proteins that are important for growth under poor carbon sources. Some of such proteins are encoded by genes that may have poor termination signals and, consequently, require efficient RFs [108]. The expression of a RF MTase by *G. diazotrophicus* cells at the stationary phase of culture, might be an indication that similar mechanism operates in the bacterium to allow growth under nutritional stress.

3.7. Putative roles for the proteins induced under low (L) level of $(NH_4)_2SO_4$

3.7.1. Nitrogen-fixation process related proteins

G. diazotrophicus was previously shown to fix nitrogen when in LGIM with starter concentration of 1 mM $(NH_4)_2SO_4$, under low % of O_2 [17]. In the present work, similar growth condition was used and we observed that, in fact, the majority of the proteins identified among those specifically induced in the cells were related to the nitrogen-fixation process. However, neither the proteins of the nitrogenase complex, NifH, NifD and NifK nor NifA, the transcriptional regulator of *nifHDK* operon, were detected. *G. diazotrophicus* NifA shows high similarity to its homologs from *B. japonicum* and *R. meliloti* [23] and degrades when oxygen-exposed [109,110]. More recently, it has been suggested that the *G. diazotrophicus* nitrogenase component Gd2/MoFe-protein also can be damaged by O_2 [29]. NifA and Gd2 activities depend on the cofactor FeMoco, which is extremely air sensitive [111]. Once this essential metal cofactor is oxidized the conjugated proteins are inactivated and then degraded [110]. Thus, we reasoned that degradation of such O_2-sensitive proteins might have occurred during the many steps of the 2D-GE sample preparation (see Materials and methods, Section 2.2, "Sample preparation and 2D gel electrophoresis").

Among the proteins detected in *G. diazotrophicus* cells grown under L condition with association to the nitrogen-fixation process we found: NifB (spot 41, Table 3, GDI0430), the nitrogenase-stabilizing/protective protein NifW (spot 35, Table 3, GDI0450) and a NifR3-like protein (spot 44, Table 3, GDI2267). NifB, is involved in the biosynthesis of FeMoCo, essential for the activity of the nitrogenase complex and other cellular functions [112]. NifW, on the other hand, may protect the nitrogenase FeMoco protein from oxidative damage [113]. NifB and NifW genes are both located in the nitrogen-fixation (*nif*) cluster of *G. diazotrophicus*, which encodes also the nitrogenases NifH, NifD, NifK and its accessory proteins. Proteins encoded by the *nif* cluster, namely, NifA, NifS, NifX and FixA were also identified in *B. japonicum* grown in microaerobiosis [114]. NifR3 from many bacteria has been annotated as a high conserved protein of unknown function, however in *R. capsulatus* its expression increases approximately five fold under nitrogen limitation, suggesting that it also might play a role in the nitrogen-fixation process [115]. Interestingly, *nifR3* is within a nitrogen metabolism gene cluster in *G. diazotrophicus* genome (*nrfA*, GDI2262; *ntrX*, GDI2263; *ntrY*, *GDI2264*; *ntrC*, GDI2265, *ntrB*, GDI2266 and *nifR3*, GDI2267) and, as in *Azospirillum*, *Rhodospirillum* and *Rhizobium* species, *nifR3* (GDI2267) is immediately upstream *ntrBC* (GDI2265 and GDI2266, respectively) [116].

3.7.2. Cell wall metabolism

Two proteins found exclusively in L grown cells were glucosamine-fructose-6-phosphate aminotransferase (*gmlS*, spot 43, Table 3, GDI1778) and a glycosyl transferase (spot 30, Table 3, GDI1624). GDI1778 gene product presents high sequence identity to many bacterial GmlS, which catalyses the formation of glucosamine 6-phosphate from fructose 6-phosphate and glutamine and, also, to the *Rhizobium* species NodM (52.1% identity, 79.4% similar), a protein essential for nodule formation [117–119]. In rhizobia *nodM* mutation decreased symbiotic nitrogen-fixation efficiency and induced morphological abnormalities in the bacteroids, suggesting that NodM is essential for the bacterial symbiont maturation [120]. *G. diazotrophicus* is a non-nodulating bacterium, consequently, the putative GmlS might be part of the wall biogenesis apparatus. The protein glucosyl transferase (GDI1624) is apparently involved in aminosugar

metabolism and might, as well, play roles in the biosynthesis of the wall.

Another protein of the cell envelope of *G. diazotrophicus* differentially expressed by cells under L growth condition was a putative peptidoglycan synthetase FtsI (spot 27, Table 3, GDI3185), also known as penicillin-binding protein 3 (PBP3) [121,122]. PBP is a family of proteins involved in the last stages of the synthesis of the murein [123] and *E. coli* PBP3 specifically catalyzes peptide cross-bridges of the septal cell wall peptidoglycan during cell division [124]. It has been shown that the levels of N, specially in the form of ammonium, induce morphological changes in *G. diazotrophicus* cells [4]. Therefore, it is possible that NodM/GlmS (spot 43, GDI1778), glycosyl transferase (spot 30, GDI1624) as well as, the outer membrane lipoprotein (spots 36–39, Table 3, GDI2185) and FtsI/PBP3 (spot27, GDI3185) play roles in the reshaping of the *G. diazotrophicus* cell wall during growth under nitrogen limited condition.

3.7.3. General metabolism

A dienelactone hydrolase, DHL (spot 29, Table 3, GDI2054) was also expressed by cells under L level of $(NH_4)_2SO_4$. It is a detoxification enzyme responsible for the degradation of chlorinated aromatic compounds in *Pseudomonas*, *Rhodococcus opacus* and *Ralstonia eutropha* [125–127]. Its differential expression in *G. diazotrophicus* cells under L condition suggests it might work in the detoxification of noxious products and that *G. diazotrophicus* has the potential to deal with aromatic compounds produced in abundance by plants in the symbiotic stage of life [128]. For the nitrogen-fixing rhizobia polyphenols in the rhizosphere function as signaling molecules to initiate plant–host infection, once inside the nodules the bacteroids metabolize these aromatic compounds to use as carbon source [114]. However, no such observation has been reported for *G. diazotrophicus*.

3.7.4. Regulators

Other proteins expressed by L condition grown cells were a putative polyhydroxyalkanoate (PHA) synthesis repressor (*phaR*, spots 31–33, Table 3, GDI3301) involved in the control of PHA biosynthesis, as discussed above (Section 3.5, "Synthesis of carbon and energy storage compounds") and a transcriptional activator protein (spot 26, Table 3, GDI1273). Besides having a sequence-specific DNA-binding motif (helix–turn–helix) the GDI1273 gene product has also a putative cAMP binding site, suggesting that it might be a transcription regulator of the Crp family. However, it shows high sequence similarity to the oxygen-responsive transcriptional regulators FNR (fumarate nitrate reductase regulator), which under anoxic conditions is in the active state. We thus hypothesized that the product of GDI1273 is a member of the CRP-FNR superfamily [129] that is, apparently, required for growth of *G. diazotrophicus* cells under deprivation of combined nitrogen and low level of O_2.

A putative member of the transcriptional regulator GntR family (spot 28, Table 3, GDI0682) was found in significant higher levels in *G. diazotrophicus* cells grown under L than in those under H condition. GntR belongs to a family of bacterial helix–turn–helix DNA-binding transcriptional regulators that comprises four subfamilies, with more than 270 members, distributed among the most diverse bacterial groups. A great number of them are of unknown function, but others, are involved in the regulation the most various biological processes [130].

3.7.5. Other functions

The putative dihydrolipoyllysine-residue acetyltransferase, also called pyruvate dehydrogenase E2 component (*aceF*, spot 34, Table 3, GDI1720) was identified in cells deprived of combined nitrogen. AceF (GDI1720) is one of the three components of the pyruvate dehydrogenase that catalyses the oxidative decarboxylation of pyruvate to acetyl-CoA and CO_2 via a series of the enzyme-bound intermediates. The enzyme has been well studied in *E. coli* whose AceF subunit is an oxidation-responsive factor of 66,095 kDa. However, it has been detected as smaller components by SDS-PAGE suggesting that is liable to cleavage [131]. The putative AceF-homolog of *G. diazotrophicus* identified in this work has the theoretical molecular weight of 38,708 kDa, but was detected on the 2D gel as a protein of about 18 kDa, an indication that, similarly to its *E. coli* counterpart, it has been submitted to a cleavage mechanism [131]. It is already known that microaerobic diazotrophs possess numerous restriction strategies to protect nitrogenases from inactivation by oxygen. Therefore, the expression of an *E. coli* AceF-homolog in *G. diazotrophicus* nitrogen-fixing cells could be one of the protection mechanisms used by the bacterium against oxidative inactivation of the nitrogenases.

The 50S ribosomal protein L25 (spot 42, Table 3, GDI0935) was also super expressed by cells under L growth condition.

3.8. Putative roles for the proteins induced under high (H) level $(NH_4)_2SO_4$

3.8.1. Protein and DNA metabolisms

Only three proteins were differentially induced in *G. diazotrophicus* grown under H condition, an integrase, (spot 45, Table 4, GDI3581), probably associated to DNA recombination [76] and two peptidases, namely, the peptidase M24 (spot 46, Table 4, GDI1990) and peptidase signal I, SPaseI (spot 47, Table 4, GDI2154). M24 and SPaseI peptidases specifically remove the N-terminal amino acids from peptides and might function in protein export [84]. Gram-negative bacteria typically have only one chromosomal type I SPase, exceptions are *P. aeruginosa* with two and *B. japonicum* with three SPases [132]. A search in the genome database of *G. diazotrophicus* leads us to two proteins annotated as putative type I SPases, the products of GDI2154 (identified in this work) and of GDI3512, suggesting that the bacterium might be among the atypical type I peptidase signal I producers.

4. Conclusion

This study can be regarded as an additional step towards establishing the cytoplasmic proteome of *G. diazotrophicus*. The bacterium was cultured in a bioreactor under low and high concentrations of combined nitrogen, controlled levels of oxygen and varying pH, conditions that allowed the expression of special sets of proteins. These were analyzed by 2D-GE and a reasonable number of protein spots were detected and used to construct master gels representing the proteomes of exponential (E) and stationary (S) phase cells under low (L) or high (H) levels of $(NH_4)_2SO_4$. Comparison of master gels showed 131 proteins (42E+32S+ 29H+28L) expressed differentially by *G. diazotrophicus*, from which 46 were identified. The groups of proteins differentially induced during the E growth phase were those expected to be functional in metabolically active bacterial cells. Particularly interesting was the increased expression of proteins that could play roles in pHi homeostasis, suggesting that the high acid tolerance of the exponential-phase *G. diazotrophicus* cells is, apparently, mediated by the products of a special set of genes. Cells from S-phase of growth, on the other hand, survived nutrient starvation and stress mainly by activating many transporters and transferases and other proteins to help the cells to cope with low levels of amino acids and carbon sources. The vast majority of proteins induced differentially by cells under low (L) level of $(NH_4)_2SO_4$ comprised those with roles in cell wall biosynthesis, confirming previous observation that the level of N, especially in the form of ammonium, affects the morphology of *G. diazotrophicus* cells [4]. Nitrogen-fixation related proteins were also identified in cells grown under L condition, as well as proteins involved in the protection of the nitrogenases complex against oxidative damage, in agreement with the results of other studies, [4,17,131]. Moreover, the results of the present work permitted to deduce some novel metabolic pathways for *G. diazotrophicus* from the sequenced genome directly, based on the tendency of functionally related bacterial genes to cluster along the chromosome. Therefore,

this study and our previous work [38] represent landmark accomplishments in the functional proteomic analysis of *G. diazotrophicus*.

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