RESEARCH ARTICLE

Proteomics analysis of a long-term survival strain of *Escherichia coli* K-12 exhibiting a growth advantage in stationary-phase (GASP) phenotype

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Received: July 30, 2015
Revised: November 24, 2015
Accepted: December 23, 2015

The aim of this work was the functional and proteomic analysis of a mutant, W3110 Bgl^+/10, isolated from a batch culture of an *Escherichia coli* K-12 strain maintained at room temperature without addition of nutrients for 10 years. When the mutant was evaluated in competition experiments in co-culture with the wild-type, it exhibited the growth advantage in stationary phase (GASP) phenotype. Proteomes of the GASP mutant and its parental strain were compared by using a 2DE coupled with MS approach. Several differentially expressed proteins were detected and many of them were successful identified by mass spectrometry. Identified expression-changing proteins were grouped into three functional categories: metabolism, protein synthesis, chaperone and stress responsive proteins. Among them, the prevalence was ascribable to the "metabolism" group (72%) for the GASP mutant, and to "chaperones and stress responsive proteins" group for the parental strain (48%).

Keywords:
Aging / *Escherichia coli* / GASP / Mass spectrometry / Microbiology

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1 Introduction

The ability of the bacterium *Escherichia coli* to grow under batch conditions during prolonged stationary phase in the absence of a carbon source is referred to as growth advantage in stationary phase, or the GASP phenomenon [1–4]. Batch cultures expressing GASP are dynamic, with growth and succession of mutant strains occurring continuously despite near-constant total population size [3, 5–7]. Four GASP mutations have been identified so far, and three of the loci are well characterized [1, 7–10]. Two mutations were identified as loss-of-function alleles of the global regulators *rpoS* and *lrp* [1, 9], the third was mapped on the *ybeJ–gltJKL* cluster encoding a high-affinity aspartate and glutamate transporter [7, 8, 10]. Although each of these genes is involved in different processes, all GASP mutations result in an increased ability to catabolize one or more amino acids as a source of carbon and energy [3]. The ability to acquire a growth advantage in stationary-phase phenotype is not only limited to *E. coli*, but it has also been demonstrated in other species, including eukaryotic microorganisms [11–14].

The GASP phenomenon demonstrates that starvation stress is a proximal cue that leads to the accumulation of beneficial mutations, also providing an explanation for the persistence of the population under resource-limited conditions [3, 15]. Consequently, it serves as a valuable model to gain insights into physiological adaptation to severe energy limitation in nature as the stressful conditions which characterize long-term stationary phase in the laboratory are similar to those of many natural environments where bacteria exist [3], as well as in understanding the interaction and dynamics

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Abbreviations: **cfu**, colony forming unit; **GASP**, growth advantage in stationary phase

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Significance of the study

The study of GASP phenomenon is important in order to understand how bacteria survive in a natural environment, where resources are scarce and competition is fierce. Such learning may give more insight into elucidation of bacterial pathogenicity, increased antibiotic resistance, spontaneous adaptation of remediation bacteria, persistence of food contaminants, biofilm formation, and aging. Moreover, the increased competitiveness of "survivors" could be exploited to develop a new class of metabolically efficient, contamination resistant host organisms for industrial biotechnology.

of bacterial population [4, 16]. Moreover, because of the acquisition of beneficial mutations, the GASP phenotype could serve as a robust chassis for synthetic biology.

It has been reported that *E. coli* bacterial cultures can persist in the prolonged stationary phase for the period of up to 5 years [7]. For this study we isolated a mutant from an *E. coli* batch culture maintained without addition of nutrients for 10 years at room temperature. Despite the fact that previous studies monitored *E. coli* proteome changes in response to various stresses and growth conditions [17–19], analysis of proteome changes in *E. coli* over such a prolonged period of starvation has not yet been performed. Monitoring these changes will hopefully help to provide a better understanding into the GASP phenomenon and its implications.

2 Materials and methods

2.1 Bacterial strains and culture conditions

The strains used in this study were *E. coli* K-12 W3110 wild type (phenotypically Bgl^-), and its derivative Bgl^+ isolated as previously described [20], where the silent *bgl* operon for the uptake and metabolism of β-glucoside sugars has been activated by transposition of IS*1* into the regulatory region *bglR* (Supporting Information Fig. S1). Bacterial cultures were maintained and grown on Luria-Bertani (LB) medium at 37°C.

2.2 Isolation and characterization of a GASP mutant

A sample was removed from a 10-year old LB unsupplemented batch culture of *E. coli* W3110 Bgl^+, serially diluted in sterile saline solution and plated on LB agar plates. After 18 h incubation at 37°C, grown colonies were counted, and some of them were picked up and streaked onto fresh LB plate for re-purification. Pure liquid cultures derived from a single colony were added with 30% sterile glycerol before storage at −80°C.

The isolated strain, subsequently named W3110 Bgl^+/10, was tested for phospho-β-glucosidase activity by measuring the hydrolysis of *p*-nitrophenyl-β-D-glucopyranoside in intact cells grown overnight in 7 mL of LB medium with and without inducer (10 mM arbutin) as previously reported [21]. Maximum cell density was measured after 14 h growth of overnight cultures diluted 500-fold into fresh LB accordingly to Vulič and Kolter [22]. The ability of W3110 Bgl^+/10 to outcompete W3110 Bgl^+ in competition assays was determined essentially as described by Zinser and Kolter [8]. Both strains were inoculated from frozen glycerol stocks into 3 mL of LB and grown in parallel overnight. These were subcultured 1:100 into fresh LB and incubated for 24 h. Competitors were mixed at about 1:150 ratio. The two populations were monitored daily by serial dilution in sterile saline (0.9% NaCl) and plating on M9 salts minimal medium [23] added with arbutin (0.5%) and LB agar plates. Bgl^+ (in this case growth on β-glucoside arbutin) was used as marker to distinguish the competitors in mixed-culture experiments, as Bgl^+ was previously shown [8, 22] and confirmed here to be neutral under these experimental conditions. Each competition assay was repeated at least three times.

2.3 Sample preparation for proteomic analysis

E. coli W3110 Bgl^+ and W3110 Bgl^+/10 were grown to early stationary phase in LB medium by monitoring the optical density at 560 nm (UV-VIS spectrophotometer, Ultrospec 2100; Amersham Pharmacia), (Supporting Information Fig. S2). Culture broth was centrifuged for 5 min at 3500 × *g* at 4°C. The pellet was washed four times with TE solution (10 mM Tris-HCl, 1 mM EDTA; pH 8.0) and then resuspended in a conventional 2D lysis buffer composed of 8 M urea, 4% (w/v) CHAPS, 1% (w/v) dithioerythritol (DTE). In order to improve cell lysis, samples were sonicated on ice for 2 min, with a pause of 1 min every 30 sec to avoid overheating, with six pulses at maximum intensity by using a Labsonic U sonicator (B. Braun Biotech, Melsungen, Germany). Then, due to their high viscosity, the samples were subjected to ten "up and down" cycles with insulin syringes. Each sample was vortexed and then centrifuged at 15 000 × *g* for 10 min at 4°C. The supernatants were recovered and stored at –80°C until use. Total protein concentration was estimated according to Bradford method [24].

2.4 2DE

2DE was performed using the Immobiline-polyacrylamide system, as previously described [25, 26]. IEF was carried out on non-linear wide-range immobilized pH gradients (IPG)

(pH 3–10; 18 cm long IPG strips; GE Healthcare, Uppsala, Sweden) and performed using the Ettan™ IPGphor system (GE Healthcare). Strips for analytical runs were rehydrated with 60 μg protein in 350 μL of lysis buffer and 0.2% (v/v) carrier ampholyte for 1 h at 0 V and for 8 h at 30 V, at 16°C. The strips were then focused according to the following electrical conditions at 16°C: 200 V for 1 h, from 300 V to 3500 V in 30 min, 3500 V for 3 h, from 3500 V to 8000 V in 30 min, 8000 V for 3 h, 10 000 V until a total of 80 000 Vh was reached.

MS-preparative strips were rehydrated with 350 μl of lysis buffer and 2% (v/v) carrier ampholyte, for 12 h at room temperature. Sample load, 600 μg protein per strip, was performed by cup loading in the IPGphor Cup Loading Strip Holders (GE Healthcare), and it was applied at the cathodic end of the strip. IEF was then achieved according to the following voltage steps at 16°C: 30 V for 30 min, 200 V for 2 h, 500 V for 2 h, from 500 V to 3500 V in 30 min, 3500 V for 5 h, from 3500 V to 5000 V in 30 min, 5000 V for 4 h, from 5000 V to 8000 V in 30 min, 8000 V for 3 h, 10 000 until a total of 100 000 Vh was reached.

After IEF, strips were equilibrated for 12 min in 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS, 0.05 M Tris–HCl pH 6.8, 2% (w/v) DTE; and for further 5 min in the same equilibration buffer except for 2% (w/v) DTE which was substituted with 2.5% (w/v) iodoacetamide.

The second dimension was carried out, at 10°C, on house-made 9–16% polyacrylamide linear gradient gels (18 cm x 20 cm x 1.5 mm) at 40 mA/gel constant current, until the dye front reached the bottom of the gel.

Analytical gels were stained with ammoniacal silver nitrate [27, 28] and they were scanned using the ImageScanner III (GE Healthcare). Instead, MS-preparative gels, previously attached covalently to a glass surface using Bind-Silane (γ-methacryloxypropyltrimethoxysilane) (LKB-Produkter AB, Bromma, Sweden), were stained with SYPRO Ruby staining (Bio-Rad Laboratories, Hercules, CA) following manufacturer's protocol and digitalized with a Typhoon 9400 laser densitometer (GE Healthcare) using the laser at 532 nm wave length.

2.5 Image analysis and statistics

Image analysis was performed on 2D silver stained gels using the ImageMaster 2D Platinum v6.0 software (GE Healthcare). For each tested condition, image analysis was performed on three different spot maps from three biological replicates of *E. coli* samples, with every replicate becoming from an independent cultivation experiment. An intra-class quality and experimental control was performed by comparing the gels belonging to the same condition, and then a differential inter-class analysis was performed to detect any statistically significant quantitative and qualitative differences. Based on a fold change, between the two sample sets, of at least ±2.5 in relative volume (%V) ratio, and on statistically analysis with two-tailed Student's *t*-test score less than 0.05, differentially expressed proteins were detected between the two examined conditions. Moreover, in order to visualize the relationships occurring among different spot maps, a PCA was performed using the statistics and analytics software package STATISTICA 8.0 (StatSoft, Tulsa, Oklahoma). For this statistical analysis, %V values of each differentially expressed spot in the gels of both analyzed conditions were considered. Spot maps were plotted in the two-dimensional space resulting in the PC1 and PC2 principal components that orthogonally divide the analyzed samples according to the two principal sources of variation in the data set.

2.6 Protein identification and functional analysis

Protein identification was carried out by peptide mass fingerprinting [29, 30] and/or peptide sequencing by tandem mass spectrometry using an Ultraflex III MALDI-TOF/TOF mass spectrometer (Bruker Daltonics, Billerica, MA). SYPRO Ruby stained differentially expressed spots were automatically excised from MS preparative gels by an ETTAN™ Spot-picker (GE Healthcare), destained in 2.5 mM ammonium bicarbonate and 50% acetonitrile, and finally they were dehydrated in acetonitrile. After spot rehydration in trypsin solution (Sigma Aldrich, St. Louis, MO), in-gel protein digestion was performed by an overnight incubation at 37°C. 1.25 μL of each protein digest was directly spotted onto the MALDI target and air-dried. Then, 0.75 μL of matrix solution (a solution of 5mg/mL CHCA in 50% v/v ACN and 0.5% v/v TFA) was added to the dried samples and allowed to dry again. Mass spectra were acquired using an Ultraflex III MALDI-TOF/TOF mass spectrometer (Bruker Daltonics, Billerica, MA), equipped with a 200 Hz smartbeam™ I laser, in reflector positive mode with a laser frequency set to 100 Hz. Spectra were analyzed by Flex Analysis software v.3.0. PMF database searching was carried out in Swiss-Prot/TrEMBL database (version 2014_10; 546 790 sequences; 194 613 039 residues) set for *E. coli* using Mascot (Matrix Science Ltd., London, UK, http://www.matrixscience.com) on-line available software. The experimental and theoretical peptide fingerprinting patterns Δmass was less than 100 ppm, and trypsin was selected as the digestion enzyme with one allowed missed cleavage. Alkylation of cysteine by carbamidomethylation was assumed as fixed modification, while oxidation as possible modification. The parameters used to accept identifications were the number of matched peptides, the extent of sequence coverage, and the probabilistic score, as reported in Supporting Information Tables S2 and S3.

Peptide digests that did not give unambiguous identifications were further analyzed performing peptide sequencing by tandem mass spectrometry. MS/MS analysis was performed by using the same above cited mass spectrometer. Two to three PMF peaks showing a high intensity were CID fragmented using Argon as collision gas, and MALDI-TOF/TOF tandem MS was performed in LIFT mode by software controlled data acquisition. Fragmented ions were an-

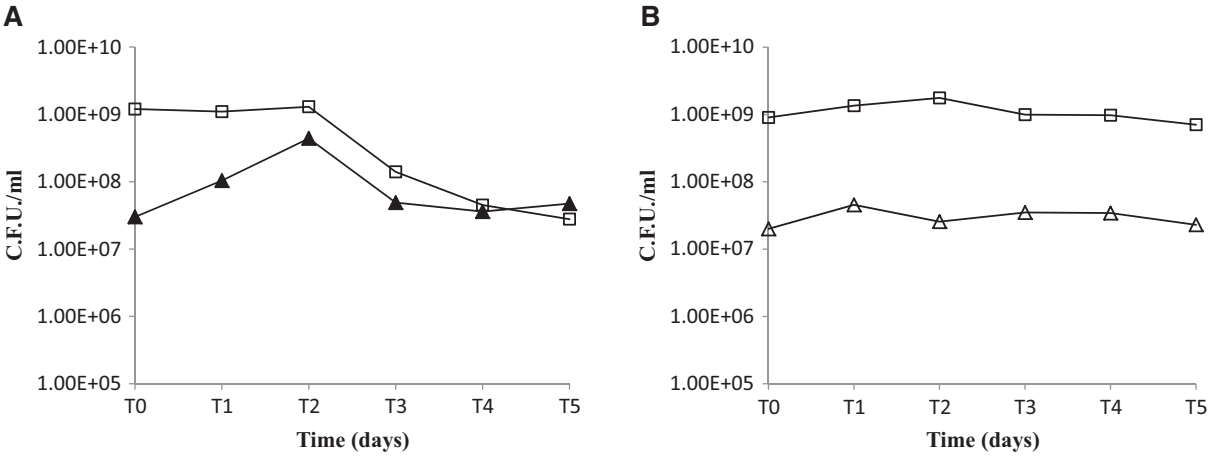

Figure 1. Mixed-culture experiments in LB medium. One-day-old cultures of both competitors were mixed at a 1:150 ratio. (**A**) W3110 Bgl^+/10 (▲) mixed with wild-type W3110 majority (□); and (**B**) W3110 Bgl^+ minority (Δ) mixed with wild-type W3110 majority (□).

alyzed using the Flex Analysis software v.3.0. The MS/MS database searching was carried out in the Swiss-Prot/TrEMBL database (version 2014_10; 546 790 sequences; 194 613 039 residues) using the on-line available MASCOT MS/MS ion search software. The following parameters were applied for database searching: taxonomy: *E. coli*, trypsin specificity, one missed cleavage allowed, peptide precursor mass tolerance: ±100 ppm, fragment mass tolerance: ±0.6 Da, peptide precursor charge state: +1, carbamidomethylation of cysteine as a fixed modification, oxidation of methionine as a possible modification. For protein identification, Mascot ion score, peptide coverage by "b" and "y" ions, and expected value were considered.

By using UniProtKB/Gene Ontology/Biological Process tool and EcoCyc database, identified differentially expressed proteins were grouped according to their biological functions into three main functional groups: metabolism, protein synthesis, and chaperones and stress responsive proteins.

2.7 Western blotting

One-dimensional Western blotting was performed according to standard procedures, as detailed in Supporting Information. Immunodetection was achieved using mouse anti-DnaK monoclonal antibody from StressGen Biotechnologies Corporation (Victoria, BC, Canada).

3 Results and discussion

3.1 Isolation and characterization of a GASP mutant

The *E. coli* K-12 strain was isolated from an LB unsupplemented batch culture of W3110 Bgl^+ kept at room temperature for 10 years. The number of viable cells of the 10-years old culture, estimated by a colony forming unit (cfu) assay, resulted in about 10^6 cfu per mL, accordingly to that previously reported for batch culture maintained for more than 5 years under similar conditions [3].

The isolated mutant was shown to maintain the phenotypic Bgl^+ trait (ability to metabolize aromatic β-glucosides) of the original strain (Supporting Information Table S1) as well as the IS*1* element insertion in the regulatory region of the *bgl* operon (not shown). It was then named W3110 Bgl^+/10. To determine whether this "survivor" expressed a GASP phenotype, it was evaluated in competition experiments in mixed bacterial cultures with the wild-type by using the Bgl^+ trait as a selectable marker on M9 minimal arbutin medium. Competitions were done in the LB medium in which Bgl^+/10 strain was originally isolated. We ran two series of mixed-culture experiments: (i) Bgl^+/10 minority mixed with wild-type (WT) W3110 majority; (ii) Bgl^+ minority mixed with a WT majority. Typical results from mixing experiments are shown in (Fig. 1). In the Bgl^+/10-WT mixes, the mutant grows and by day 5 it takes over (Fig. 1A), whereas in the Bgl^+-WT mixes (Fig. 1B) the dynamic of the minority and majority populations is the same. Likely, in the former mix, the WT stops dividing before Bgl^+/10 as the nutrients are becoming exhausted at the end of the growth phase. Indeed, Bgl^+/10 attains higher cell density than WT when growing in pure culture in fresh LB (Supporting Information Fig. S3). The behavior of Bgl^+/10 in competition experiments is then consistent with a GASP phenotype [1, 3].

3.2 Proteomic analysis

A functional proteomic approach, consisting of 2DE, mass spectrometry and dedicated functional analysis bioinformatic tools, was applied to compare the protein expression profile of W3110 Bgl^+/10 and W3110 Bgl^+. *E. coli* W3110 was not included in this analysis as it was previously shown that, under the same conditions, its proteomic profile did not differ

significantly from that of W3110 Bgl^+ [20]. For each strain, three different biological replicates were subjected to 2DE for a total of six produced gels. Gel images were acquired and analyzed using ImageMaster 2D Platinum v.6.0 software. Representative silver stained electropherograms of W3110 Bgl^+ and of W3110 Bgl^+/10 samples are reported in Fig. 2A and B, respectively. Approximately 1500 spots were well detected in each gel and all analyzed gels showed a quite similar protein pattern, thus supporting the experimental reproducibility. According to statistics and to relative volume (%V) ratio threshold, as reported in detail in Materials and Methods, 92 different protein spots were found as quantitative and/or qualitative significant differences occurring between the parental strain W3110 Bgl^+ and its derivative Bgl^+/10. Based on these differentially expressed protein spots, a PCA analysis was performed in order to examine the correlations between W3110 Bgl^+/10 and its parental strain and to provide a biological validation to our 2DE analysis. PCA revealed distinct expression patterns for the two tested conditions (Supporting Information Fig. S4) and demonstrated a consistent reproducibility among the biological replicates. Indeed, spot maps properly segregate in two different experimental groups (encircled by different colors, Supporting Information Fig. S4). The first principal component (PC1) accounted for the 55.42% of the variance in the data, while the second principal component for the 41.03% of variation.

Sixty-five of the 92 differentially expressed protein spots were unambiguously identified by MALDI-TOF and/or MALDI-TOF/TOF mass spectrometry (Supporting Information Tables S2 and S3). In particular, quantitative computer-aided image analysis pointed out 75 differentially expressed protein spots between the two strains. Thirty-six were stronger expressed in W3110 Bgl^+ and 39 in W3110 Bgl^+/10, among these 53 were identified by MS (Supporting Information Table S2). Qualitative image analysis, as allowed by the applied analytical procedure, detected 17 protein spots typical of W3110 Bgl^+/10, and 12 of them were identified (Supporting Information Table S3). Hereinafter proteins identified either quantitatively or qualitatively will be reported together. The 65 differentially expressed spots identified by MS correspond to 52 unique proteins, as different isoforms (multiple spots) of a same protein, such as Periplasmic oligopeptide-binding protein (OppA), result to significantly change in abundance between W3110 Bgl^+ and W3110 Bgl^+/10. On the other hand, it is important to note that in some isoelectric series, such as those to which spots 2, 30, and 45 belong, only one spot was detected as significantly changed, suggesting an important role played by specific post-translational modifications between the two analyzed conditions. Furthermore, we also identified by MS, as reported in Supporting Information Tables S2 and S3, some protein spot fragments whose expression is stronger in W3110 Bgl^+/10 with respect to W3110 Bgl^+. These fragments could be easily identified based on the partial sequence coverage obtained by MS and on their position in the gel, with a molecular weight lower than expected.

Based on UniProtKB/Gene Ontology/Biological Process tool and EcoCyc database, all proteins identified by MS were assigned to three main functional groups (Table 1; Fig. 3).

A functional classification of the identified proteins showed that the highest proportion was involved in metabolism and translation in W3110 Bgl^+/10 (82%, Fig. 3), while chaperones and stress-induced proteins were prevalent in W3110 Bgl^+ (48%, Fig. 3).

Among proteins stronger expressed in W3110 Bgl^+/10 included in the "metabolism" group there are two periplasmic components of the ATP-Binding Cassette (ABC) superfamily transporters: the periplasmic oligopeptide-binding protein (encoded by *oppA*) implicated in peptides binding and recycling [31], and the glutamate/aspartate periplasmic-binding protein (encoded by *gltI,* synonym *ybeJ*) engaged in aspartate and glutamate transport [32]. A transposon 'hop' coupled with a site-specific inversion in the *ybeJ-gltJKL* locus was earlier identified as a GASP mutation [3, 10]. Both proteins, then, play a role in amino acid scavenging which has been recognized an important role in conferring a stationary phase fitness gain [3, 9]. Proteins involved in the transport of sugars as ManX and MalK show also high expression levels. The *manXYZ* operon of *E. coli* encodes a sugar transporter of the phosphoenol pyruvate (PEP)-dependent phosphotransferase system, which is capable of transporting many sugars, including glucose, mannose and the aminosugars, glucosamine and N-acetylglucosamine [33]; *malKFGE* encodes a member of the ABC superfamily of transporters capable of transporting malto-oligosaccharides up to seven glucose units long [34]. It was reported that the constitutive expression of the maltose regulon enhanced *E. coli* fitness in severely glucose-limited environment [35]. In fact, the most frequent mutations improving the scavenging capabilities of *E. coli* involve regulatory mutations affecting the expression of transport components for carbohydrate uptake under prolonged hunger conditions [36]. The data suggest the coexistence in the same strain of more beneficial adaptive traits, some of them already found involved with a GASP phenotype, which confer a fitness advantage and delay the entry into stationary phase.

The identified proteins whose presence was increased in W3110 Bgl^+ result mainly related to the functional group of "chaperones and stress responsive proteins" (48%, Fig. 3). The universal stress protein UspD is encoded by *uspD* which is induced in stationary phase and by a variety of stresses causing growth arrest of cells [37]. Similarly, *osmC* and *osmY* (encoding for OsmC and OsmY, respectively), besides being induced under conditions of hyperosmotic stress, are also induced upon entry into stationary phase [38, 39], as well as increasing expression levels in the stationary growth phase have been also observed for *btuE* and *wrbA* [40, 41].

Interestingly, among the few proteins ascribable to the functional group of "chaperones and stress responsive proteins" differentially expressed in W3110 Bgl^+/10, there are

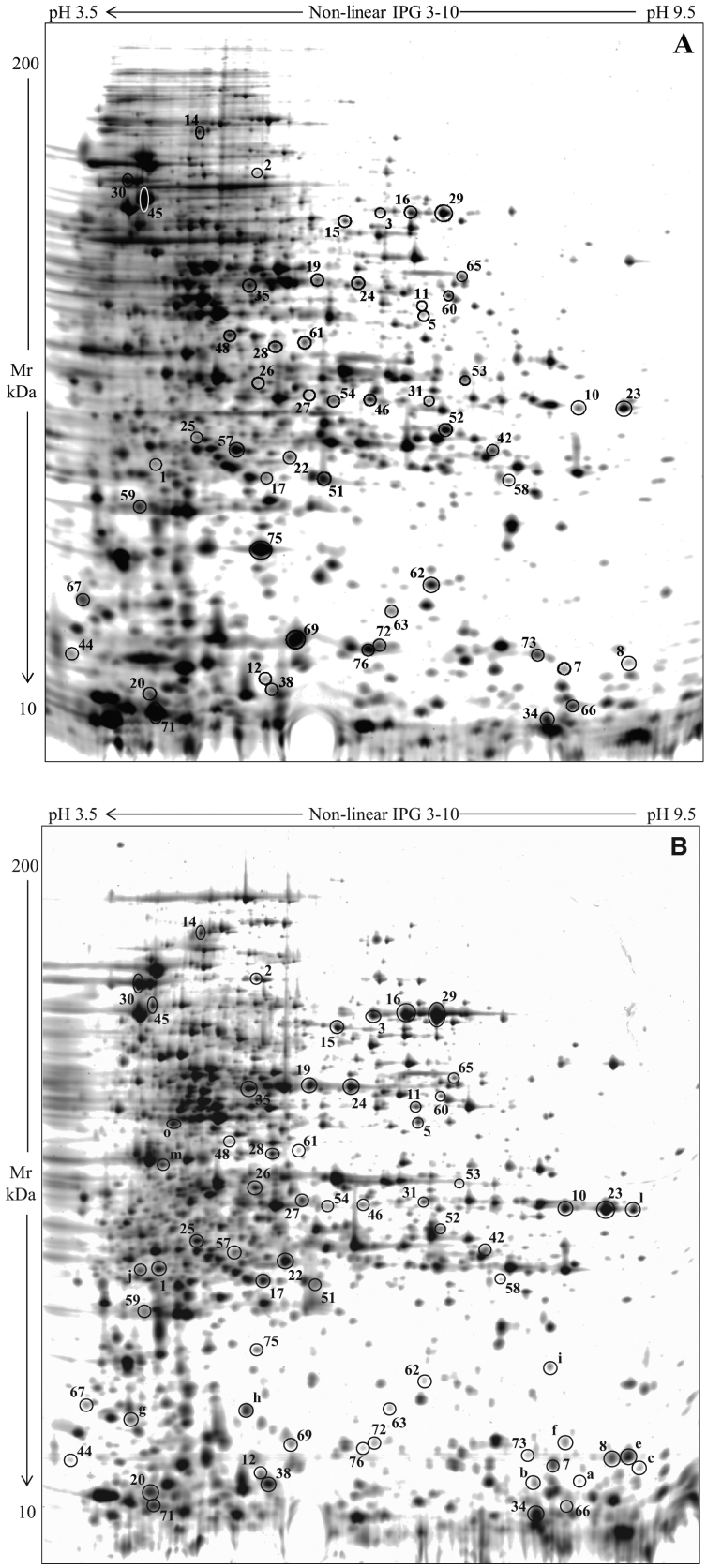

Figure 2. Representative silver stained electropherograms of *E. coli* W3110 Bgl^+ (A) and *E. coli* W3110 Bgl^+/10 (B) samples where the MS identified differentially expressed protein spots between the two analyzed conditions are reported. Quantitative differences are visualized by numbers, while the qualitative ones by letters. Numbers and letters correspond to those reported in Table 1 and in Supporting Information Tables S2 and S3.

Table 1. MS identified proteins stronger expressed in *E. coli* W3110 Bgl$^+$/10 or in W3110 Bgl$^+$ and their physiological functions

Spot No.	Protein description	AC[a)]	Gene name	Functional group
Bgl$^+$/10				*Metabolism*
2	2-Hydroxy-3-oxopropionate reductase	P0ABQ2	*garR*	Carbon compound utilization
28	Adenosine deaminase	P22333	*add*	Purine ribonucleotides and deoxyribonucleotides metabolism
20	Adenylate kinase	P69441	*adk*	Purine ribonucleotides biosynthesis
h	ATP phosphoribosyltransferase	P60757	*hisG*	Amino-acid biosynthesis
11, 38	ATP synthase subunit alpha	P0ABB0	*atpA*	Energy metabolism
5	D-Lactate dehydrogenase	P06149	*dld*	Energy metabolism
j	Enolase	P0A6P9	*eno*	Energy metabolism
10, 23, l	Glutamate/aspartate periplasmic-binding protein	P37902	*gltI*	Amino-acid transport
o	Histidinol-phosphate aminotransferase	P06986	*hisC*	Amino-acid biosynthesis
17	Imidazole glycerol phosphate synthase subunit HisH	P60595	*hisH*	Amino-acid metabolism
14	Isocitrate dehydrogenase	P08200	*icd*	Energy metabolism
m	Isocitrate lyase	P0A9G6	*aceA*	Energy metabolism
g	Lactaldehyde dehydrogenase	P25553	*aldA*	Carbon compound utilization
a	Maltose/maltodextrin import ATP-binding protein MalK	P68187	*malK*	Sugar transport
3, 7, 8, 15, 16, 19, 24, 29, 35	Periplasmic oligopeptide-binding protein	P23843	*oppA*	Amino-acid transport
27	PTS system mannose-specific EIIAB component	P69797	*manX*	Sugar transport
22	Purine nucleoside phosphorylase DeoD-type	P0ABP8	*deoD*	Purine ribonucleotides and deoxyribonucleotides metabolism
i	Putative NAD(P)H nitroreductase YdjA	P0ACY1	*ydjA*	Oxidation-reduction process
f	Serine hydroxymethyltransferase	P0A825	*glyA*	Amino-acid biosynthesis
31	Succinyl-CoA ligase	P0AGE9	*sucD*	Energy metabolism
b	Triosephosphate isomerase	P0A858	*tpiA*	Energy metabolism
				Chaperones and stress responsive proteins
26	Alkyl hydroperoxide reductase subunit F	P35340	*ahpF*	Response to reactive oxygen species
2	Chaperone protein ClpB	P63284	*clpB*	Response to heat damage
30	Chaperone protein DnaK	P0A6Y8	*dnaK*	Response to heat damage
1	FKBP-type 22 kDa peptidyl-prolyl cis-trans isomerase	P0A9L3	*fklB*	Protein folding
12	Uncharacterized protein YqiC	Q46868	*yqiC*	Promotion of stress-induced mutagenesis (SIM) response
				Protein synthesis
34	50S ribosomal protein L10	P0A7J3	*rplJ*	Translation
e	Elongation factor Tu 2	P0CE48	*tufB*	Translation
c	Transcriptional regulatory protein OmpR	P0AA16	*ompR*	Transcription
Bgl$^+$				
				Metabolism
52	2,5-Diketo-D-gluconic acid reductase A	Q46857	*dkgA*	Carbon compound utilization
61	ATP-dependent 6-phosphofructokinase isozyme 1	P0A796	*pfkA*	Energy metabolism
46, 54	Cytidine deaminase	P0ABF6	*cdd*	Pyrimidine nucleotide metabolism

Table 1. Continued

Spot No.	Protein description	AC[a)]	Gene name	Functional group
66	Low specificity L-threonine aldolase	P75823	*ltaE*	Amino-acid biosynthesis
71	Nucleoside diphosphate kinase	P0A763	*ndk*	Nucleotides biosynthesis
57	Putative ABC transporter arginine-binding protein 2	P30859	*artI*	Amino-acid transport
67	Regulator of nucleoside diphosphate kinase	P0AFW4	*rnk*	Nucleotides biosynthesis
60	*sn*-Glycerol-3-phosphate-binding periplasmic protein UgpB	P0AG80	*ugpB*	Phospholipid biosynthesis
42	Succinate dehydrogenase iron-sulfur subunit	P07014	*sdhB*	Energy metabolism
48	Uncharacterized oxidoreductase YajO	P77735	*yajO*	Oxidation-reduction process
				Chaperones and stress responsive proteins
45	60 kDa chaperonin	P0A6F5	*groL*	Folding
58	LexA repressor	P0A7C2	*lexA*	Response to DNA damage
53	Malate dehydrogenase	P61889	*mdh*	Promotion of stress-induced mutagenesis (SIM) response
51	NAD(P)H dehydrogenase (quinone)	P0A8G6	*wrbA*	Response to oxidative stress
75	Osmotically-inducible protein Y	P0AFH8	*osmY*	Response to osmotic stress
65	Periplasmic AppA protein	P07102	*appA*	Response to anoxia
69	Peroxiredoxin OsmC	P0C0L2	*osmC*	Response to oxidative stress
73	Universal stress protein D	P0AAB8	*uspD*	Response to oxidative stress and DNA damage
76	Universal stress protein F	P37903	*uspF*	Response to oxidative stress
62	UPF0234 protein YajQ	P0A8E7	*yajQ*	Response to DNA damage
59	Vitamin B12 transport periplasmic protein BtuE	P06610	*btuE*	Response to oxidative stress
				Protein synthesis
63, 72	Ferric uptake regulation protein	P0A9A9	*fur*	Transcription
44	UPF0381 protein YfcZ	P0AD33	*yfcZ*	Unknown function

a) Accession number in UniProtKB database.

molecular chaperones components of a stress-induced multi-chaperone system DnaK and ClpB (encoded by *dnaK* and *clpB*, respectively). DnaK is the major bacterial Hsp70, a family of highly conserved 70-kDa heat shock proteins implicated in protein folding, repair and degradation. Its protective role during heat shock is well documented and many other stresses can also induce its production [42]. The expression profile of the DnaK protein was verified here by immunoblotting of whole-cell extracts with monoclonal anti-DnaK antibody. In both strains two bands reacted with anti-DnaK antibody corresponding to a polypeptide with expected molecular weight of 70 kDa, and with an apparent molecular

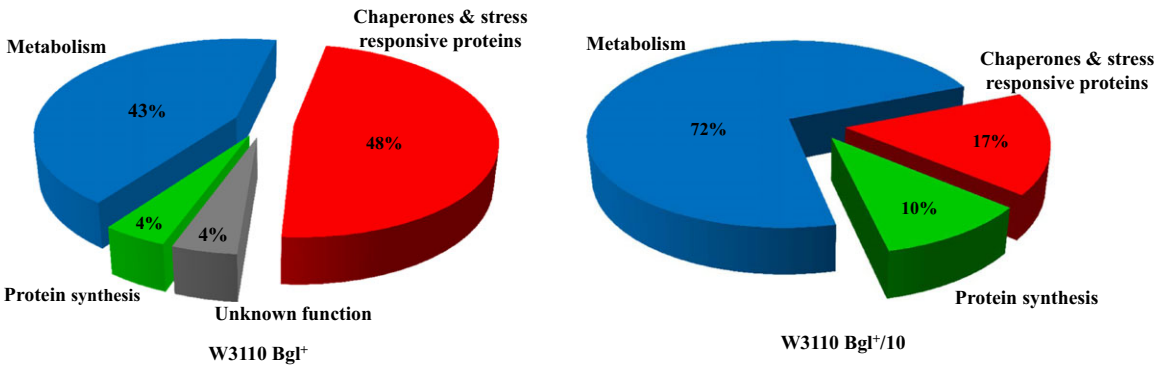

Figure 3. Functional distribution of MS identified differentially expressed proteins. 2DE and MS identified expression-changing proteins were grouped, using UniProtKB/Gene Ontology/Biological Process tool and EcoCyc database, into three main functional groups.

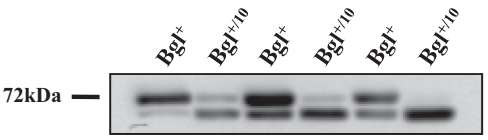

Figure 4. 1D Western blot validation of DnaK protein expression pattern. Whole-cell protein lysates from W3110 Bgl^+ ($n = 3$) and W3110 Bgl^+/10 ($n = 3$) were immunostained with monoclonal anti-DnaK antibody.

weight 1–2 kDa higher, respectively (Fig. 4). While the former band (corresponding to the major form of DnaK) showed always higher intensity in W3110 Bgl^+/10 than in W3110 Bgl^+, thus validating proteomic data, the latter followed the opposite trend. It is well known that DnaK undergoes to reversible phosphorylation which decreases its electrophoretic mobility and isoelectric point [43], thus our data might indicate a prevalence of the phosphorylated form of DnaK in W3110 Bgl^+. Highly phosphorylated proteins accumulated during heat shock, stationary phase and starvation for a carbon source, iron, or amino acids, all of which are known to induce specific stress responses [44]. Recently, the phosphoproteome analysis has shown a global increase of protein phosphorylation levels in the late stationary phase, pointing to a specific role of this modification in later phases of growth [18].

Altogether the results obtained indicate that the two strains oppose, at the conditions applied, different survival strategies, while W3110 Bgl^+ invests energy in enhancing stress resistance and restrain growth, W3110 Bgl^+/10 continues to proliferate by squandering resources and postponing onset of stationary phase.

4 Concluding remarks

Here we present the characterization of a mutant expressing a GASP phenotype isolated from an aged culture of the *E. coli* W3110 Bgl^+ strain. Data from both *in vitro* and proteomic analysis show an enhanced metabolic activity of the mutant compared to the parental strain according to the acquired observation that, under starvation, GASP phenotype cells show better ability to adapt and can scavenge poor nutrient sources formed by the dead cells [3]. Previous data indicate that three of the four known GASP mutations coexist in a single survivor significantly expanding its metabolic capacity [7]. Similarly, we have found more beneficial metabolic traits associated to W3110 Bgl^+/10. Moreover, the change in the protein abundance of the major form of DnaK in this survivor compared to the parental strain evokes a suggestive scenario given the well-known role of Hsp70 proteins in lifespan extension [45]. Further studies are required to better characterize W3110 Bgl^+/10. Nevertheless, this is, to our knowledge, the first proteomic investigation of an *E. coli* strain derived from a situation of surprising resilience of 10-years batch culture regime.

This work was partially supported by the Italian Ministry of Education, University and Research (project MIUR-PRIN 2008BPLF7Y) to LM.

The authors have declared no conflict of interest.

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