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ORIGINAL ARTICLE

LC–MS/MS proteomic analysis of starved *Bacillus subtilis* cells overexpressing ribonucleotide reductase (*nrdEF*): implications in stress-associated mutagenesis

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Abstract The non-appropriate conditions faced by nutritionally stressed bacteria propitiate error-prone repair events underlying stationary-phase- or stress-associated mutagenesis (SPM). The genetic and molecular mechanisms involved in SPM have been deeply studied but the biochemical aspects of this process have so far been less explored. Previous evidence showed that under conditions of nutritional stress, non-dividing cells of strain *B. subtilis* YB955 overexpressing ribonucleotide reductase (RNR) exhibited a strong propensity to generate true reversions in the *hisC952* (amber), *metB5* (ochre) and *leuC425* (missense) mutant alleles. To further advance our knowledge on the metabolic conditions underlying this hypermutagenic phenotype, a high-throughput LC–MS/MS proteomic analysis was performed in non-dividing cells of an amino acid-starved strain, deficient for NrdR, the RNR repressor. Compared with the parental strain, the level of 57 proteins was found to increase and of 80 decreases in the NrdR-deficient strain. The proteomic analysis revealed an altered content in proteins associated with the stringent response, nucleotide metabolism, DNA repair, and cell signaling in amino acid-starved cells of the Δ*nrdR* strain. Overall, our results revealed that amino acid-starved cells of strain *B. subtilis* Δ*nrdR* that escape from growth-limiting conditions exhibit a complex proteomic pattern reminiscent of a disturbed metabolism. Future experiments aimed to understand the consequences of disrupting the cell signaling pathways unveiled in this study, will advance our knowledge on the genetic adaptations deployed by bacteria to escape from growth-limiting environments.

Keywords *Bacillus subtilis* · Ribonucleotide reductase · Amino acid starvation · Stress-associated mutagenesis

Introduction

Upon detecting inappropriate conditions for growth, *B. subtilis* activates a diversity of cellular responses controlled by global regulators, including the synthesis of spores, the general stress response, competence and stress-associated mutagenesis (Kovác 2016; Ambriz-Aviña et al. 2016). In replicating bacteria, the proper metabolic conditions support the correct operation of the repair machinery to faithfully eliminate genetic insults, thus preserving the fidelity of its genetic information (Barajas-Ornelas et al. 2014). In contrast, when growth and/or replication ceases bacteria frequently process DNA insults in an error-prone manner conferring on microorganisms a chance to overcome growth arrest and to proliferate; this process is called stationary-phase- or stress-associated mutagenesis (SPM) (Sung and Yasbin 2002; Gómez-Marroquín et al. 2016). In distinct bacteria, including *B. subtilis*, the transcriptional

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factor NrdR regulates the expression levels of *nrdEF* encoding ribonucleotide reductase (RNR) in response to nucleotide concentration (Rodionov and Gelfand 2005; Grinberg et al. 2006; Torrents et al. 2007; Case et al. 2011). RNR catalyzes the conversion of ribonucleotide diphosphates (NDPs) to the corresponding deoxyribonucleotide triphosphates (dNTPs). dNTPs are necessary for DNA replication and repair; therefore, this enzymatic reaction is considered a key step for nucleotide metabolism.

A recent report revealed that a null *nrdR* (*ytc*G) mutant of strain *B. subtilis* YB955 [*hisC952* (amber) *metB5* (ochre) *leuC427* (missense)] increased the production of His^+ Met^+ and Leu^+ prototrophs in reference to the NrdR-proficient strain, under conditions of prolonged nutritional stress (Castro-Cerritos et al. 2017). Interestingly, in reference to the parental strain; in starved cells of the Δ*nrdR* strain, the expression levels of *nrdEF* increased several fold and produced an elevated proportion of colonies with a His^+ Met^+ Leu^+ phenotype. Whereas a great proportion of the His^+ and Met^+ revertants have shown to be produced from suppression mutations in YB955 (Sung and Yasbin 2002), in the NrdR-deficient strain the His^+ Met^+ and Leu^+ prototrophs arose from true reversions in the three mutant alleles (Castro-Cerritos et al. 2017). Previous results have reported a positive correlation between levels of RelA, which controls the stringent response, and the rates of reversion to distinct amino acid auxotrophies in *Escherichia coli* and *B. subtilis* (Wright 1996; Rudner et al. 1999). Altogether, these results point to the existence of an interaction between the stringent response, dNTP metabolism, and mutagenesis.

A high-throughput LC–MS/MS screening analysis of the protein content in amino acid-starved cells of *B. subtilis* overexpressing RNR was performed in this work to gain further knowledge on the metabolic conditions leading to a hypermutagenic phenotype in this microorganism.

Methods

Cell harvesting and protein extraction

Bacillus subtilis strains YB955 (*hisC*952 *metB*5 *leuC427 xin-1 Spβ*^{SENS}) (Sung and Yasbin, 2002) and its derivative PERM1202 (YB955, Δ*ytcG::neo;* Neo^R) (Castro-Cerritos et al. 2017) were propagated in 20 ml of Penassay broth (PAB) (antibiotic A3 medium; Difco Laboratories, Sparks, MD) to 90 min after T_0, namely the time point in the culture when the slopes of the logarithmic and stationary phases of growth intercepted. The cultures were centrifuged at 10,000×*g* for 10 min and resuspended in 10 ml of 1X Spizizen Minimal Salts (SMS) (Spizizen 1958). Aliquots of 100 µl were plated onto Spizizen minimal medium with growth-limiting amounts of histidine, methionine, and leucine. (SMM; 1.5% agar, 1X SS, 0.5% glucose, 50 µg/ml isoleucine, 50 µg/ml glutamic acid, 200 ng/ml histidine, 200 ng/ml methionine and 200 ng/ml leucine). At day five of incubation, the non-revertant background was scraped off the agar medium with 2 ml of 1X SS and recovered by centrifugation. Cell lysis was carried out by initial sample boiling and repeated freeze/thaw cycles combined with mechanical disruption with glass beads in aqueous medium, cell debris and beads were removed by centrifugation (16,000×*g* for 10 min at 4 °C). The protein content in the cell-free extract was determined by the bicinchoninic acid method, using the Micro BCA Protein Assay Kit (Thermo Scientific; Waltham, MA, USA). Three independent biological replicates were used for protein extraction and posterior LC–MS/MS analysis.

Sample preparation for proteomic analysis

Pre-treatment of protein extracts and hydrolysis were carried out as was previously published (Karasinski et al. 2017) with some variations. Briefly, each sample (200 µg) was solubilized in 100 µl of 100 mM ammonium bicarbonate buffer (pH 8) and reduced with 20 µl of 50 mM DTT during 1 h at room temperature. Samples were then alkylated with 20 µl of 100 mM iodoacetamide at room temperature protected from light and reduced again with 20 µl of 50 mM DTT. For hydrolysis, 20 µl of trypsin (0.25 µg/µl) were added to samples and proteolysis was allowed to proceed at 37 °C overnight; the reaction was stopped with 20 µl of 50% (v/v) formic acid. The samples were conserved at −20 °C and before chromatographic analysis centrifuged at 18,000×*g* (20 min/4 °C).

Liquid chromatography and mass spectrometric (LC–MS/MS) analysis

Protein digests were analyzed by capLC-ESI-QTOF-MS using an Ultimate 3000 RLSC system (Thermo Scientific Dionex) coupled to a maXis Impact ESI-QTOF mass spectrometer (Bruker Daltonics), as previously described (Karasinski et al. 2017), with slight modifications. In brief, 5 µl of tryptic peptides were loaded onto a trap column (Acclaim PepMap100 C18, 5 µm, 100 Å, 300 µm i.d. × 5 mm, Thermo Scientific) using 0.1% (v/v) formic acid and acetonitrile 2% (v/v) at a flow rate of 15 µl/min during 2 min. Afterwards, the flow was switched to the reversed phase capillary column Halo C18 (150 × 0.3 mm, 2.7 µm). The separation was carried out setting the column thermostat at 40 °C and a flow rate of 3 µl/min using two mobile phases (A—0.1% aqueous formic acid; B—0.1% formic acid in acetonitrile) with a linear gradient from 2 to 80% B during 120 min. The column exit was connected to

ESI source and the lock-mass standard *m/z* 1221.9907 was introduced constantly to the ion source. ESI was operated in a positive mode with an ion spray voltage of 4500 V, vend plate offset 500 V, dry gas 4 L/min, drying temperature 180 °C and nebulizing gas pressure 0.4 bar. MS data were obtained with acquisition rate 2 Hz within the *m/z* range 300–2000. MS/MS data were acquired using Bruker's "Impact—Protein identification-Instant Expertise—Auto MSMS" protocol (scan mode, Auto MS/MS; mass range 150–2200 *m/z*; spectra rate 2 Hz; charge state 2–5).

Peptide/protein identification

LC–MS/MS data (.raw files) were processed using the MaxQuant software (Cox and Mann 2008) (version 1.5.5.1). Peptide/protein identification was performed against UniProtKB FASTA database containing proteins of *B. subtilis* and using default MaxQuant settings for Bruker QTOF instrument type, with the following parameters: specific enzyme digestion, trypsin; variable modification, oxidation (M); fixed modification, carbamidomethyl (C); label-free quantification, LFQ; and match between runs, checked.

Statistical analysis

Bioinformatic analysis of the output files of MaxQuant (proteinGroups.txt file) was managed by Perseus software (Tyanova et al. 2016) (version 1.5.5.3). Label-free intensity analysis was selected for each individual sample. A two-sample Student's *t* test based on triplicate of each sample group was performed on $\log_2$-transformed intensity values. To classify proteins as variant and non-variant in scatter plot, Student *t* test difference >1 (twofold change) and *p* value <0.05 were chosen as criteria.

Results

To investigate how deregulation of dNTP synthesis affects proteome in non-growing *B. subtilis* cells during long periods of nutritional stress, the strains PERM1202 (Δ*nrdR*) and YB955 (parental) were starved for histidine, methionine, and leucine for 5 days. After this period, cells from both strains were collected and processed to determine their proteomic profiles employing a high-throughput LC–MS/MS approach. Results of these analyses allowed us to identify 749 proteins in water-soluble extracts of *B. subtilis* lysates. Of note, although a similar number (~800) of soluble proteins were identified in *E. coli* (Ishihama et al. 2008; Naveen and Hsiao 2016), such a number was superior to the 465 soluble proteins detected in *B. subtilis* subject to glucose starvation (Maaβ et al. 2014). In this study, proteins exhibiting more than a twofold difference between the parental and NrdR-deficient strains as well as those detected in *ΔnrdR* but not in YB955 and vice versa were reported. Using these criteria, in reference to the parental strain, the level of 57 proteins was found to increase and of 80 decreased (or depleted) in the NrdR-deficient strain (Fig. 1; Table 1, Table S1).

It has been previously shown that NrdR deficiency induced expression of RNR-encoding genes (Castro-Cerritos et al. 2017). In agreement with this observation, NrdE, the major subunit of RNR was increased over 14-fold in the Δ*nrdR* strain (Table 1). Of note, NrdF, the second RNR subunit, was not detected in the parental strain; however, the levels of this protein were similar to NrdE (namely, 13.5 vs. 13.8) in amino acid-starved cells that overexpressed RNR (results not shown). The proteins that were up- or downregulated in the null *nrdR* mutant possibly impacting SPM (Table 1) are described below based on their cellular functions.

Proteins of nucleotide metabolism

Our results revealed that the concentrations of several proteins involved in dNTP synthesis in addition to NrdEF including enzymes involved in previous steps of dNTP synthesis such as adenylate kinase and thymidylate kinase (Kobayashi et al. 2003) were not affected by the absence of NrdR (data not shown). However, PyrH, GmK, and CmK (Kobayashi et al. 2003) were found in the parental strain YB955 but not in the Δ*nrdR* strain.

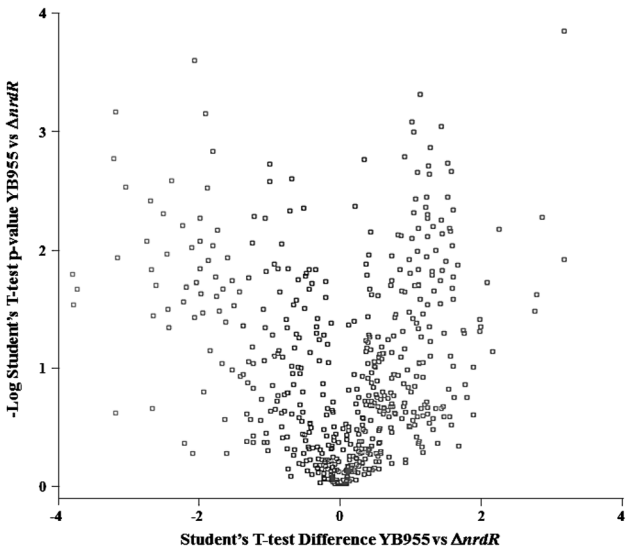

Fig. 1 Scatter plot representing results of the label-free quantification between strains YB955 (parental) and PERM1202 (*ΔnrdR*). Difference of $\log_2$ proteins intensities were plotted against negative logarithmic *p* values of the Student's *t* test performed from each sample group

Table 1 Differential proteins found in strains *B. subtilis ΔnrdR* and YB955

Fold change	Protein	Function
Nucleotide metabolism		
14.6	NrdE	Ribonucleotide reductase Ib (alfa subunit)
2.2	PurK	Phosphoribosylaminoimidazole carboxylase
−2.5	Prs[c]	Phosphoribosylpyrophosphate synthetase
−2.7	PurE	Phosphoribosylaminoimidazole carboxylase
ND[a]	NrdF	Ribonucleotide reductase Ib (beta subunit)
ND[b]	PyrH[c]	Uridylate kinase
ND[b]	Gmk	Guanylate kinase
ND[b]	Cmk	Cytidylate kinase
ND[b]	PurT	Phosphoribosylglycinamide formyltransferase
mRNA degradation		
ND[a]	YugI[c]	Putative polyribonucleotide phosphorylase
14.7	YhaM	3′–5′ exoribonuclease
Stringent response/CodY regulon		
4.9	Oppb	Oligopeptide ABC transporter
4.7	YuiA	Unknown
3.6	Ptsh	Phosphocarrier protein
2.9	Hag	Flagellin protein
2.8	UreC	Urease (alpha subunit)
2.5	InfA	Translation initiation factor IF-1
2.5	RplF	Ribosomal protein L6
2.4	RpmE	Ribosomal protein L31
2.2	RplU	Ribosomal protein L21
2.1	UreA	Urease (gamma subunit)
2.1	Vpr	Minor extracellular serine protease
2.1	AtpC	ATP synthase
2.0	IspA	Intracellular serine protease
−2.1	RocD	Ornithine transaminase
−2.1	PtsI	Enzyme I, general component of the HPr
−2.2	RocF	Arginase
−2.3	RocA	3-Hydroxy-1-pyrroline-5-carboxylate dehydrogenase
−2.3	ClpY	Two-component ATP-dependent protease
−2.3	CodY	Transcriptional pleiotropic repressor
−2.5	IlvD	Dihydroxy-acid dehydratase
−2.9	ArgF	Ornithine carbamoyltransferase
−4.2	YwaA	Branched-chain amino acid aminotransferase
ND[b]	LeuA	2-Isopropylmalate synthase
ND[b]	ThrB	Homoserine kinase
ND[b]	RplJ	Ribosomal protein L10
ND[b]	FliF	Flagellar basal-body M-ring protein
GTP/ppGpp binding proteins		
−7.2	InfB[c]	Translation initiation factor IF-2
ND[b]	Obg	GTP-binding protein
c-di-AMP-interacting proteins		
4.3	YfkN	c-di-AMP phosphodiesterase
3.9	DarA	c-di-AMP-binding PII-like protein
2.6	CdaR	Effector protein controlling CdaA diadenylate cyclase activity
−3.4	DisA	DNA integrity scanning protein
DNA repair		
−2.2	Nfo	Type IV apurinic/apyrimidinic endonuclease

Table 1 continued

Fold change	Protein	Function
−2.4	RecA	Multifunctional protein
−8.9	DinB	Nuclease inhibitor
ND[b]	SbcC	DNA exonuclease

[a] ND. Protein detected only in soluble extracts of PERM1202 (*ΔnrdR*)

[b] ND. Protein detected only in soluble extracts of YB955

[c] These proteins are also part of stringent response

Enzymes involved in mRNA degradation

Interestingly, in reference to YB955, the parental strain, a 14.7-fold increase in the concentration of YhaM, a 3′–5′ exoribonuclease (Oussenko et al. 2002) was detected in the RNR-overproducing strain suggesting an increased degradation of mRNAs in this strain.

Proteins regulated by CodY and stringent response

Notably, in agreement with a decreased synthesis of CodY (~2.3-fold), levels of enzymes involved in protein catabolism as Vpr, IspA, UrecC, and UreA were derepressed; however, additional CodY-regulated enzymes involved in amino acid biosynthesis, including, ArgF, LeuA, YwaA, and ThrB, either decreased (≥2.5-fold) or were not found in the Δ*nrdR* strain. In addition, stringent response proteins, including the ribosomal proteins RplF, RpmE, RplU and the translation InfA factor increased around twofold its synthesis in the Δ*nrdR* mutant (Table 1). In total, a group of 30 proteins involved in the stringent response and CodY regulation resulted affected in the starved NrdR-deficient cells (Table 1). Since the stringent response and CodY derepression diminishes expression of ribosomal genes and increase transcription of genes involved in amino acid biosynthesis and degradation operons (Eymann et al. 2002; Molle et al. 2003; Geiger and Wolz 2014), our results revealed the existence of an altered stringent response in the Δ*nrdR* strain.

Proteins that bind second messengers

Interestingly, the amount of the GTP-binding protein InfB decreased ~sevenfold in the NrdR-deficient strain (Table 1). InfB corresponds to the ribosomal elongation factor EF2 in *B. subtilis* (Shazand et al. 1990). This protein not only binds and hydrolyzes GTP but also interacts with the alarmone (p)ppGpp; working thus as a metabolic sensor that regulates translation and stress responses during starvation and/or alterations on GTP levels (Milon et al. 2006).

The concentrations of proteins that interact with the second messenger c-di-AMP were found altered in the Δ*nrdR* strain. Thus, in the NrdR-deficient strain, the diadenylate cyclase DisA was downregulated (3.4-fold decrease). On the other hand, the concentrations of CdaR and YfkN increased 2.6- and 4.3-fold, respectively; the former has been involved in the synthesis of c-di-AMP whereas the last one participates in the degradation of this messenger (Chambert et al. 2003; Mehne et al. 2013). In addition, the concentration of the c-di-AMP-interacting DarA protein (Gundlach et al. 2015) increased 3.9-fold in the *ΔnrdR* mutant.

Proteins involved in DNA repair and mutagenesis

Our study revealed that DNA repair proteins like the endonuclease Nfo decreased 2.2-fold in nutritionally stressed cells overexpressing RNR. Interestingly, RecA that controls the SOS response and plays an important role in homologous recombination repair decreased 2.3-fold in the Δ*nrdR* strain; in addition, DinB a nuclease inhibitor was substantially decreased (~ninefold) in the *ΔnrdR* mutant; it has been shown that *dinB* expression is regulated by DinR the LexA homolog in *B. subtilis* (Au et al. 2005).

Discussion

Here, we show that non-growing Δ*nrdR B. subtilis* cells subjected to prolonged amino acid starvation display a differential proteomic pattern indicative of a disturbed metabolic status. The correlations of this altered proteome with the strain's propensity to revert amino acid auxotrophies under conditions of limited growth are further discussed. A recent report revealed that disruption of NrdR promoted mutagenesis in starved *B. subtilis* cells (Castro-Cerritos et al. 2017). As the absence of NrdR increased the expression of *nrdEF*, a rise in dNTP pools was expected to take place in these nutritionally stressed cells (Castro-Cerritos et al. 2017). However, metabolic conditions operating in growth-limited bacteria may be inappropriate for dNTPs synthesis; in support of this notion, in *E. coli*, the levels of NTPs decrease during the stationary phase of growth (Buckstein et al. 2008). Furthermore, as our proteomic analysis revealed, the concentration of guanylate kinase, cytidylate kinase and uridylate kinase was downregulated

in NrdR-deficient cells (Table 1). However, we did not detect similar effects for adenylate kinase or thymidylate kinase, necessary for the synthesis of ADP and TDP (data not shown). Interestingly, the 3′ → 5′ exoribonuclease YhaM (Oussenko et al. 2002) was highly upregulated in the starved *nrdR*-deficient cells (Table 1). Based on these observations, it is feasible to propose that YhaM-dependent hydrolysis of mRNAs and a biased recycling of NMPs for salvage dNTP synthesis may potentially bias dNTP pools and promote error-prone repair mutagenic events in starved cells of the Δ*nrdR* strain.

It has been recently reported that accumulation of apurinic/apyrimidinic (AP) sites and error-prone processing of these lesions constitutes a mechanism of stationary phase-associated mutagenesis (SPM) in *B. subtilis* (Barajas-Ornelas et al. 2014). In agreement with this report, the levels of Nfo, a repair enzyme involved in processing of AP sites (Salas-Pacheco et al. 2003; Urtiz-Estrada et al. 2003), were significantly diminished in starved *B. subtilis* deficient for NrdR (Table 1). Interestingly, DisA, a protein that scans the chromosome and pauses in DNA lesions during sporulation and germination/outgrowth in *B. subtilis* (Bejerano-Sagie et al. 2006; Campos et al. 2014) was downregulated in the NrdR-deficient strain. The absence of *disA* in AP-endonuclease-deficient outgrowing spores increased oxidative-induced DNA lesions, including 8-OxoG and AP sites (Campos et al., 2014). As noted above and in additional reports, in growth-limited *B. subtilis* cells, these types of lesions promote SPM (Vidales et al. 2009; Debora et al. 2011; Barajas-Ornelas et al. 2014; Gómez-Marroquín et al. 2015). Of note, the recombination protein RecA was found to be downregulated in the Δ*nrdR* strain. It is possible that lower levels of RecA could be associated with increased dATP levels, a condition reported to decrease the expression of SOS-regulated genes in *E. coli* (Maslowska et al. 2015). Congruent with these observations, compelling evidence (Sung and Yasbin 2002) have ruled out the contribution of RecA to *B. subtilis* SPM.

As described above, nucleotide monophosphate kinases, key enzymes involved in supplying substrates for dNTP and NTP synthesis (Kobayashi et al. 2003), were differentially regulated in the Δ*nrdR* mutant, a metabolic condition that may theoretically generate a bias in the dNTP/NTP pools. Modifications on GTP levels or GTP/ATP ratios cause changes in expression pattern of genes regulated by stringent response (Kriel et al. 2012; Bittner et al. 2014; Kriel et al. 2014; Pulschen et al. 2017). Furthermore, in reference to strain YB955, our proteomic analysis in the Δ*nrdR* mutant revealed a 2.3-fold reduction in the concentration of CodY, the GTP-sensing transcriptional repressor (Blagova et al. 2003) that was paralleled by changes in the concentration of 30 proteins belonging to this stress response and that generated by (p)ppGpp (Fig. 1; Table 1). Taken together, these evidences strongly support the idea that the metabolic conditions faced by starved cells of the NrdR-deficient stress modify the response generated by CodY and the stringent response in *B. subtilis*.

The absence of NrdR in the amino acid-starved cells of *B. subtilis* altered the concentration of ci-di-AMP-interacting proteins (Fig. 1; Table 1). Interestingly, in *Listeria monocytogenes* the repressor NrdR was able to bind c-di-AMP (Sureka et al. 2014); however, the impact of this interaction in regulating dNTP synthesis is not currently understood. The levels of DarA, another c-di-AMP-binding protein increased in the Δ*nrdR* mutant, although the true targets of this signal transduction protein have remained elusive (Commichau et al. 2015; Gundlach et al. 2015) its function has been speculated to be associated with the regulation the thymidylate kinase-encoding gene *tmK*, a function essential for dTTP synthesis (Commichau et al. 2015). It does not escape to our attention that this regulatory circuit may also contribute to biasing the dNTP/NTP pools in the NrdR-deficient strain. Moreover, in this mutant, besides DisA and DarA, the c-di-AMP phosphodiesterase YfkN and the diadenylate cyclase regulator CdaR (Pham et al. 2016) were also upregulated. Altogether, these evidences unveil a link between dNTP metabolism, the stringent response, and c-di-AMP in amino acid-starved *nrdR*-deficient *B. subtilis* cells.

In *B. subtilis* the alarmones guanosine tetraphosphate and guanosine pentaphosphate [(p)ppGpp] play a central role in the metabolism of GTP; it has been shown that these alarmones inhibit the activity of proteins involved in the synthesis of GTP (Kriel et al. 2012). In this study, under conditions of amino acid starvation, the levels of several GTP-binding proteins were found to be affected by the absence of NrdR, including CodY, the GTPase Obg, and the translation factor InfB; the last two bind (p)ppGpp and participate in ribosome maturation in a way dependent of GTP concentration (Buglino et al. 2002; Milon et al. 2006).

Our proteomic analysis revealed an altered stringent response in the Δ*nrdR* mutant. Therefore, an altered GTP/ATP ratio conducting to changes in expression pattern of genes regulated by this response may potentially contribute to the hypermutagenic phenotype observed in starved NrdR-deficient cell of *B. subtilis* (Castro-Cerritos et al. 2017). In support of this proposal, it has been shown that mutation rates in genes involved in amino acid biosynthesis are modulated by (p)ppGpp levels in both, *E. coli* and *B. subtilis* (Wright 1996, 1997). In the last one, the mutagenic effects have been attributed to activation of the stringent response (Rudner et al. 1999) and alterations in GTP levels.

Taken collectively, our results revealed that amino acid-starved cells of the hypermutagenic strain *B. subtilis* Δ*nrdR* hold a complex proteomic pattern reminiscent of a disturbed metabolism in pathways related to (i) dNTP

synthesis, (ii) stringent response, (iii) cell signaling, and (iv) DNA repair.

Our finding showing deregulation of cell-signaling proteins in starved bacteria overexpressing RNR is an interesting subject which deserves to be further explored. Therefore, experiments aimed to understand the role played by the second messengers GTP and c-di-AMP and its interacting proteins in *B. subtilis* SPM are guaranteed. In a broader context, our study provides novel elements to understand how microorganisms deploy genetic and biochemical strategies to contend with the stressful conditions that limit its growth (Rosario-Cruz and Boyd 2016), including antibiotic resistance.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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