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Clp-Dependent Proteolysis Down-Regulates Central Metabolic Pathways in Glucose-Starved *Bacillus subtilis*▽†

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Entry into stationary phase in *Bacillus subtilis* is linked not only to a redirection of the gene expression program but also to posttranslational events such as protein degradation. Using ^{35}S-labeled methionine pulse-chase labeling and two-dimensional polyacrylamide gel electrophoresis we monitored the intracellular proteolysis pattern during glucose starvation. Approximately 200 protein spots diminished in the wild-type cells during an 8-h time course. The degradation rate of at least 80 proteins was significantly reduced in *clpP*, *clpC*, and *clpX* mutant strains. Enzymes of amino acid and nucleotide metabolism were overrepresented among these Clp substrate candidates. Notably, several first-committed-step enzymes for biosynthesis of aromatic and branched-chain amino acids, cell wall precursors, purines, and pyrimidines appeared as putative Clp substrates. Radioimmunoprecipitation demonstrated GlmS, IlvB, PurF, and PyrB to be novel ClpCP targets. Our data imply that Clp proteases down-regulate central metabolic pathways upon entry into a nongrowing state and thus contribute to the adaptation to nutrient starvation. Proteins that are obviously nonfunctional, unprotected, or even "unemployed" seem to be recognized and proteolyzed by Clp proteases when the resources for growth become limited.

In its natural environment, the free-living and plant-associated gram-positive soil bacterium *Bacillus subtilis* often encounters poor nutrient supply. Upon entering the nongrowing state, e.g., owing to glucose exhaustion, general stress and starvation proteins are induced. The general stress response comprises the induction of σ^B-dependent genes, the stringent response, and sporulation, whereas the glucose starvation-specific response additionally consists of proteins for the utilization of alternative carbon sources and gluconeogenesis (6). The major physiological role of the stringent response is to prevent the continued synthesis of proteins no longer required for nongrowing cells. Almost 1,000 genes are no longer expressed when cells enter the stationary phase induced by glucose starvation, most of them being under negative stringent control (6, 13, 25). The issue arises of whether proteins that are no longer required and that are available in excess in nongrowing cells are stable. Glucose starvation not only affects the synthesis rates of adaptive proteins but also elicits the breakdown of already existing proteins.

Apart from the ATP-dependent proteases LonA (42, 44), LonB (30, 45), FtsH (51), and HslUV (also called ClpYQ; see reference 20), the Clp proteases represent the main intracellular proteolytic systems in *B. subtilis* (14, 15, 27, 28, 34–36, 40, 46). Clp proteins form hetero-oligomeric complexes with AAA^+ ATPases (ATPases associated with a variety of cellular activities) and a proteolytic component. The ATPases possess chaperone activity and interact as hexameric rings with the proteolytic component ClpP composed of two heptameric rings (21, 47) to degrade selected proteins (reviewed in reference 16). Three different Clp ATPases exist in *B. subtilis*, ClpC, ClpE, and ClpX, all of which can form complexes with ClpP (14). The ATPases, assisted by adaptor proteins, recognize substrates and deliver them in an unfolded state in an ATP-dependent process to ClpP for degradation (16). Clp-mediated proteolysis is important not only during heat stress to remove denatured and aggregated proteins (3, 15, 27–29, 34, 48) but also for processes observed during stationary phase such as extracellular enzyme synthesis, motility, competence development, and sporulation (11, 35, 36, 40, 46).

A few specific Clp substrates in *B. subtilis* have already been identified and characterized. ComK, the transcriptional activator of competence genes (46), and SpoIIAB, the anti-sigma factor of the sporulation sigma factor σ^F (40), are degraded by ClpCP. CtsR, the transcriptional repressor of the class III heat shock genes, previously has been shown to be degraded by ClpCP and ClpEP (24, 29, 34). HrcA, the repressor of class I heat shock genes, is degraded primarily by ClpXP when fused to an artificial *ssrA* degradation tag (50). Spx, a transcriptional regulator that inhibits activator-stimulated transcription by interaction with the C-terminal domain of the α subunit of the RNA polymerase (e.g., *comA* and *resD*) but activates transcription of certain genes in response to oxidative stress (e.g., *trxA* and *trxB*), previously was identified as a ClpXP substrate in *B. subtilis* (37, 38). However, not only transcriptional regulators but also a biosynthetic enzyme such as MurAA, catalyzing the first committed step in cell wall biosynthesis, has been discovered to be a bona fide ClpCP substrate (26).

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In this study, we monitored the stability of exponential-phase intracellular *B. subtilis* proteins during glucose-limited growth arrest for 8 h in the wild-type cells and in *clpP*, *clpC*, and *clpX* mutant cells by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE). Nearly one-fifth of all detected protein spots on a 2D gel with ca. 1,000 spots significantly decreased in intensity in the course of the stationary phase, and for ca. 80 of these spots the decrease was attenuated in the *clpP* mutant results. Among the Clp substrate candidates, enzymes of amino acid and nucleotide metabolism were clearly overrepresented. Intriguingly, several of the putative Clp substrates are first-committed-step enzymes of biosynthetic pathways for glutamate, aromatic amino acids, branched-chain amino acids, cell wall synthesis, purines, and pyrimidines. We demonstrated by immunoprecipitation that GlmS, IlvB, PurF, and PyrB are novel ClpCP substrates. In summary, our data imply that Clp-dependent proteolysis in *B. subtilis* efficiently shuts down central metabolic pathways under nongrowth conditions.

MATERIALS AND METHODS

Pulse-chase labeling and 2D PAGE. *B. subtilis* 168 cells and isogenic *clpP*, *clpC*, and *clpX* mutants (QB4916, BUG8, and chromosomal DNA of BEK90 transformed into the strain 168 background; see reference 14) were grown in Belitsky minimal medium without citrate supplemented with 4.5 mM glutamate until the mid-exponential phase (optical density at 500 nm, 0.5), and then 30 ml of culture was labeled with 500 μCi L-[^{35}S]-labeled methionine for 10 min followed by a chase with a 600,000-fold molar excess of cold methionine. This excess of cold methionine was sufficient to prevent further incorporation of radioactive methionine, as in reverse experiments (chase-pulse) no radioactive methionine was incorporated into proteins. To allow growth and labeling of the *clp* mutant strains, 0.01% yeast extract had to be added to the minimal medium. Samples of 4 ml each were taken immediately (t_0), 30 min, 1 h, 2 h, 4 h, 6 h, and 8 h after addition of cold methionine. Cells were centrifuged (10,000 × *g*, 4°C, 10 min), and washed in 500 μl and resuspended in 400 μl ice-cold TE buffer (10 mM Tris [pH 8.0], 1 mM EDTA) containing 1 mM phenylmethylsulfonyl fluoride (PMSF). After sonication for 3 1-min pulses at 55 W in an ice bath (Labsonic U; Braun), cell debris was removed by centrifugation (10,000 × g, 4°C, 30 min), and aliquots of the supernatant were used for measuring protein concentrations (Roti-Nanoquant; Roth) and incorporated radioactivity after precipitation with 10% trichloroacetic acid by scintillation counting (Tricarb 2900 TR; PerkinElmer). Two-dimensional PAGE was carried out using Immobiline dry strips (IPG; Amersham Biosciences) (pH4 to pH7) loaded with 80 μg of radiolabeled protein extract (6). The radioactivity of the t_0 sample was used to define the exposure time of a whole-gel series (all gels for the same time period). The dried gels were exposed to storage phosphor screens that were scanned using an SI PhosphorImager (Molecular Dynamics) or a Storm 840 system (Molecular Dynamics) at a resolution of 200 μm. Samples were prepared and analyzed in parallel in two independent experiments.

Dual-channel imaging, warping, and spot quantitation. Dual-channel imaging allowed visualization of changes in the protein pattern (5, 6). To avoid spot mismatches, distorted gels were adjusted using Delta2D 3.4 software (Decodon). The spots of the first 2D gel image from cells immediately after pulse-chase were artificially false-colored red, and the gels of subsequent time points were false-colored green. After warping and image superimposition, stable proteins appeared yellow, while spots of instable proteins turned red during the chase. Combining the dual-color gel images from the wild-type results in a successive series yielded a time-lapse "2D-PAGE degradation movie" (see video S1 in the supplemental material).

The Delta2D software was also used to quantify spot intensities. Quantitation datasets for each gel series were generated by warping the consecutive gels with respect to the respective t_0 gel. Before this the t_0 gels had been carefully compared with a comprehensive *B. subtilis* 2D master gel (12) so that protein names could be assigned to most spots. For each of the matched sets the software created an artificial fused union gel in which the outlines of all congruent, colocalized spots were merged into single outlines. These were then back-transferred to the constituent gels and served as a basis for the subsequent quantitation of relative spot intensities. Quantitation data were exported to Excel software (Microsoft), and the normalized spot volumes were calculated and corrected for the cell growth accordingly.

Purification of His$_6$-tagged IlvB and GlmS and antisera production. For overproduction of *B. subtilis* IlvB and GlmS in *Escherichia coli* BL21(DE3)pLysS, the genes were amplified by PCR using Platinum *Pfx* DNA polymerase (Invitrogen) and the following forward and reverse primer pairs (restriction enzyme recognition sites underlined): primers IKilvB-Bam-for (5′-CGGGATCCATGG GGACTAATGTACAGGT-3′) and IKilvB-Kpn-rev (5′-GGGGTACCCCTCA AGGTTTCACCCCCACCA-3′ and primers IKglmS-Bam-for (5′-CGGGATCC ATGTGTGGAATCGTAGGTTA-3′ and IKglmS-Kpn-rev (5′-GGGGTACCC CTTACTCCACAGTAACACTCT-3′).

The obtained PCR fragments were digested with BamHI or KpnI and cloned into pRSETA overexpression vectors (Invitrogen) containing the codons for six N-terminal histidine residues and a linker region fused to the target protein. The correctness of the coding sequence was confirmed by DNA sequencing (Agowa GmbH, Berlin, Germany). Recombinant His-tagged proteins (His$_6$-IlvB and His$_6$-GlmS) were expressed in *E. coli* after addition of 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) for at least 1 h and purified by nickel-nitrilotriacetic acid chelate affinity chromatography under denaturating (His$_6$-IlvB) or native (His$_6$-GlmS) conditions according to standard procedures of the manufacturer (Qiagen). The purified His$_6$-IlvB and His$_6$-GlmS proteins were used for custom antiserum production in rabbits by a standard immunization protocol (Pineda, Germany). Antibodies were prepared as previously described for MurAA (26), PurF (17), and PyrB (18).

Pulse-chase labeling and immunoprecipitation. For assaying the stability of GapA, GlmS, IlvB, MurAA, PurF, and PyrB in wild-type cells and *clp* mutants, cells were grown in glucose-limited (0.05%) Belitsky minimal medium without citrate supplemented with 0.01% yeast extract at 37°C. At an optical density at 500 nm of 0.5, cells were labeled with L-[^{35}S]-labeled methionine (16.7 μCi/ml). After 10 min of labeling, the radioactive methionine was chased by addition of a 600,000-fold molar excess of cold methionine and samples of 4 ml were taken at 0, 1, 2, 4, 6 and 8 h. After centrifugation (10,000 × g at 4°C for 10 min), cells were resuspended in 53.3 μl of lysis buffer (50 mM Tris-HCl [pH 7.5], 5 mM EDTA, 4 mg/ml [wt/vol] lysozyme, 1.4 mM PMSF) and incubated for 20 min at 37°C. For complete cell lysis 8 μl of 10% (wt/vol) sodium dodecyl sulfate (SDS) was added and the samples were incubated for 5 min at 95°C. Then, 720 μl of KI buffer (50 mM Tris-HCl [pH 8.0], 1 mM EDTA, 150 mM NaCl, 1% [vol/vol] Triton X-100, 1.4 mM PMSF) was added and samples were incubated on ice for 15 min. After centrifugation (10,000 × g, 4°C, 45 min) the supernatants were incubated with specific polyclonal antisera (diluted 1:30) overnight with slow-tilt rotation at 4°C. A suspension of 40 μl of protein A-coated Dynabeads (Dynal) equilibrated with KI buffer was added to each sample for an additional incubation time of 2 h. The beads were washed three times in 500 μl of KI buffer and finally boiled in 10 μl of SDS sample buffer for 5 min at 95°C. The samples were separated by SDS-PAGE using Mini-Protean cells (Bio-Rad) with an appropriate marker (PageRuler prestained protein ladder; Fermentas). After electrophoresis gels were vacuum-dried and exposed to phosphor screens (Molecular Dynamics) overnight. Autoradiographs were scanned with an SI PhosphorImager (Molecular Dynamics) or a Storm 840 system (Molecular Dynamics). Antigen-specific signals were evaluated by size comparisons with the transferred marker, quantitated with ImageQuant (Molecular Dynamics), and used to calculate regression curves for half-life determinations.

RESULTS

Entry into glucose-limited stationary phase triggers extensive intracellular protein degradation in wild-type *B. subtilis*. To detect protein degradation during glucose starvation we performed radioactive pulse-chase labeling of exponentially growing cells followed by a 2D separation between pI4 and pI7 of consecutive samples drawn between 0 and 8 h of chase. Most of the metabolic enzymes are visualized in this main proteomic window (12). The normalized spot volumes of the matched 2D autoradiographs were used to distinguish between degradation and stabilization. For visualization we superimposed false-colored gels of the wild-type series to generate a time-lapse "protein degradation movie" with red-turning spots as putative degradation candidates (see video S1 in the supplemental material).

After quantitation nearly 200 protein spots appeared to decrease in intensity during the time span in the wild-type cell assay. More than 70% of these showed a reduction in intensity of at least a third (i.e., from 100% at t_0 to an at least 66.6% normalized spot volume after 8 h), among them spots representing many anabolic enzymes (Fig. 1). Enzymes involved in amino acid, cell wall, and nucleic acid synthesis were preferentially affected, at least in the investigated "2D window" (Table 1), but enzymes for vitamin biosynthesis, aminoacyl-tRNA synthetases, the large subunit of the glutamate synthase (GltA), the pyruvate carboxylase (PycA), and the cold shock protein CspD also decreased. Furthermore, some general stress proteins, the glucose-inhibited division proteins (Gid, GidA), the chromosome replication initiation protein DnaA, the chemotaxis protein CheW, and the glutamate dehydrogenase protein RocG, as well as an inactive glutamate dehydrogenase (GudB; see reference 4), seemed to be unstable. Moreover, methylthioribose recycling enzymes, proteins involved in surfactin production and competence (SrfAC), and SpoVG, which is required for spore cortex synthesis, were degraded. Likewise, several proteins of unknown function were found to be diminished, namely, YhaM, a member of a newly identified 3′-to-5′ exonuclease family (39); YkqC, a recently identified essential RNase RNase J1 that processes 16S rRNA (8) and colocalizes with ribosomes (19); YkuU, which exhibits similarity to 2-cys peroxiredoxins; YugI, a putative polyribonucleotide nucleotidyltransferase; YurP, a putative glutamine-fructose-6-phosphate transaminase involved in the metabolism of α-glycated amino acids (49); and Zwf, a glucose-6-phosphate dehydrogenase, as well as other as-yet-unidentified proteins (represented by D-labeled spots; see Fig. 1 to 4). In some cases modified forms of the same enzyme, probably deformylated variants with and without start methionine and formylated forms (2), for example, GltA, GltA1, and GltA2, IlvB1 and IlvB2, LeuC1 and LeuC2, MetE1 and MetE2, PurB1 and PurB2, PycA, PycA1, and PycA2, ThiC1 and ThiC2, and ThrS1 and ThrS2, were found to have been degraded.

The most clearly degraded putative degradation candidates are listed in Table 1 according to their functional categories.

Protein degradation is attenuated in *clp* gene mutants. To ascertain whether the Clp protease is involved in the observed broad protein breakdown pattern and to identify novel Clp substrate candidates the pulse-chase labeling experiment was repeated with isogenic *clp* mutants, i.e., single-knockout mutants of the *clpP* peptidase and the *clpC* and *clpX* ATPase genes. The ClpE ATPase was not synthesized in the wild-type experiments under the tested conditions, and the results seen with a *clpE* mutant gel series resembled those seen with the wild-type series, which indicates that ClpE is not involved in the degradation of proteins during glucose starvation (data not shown). Spots were regarded as representing greater stability compared to the wild-type results when the normalized and corrected spot volume remained at 66% or more in a *clp* mutant gel series over the 8-h time course. Some proteins showed up as being degraded independently of Clp or were modified or disappeared from the cytosolic fraction, because the spots in this category decreased in both the wild-type and the *clp* mutant cell results. Examples are CspD, the acidic Efp spot, GudB, MoeA, SpoVG, MetC2 (see reference 1), and YurU (Fig. 1, 2, 3, and 4; Table 1). For many proteins a relatively high variability of the spot intensity values was observed, with the result that numerous spots did not pass the 66% stabilization threshold in the *clp* mutants. However, we concluded that approximately 80 protein species are Clp substrates, as assessed by significantly slower degradation kinetics in the *clpP* mutant and either of the *clpC* and *clpX* mutant results. With respect to the ATPase involved in degradation, most of the ClpP-dependent spots appeared to be linked to ClpC and some showed overlapping substrate specificity with respect to ClpC and ClpX (e.g., for GyrA, 90% stabilization in a *clpC* mutant and 100% stabilization in a *clpX* mutant) (Table 1). Almost no protein spot was able to be assigned to ClpXP alone, at least not within the 2D PAGE window investigated under the conditions tested. We tried to corroborate this specificity overlap by constructing a *clpC*/*clpX* double mutant and assessing protein stability in this mutant. Unfortunately, the double mutant was not viable in minimal medium. Several putative Clp substrates are involved in the metabolism of amino acids and related molecules (CarB, AroA, GlmS, GltA, IlvB, IlvC, IlvD, LeuC, LeuD, LysCα, LysCβ, MetE, MetI [see reference 1], and ThrC) and in the metabolism of nucleotides and nucleic acids (PurB, PurF, PurL, PurQ, PurS, and PyrB). The spots corresponding to about 30 potential substrates have not been identified so far using the *B. subtilis* proteome (D-labeled spots), because these spots are difficult to localize on stained 2D gels. The remaining spots represented proteins from various functional categories (Table 1).

Clp-dependent proteolysis shuts down central biosynthetic pathways. Intriguingly, some of the identified Clp substrate candidates catalyze first committed steps of biosynthetic pathways: GlmS and MurAA supply precursors of cell wall synthesis; AroA carries out the first reaction for formation of the aromatic amino acids whereas IlvB initiates the biosynthesis of the branched-chain amino acids and GltA converts alpha-ketoglutarate into glutamate and seems also involved in biofilm formation (9); and CarB and PyrB mark the beginning of pyrimidine biosynthesis and PurF marks that of purine biosynthesis. PycA fuels the anaplerotic shunt by which pyruvate is converted into oxaloacetate to replenish the citric acid cycle.

In order to assess the influence of the Clp proteins on the stability of some of these crucial proteins in vivo we used freshly produced or available antisera to immunoprecipitate GlmS, MurAA, IlvB, PurF, and PyrB from wild-type and *clp* mutant cell extracts in a pulse-chase setup. In all five cases the autoradiographs showed stabilization in the *clpP* and *clpC* mutant assays as opposed to degradation in the wild-type and the *clpX* mutant assays (Fig. 5). The half-live of all of these enzymes was roughly 1.5 h in wild-type cells during the early stationary phase, as determined by analysis of the quantified band intensities (Fig. 5).

DISCUSSION

Protein degradation is increasingly acknowledged as an essential posttranslational mechanism to exert regulatory switches and readjust the distribution of cellular resources. In *B. subtilis*, Clp proteases are the main components of ATP-dependent degradation, and *clp* mutants show highly pleiotropic phenotypes (15, 28, 34, 35). So far, denatured proteins or specific regulatory proteins (e.g., ComK, SpoIIAB, CtsR, Spx)

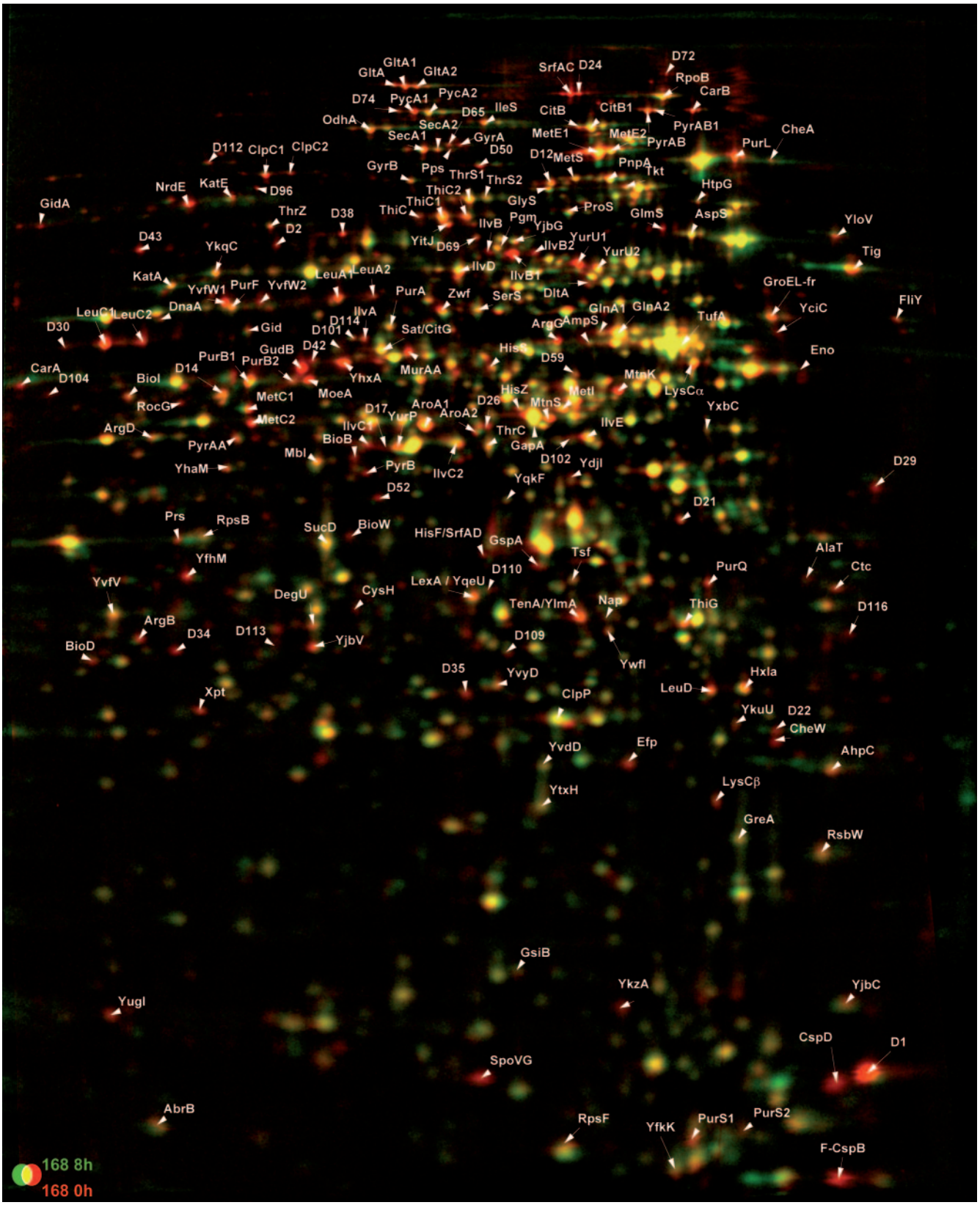

FIG. 1. Pulse-chase experiment followed by 2D PAGE of *B. subtilis* 168 wild-type cells. A false-colored gel-pair image is shown (red, 0 h after labeling; green, 8 h after labeling).

TABLE 1. Selected degradation candidates during growth into the glucose-limited stationary phase identified by pulse-chase labeling and 2-D PAGE

Functional category and enzyme	Stability (% remaining protein after 8 h of pulse-chase labeling)			
	Wild type	Δ*clpP*	Δ*clpC*	Δ*clpX*
Cell wall biosynthesis				
GlmS	12	85	71	20
MurAA	22	49	75	16
Amino acid biosynthesis				
ArgB	38	54	80	100
AroA1	41	68	93	28
CarB	6	97	71	22
IlvA	17	61	84	26
IlvB1	8	74	39	17
GltA1	11	80	82	64
GltA2	22	91	>100	51
LeuA1	19	71	30	47
LeuA2	20	56	27	83
LeuC1	9	69	41	18
LeuC2	14	67	50	39
LeuD	28	77	63	38
LysCα	31	91	76	22
LysCβ	21	79	73	20
MetE1	39	75	75	70
Nucleic acid biosynthesis				
NrdE	24	38	70	25
PurB1	28	75	>100	>100
PurB2	34	100	69	100
PurF	36	72	77	10
PurL	30	95	61	17
PurQ	19	44	>100	13
PyrB	21	61	64	49
Xpt	26	79	ND	21
Vitamin biosynthesis				
BioB	18	43	56	19
ThiC1	22	69	90	40
ThiC2	28	62	>100	47
ThiD (YjbV)	39	57	74	52
Aminoacyl-tRNA synthetases				
IleS	49	87	69	100
ThrS	47	66	68	94
ThrZ	27	65	>100	ND
Glycolysis				
PycA1	10	45	50	56
Ribosomal proteins				
Efp	32	35	40	41
Tig	47	>100	96	78
TufA	50	45	65	76
DNA replication				
GyrA	24	89	90	>100
GyrB	48	60	53	80
RNA polymerase subunits				
RpoB	35	84	>100	73
Clp ATPase				
ClpC1	12	83	x	67
ClpC2	22	95	x	98
Cold shock				
F-CspB	10	59	11	38
CspD	12	23	19	14
General stress response				
Ctc	50	98	67	48
OsmC(YkzA)	21	61	23	9
Secretion				
SecA1	32	62	76	84
SecA2	39	82	93	>100
Chemotaxis				
CheW	9	>100	ND	59
Inactive glutamate dehydrogenase				
GudB	2	28	18	18
Methionine salvage pathway				
MtnK (YkrT)	19	93	45	22
MtnS (YkrS)	41	64	60	37
Sporulation				
SpoVG	10	19	5	20
Unknown function				
YfhM	24	62	100	93
YhaM	40	75	99	65
YhxA	11	65	90	72
YkqC	49	72	>100	100
YloV	47	>100	59	43
YugI	19	45	67	43
YurU1	18	31	10	20
YvfW2	19	48	74	45
Degradation candidates				
D112	26	72	79	72
D116	42	100	56	26
D12	26	100	51	54
D34	14	75	23	65
D38	12	85	100	26
D42	18	71	49	12
D43	8	>100	36	87
D50	22	82	49	>100
D65	19	89	63	100
D74	28	54	>100	>100

have been identified as targets for proteolysis. In this report we suggest that proteins no longer required by the cells and, as a consequence, no longer protected against proteolysis form a third class of Clp target proteins. This study was aimed at pinpointing proteins that are degraded after entry into glucose-limited stationary phase and at assessing which of these proteins are degraded by the Clp protease.

Under our experimental conditions approximately 200 protein spots diminished in the wild-type strain assays during an 8-h time course. Filtering out those spots with results showing a similar decrease in the *clp* mutant assays revealed at least 80 spots to be potential Clp substrates, i.e., the proteins were stabilized or less quickly degraded in the *clpP* peptidase gene and in either the *clpC* or the *clpX* ATPase gene mutant cells

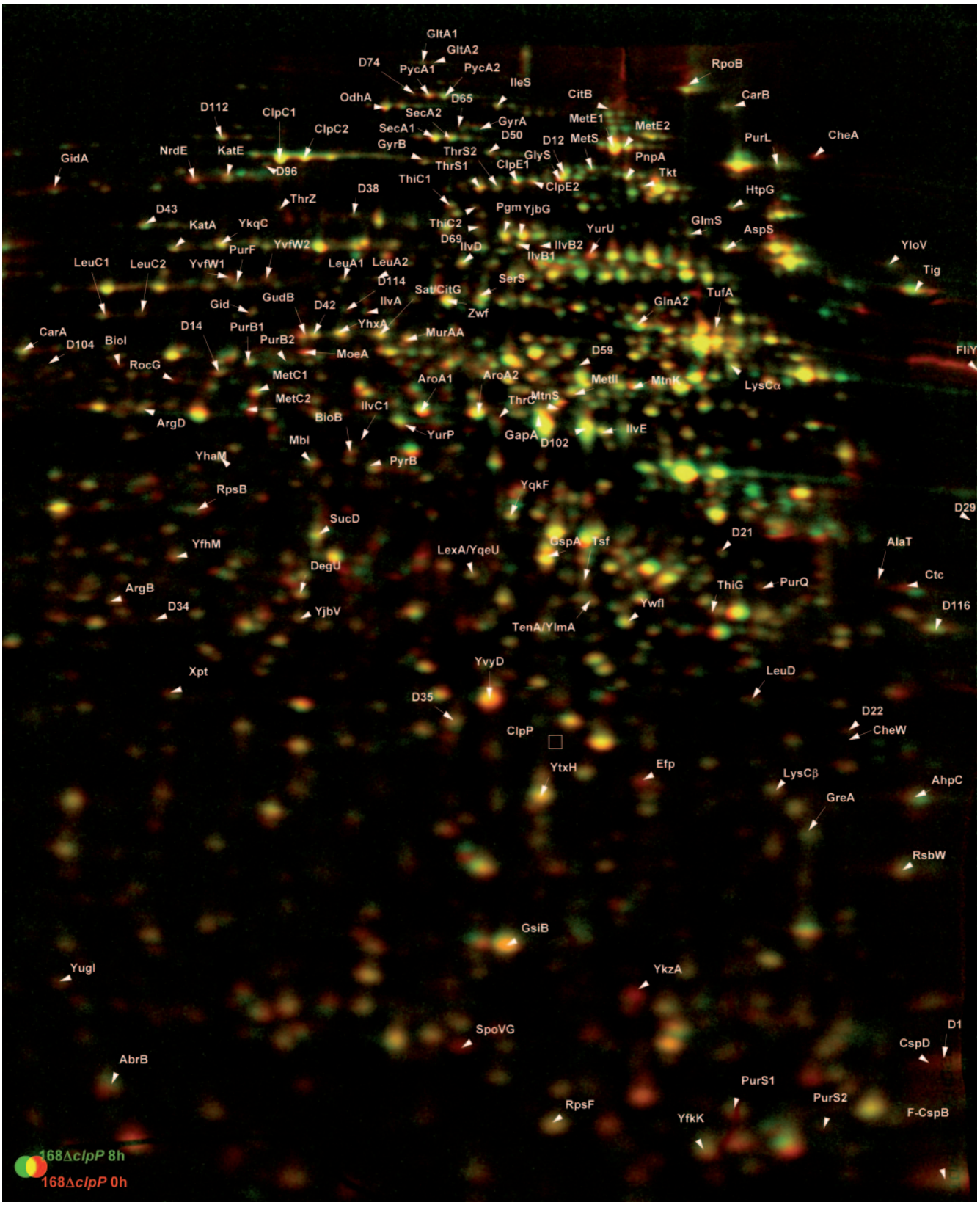

FIG. 2. Pulse-chase experiment followed by 2D PAGE of the isogenic Δ*clpP* mutant cells. A false-colored gel-pair image is shown (red, 0 h after labeling; green, 8 h after labeling).

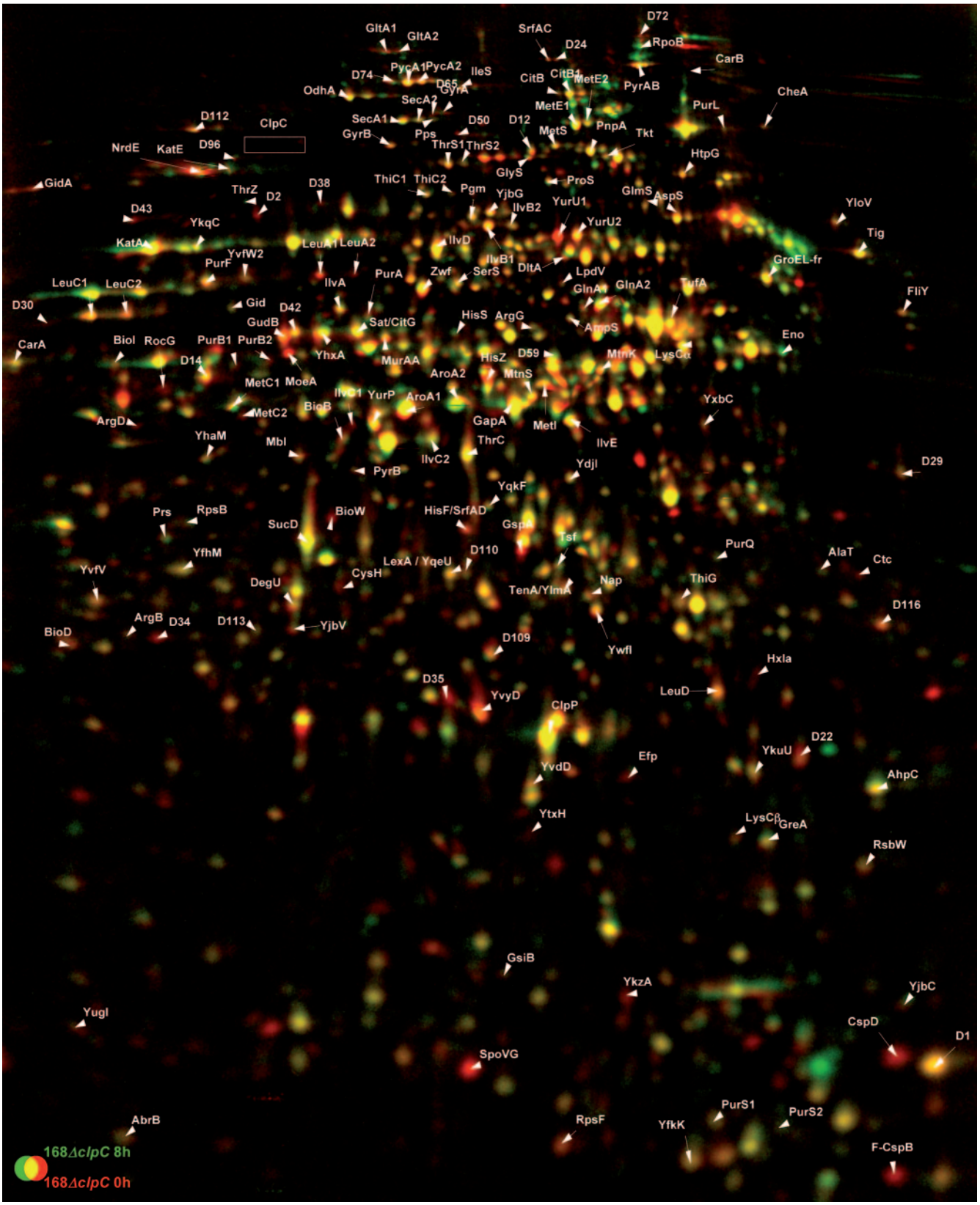

FIG. 3. Pulse-chase experiment followed by 2D PAGE of the isogenic Δ*clpC* mutant cells. A false-colored gel-pair image is shown (red, 0 h after labeling; green, 8 h after labeling).

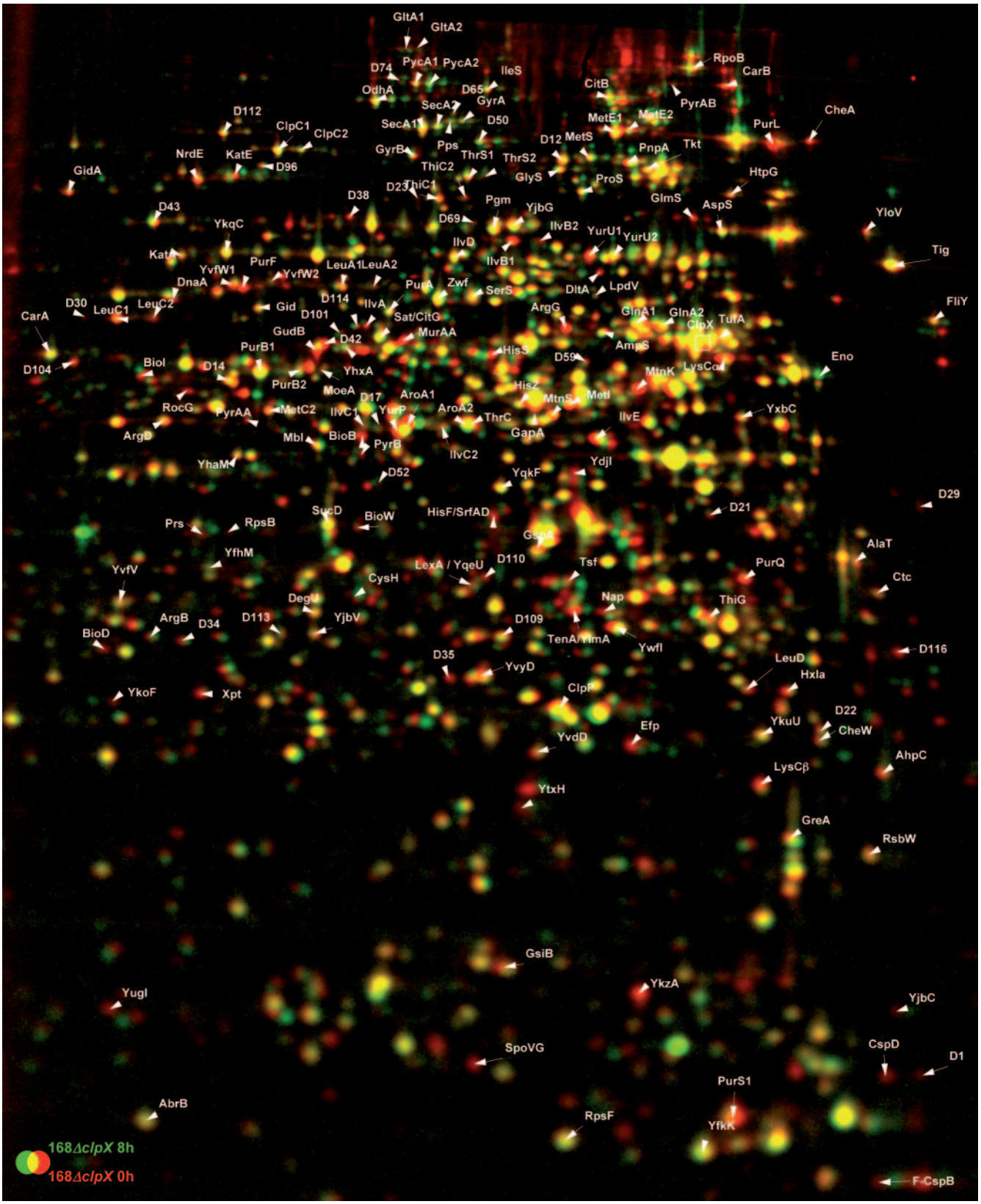

FIG. 4. Pulse-chase experiment followed by 2D PAGE of the isogenic Δ*clpX* mutant cells. A false-colored gel-pair image is shown (red, 0 h after labeling; green, 8 h after labeling).

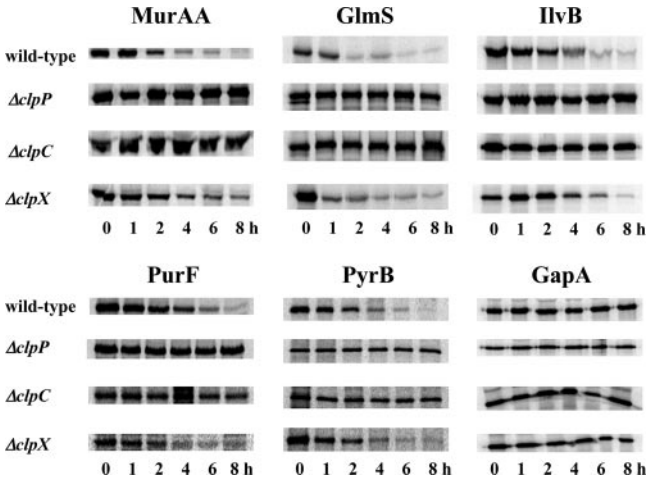

FIG. 5. Pulse-chase labeling and immunoprecipitation of MurAA, GlmS, IlvB, PurF, PyrB, and GapA during glucose starvation in wild-type cells and different *clp* mutant cell backgrounds.

than in the wild-type cells. The greatest number of proteins appeared to be degraded by ClpCP alone, but numerous others were degraded by both ClpCP and ClpXP. Not a single spot was found that had been exclusively stabilized in a *clpP* and a *clpX* mutant in the analytical window examined. In terms of functional categories the metabolism of amino acids and related molecules and the metabolism of nucleotides are clearly overrepresented among the potential Clp substrates.

An obvious explanation for the high degree of protein breakdown demonstrated by our wild-type pulse-chase gel series assays relates to the fact that under nongrowing conditions the need for anabolic resources targeted for growth and proliferation is minimized. Transcription of the genes for many of the proteins found to be degraded in this study has been shown to be down-regulated in the stationary phase (25), and the mRNAs for selected substrate candidates (MurAA, IlvB, and AroA and MetE) diminished in wild-type cells as well as *clp* mutant cells (data not shown).

Biosynthetic enzymes are often incorporated into large multiprotein complexes to optimize the synthesis and channeling of precursor and intermediate molecules. It has been suggested that during the absence of substrates or cosubstrates a catalytic center or a metal binding site of a protein may be damaged; similarly, a protein can undergo structural perturbation without other components that normally hold it in native conformation (7, 17, 18, 31–33, 41). These proteins could display a nonfunctional, unprotected, or even "unemployed" state, and it can be hypothesized that ATP-dependent proteases recognize these idle and defective proteins and degrade them.

However, other proteins that appear to be equally dispensable at high concentrations during nongrowth appeared stable under the circumstances tested; examples include glyceraldehyde-3-phosphate dehydrogenase (GapA; see Fig. 5 and Fig. 1 to 4), citric acid cycle enzymes such as isocitrate dehydrogenase (Icd), malate dehydrogenase (Mdh), and succinyl-coenzyme A synthetase (SucD), subunits of the pyruvate dehydrogenase (PdhA, PdhC, PdhD), phosphoglycerate dehydrogenase (SerA), an enzyme involved in serine biosynthesis, and the ribosomal proteins EF-Tu, FusA, RpsB, and RpIJ. Either these proteins are degraded at an average rate (in which case the normalization per time point serves to level out the decrease) and only appear stable or they really remain unaffected by the breakdown of idle and defective proteins and are still potentially functional. Several additional aspects may have an impact on protein stability, among them the nutritional state of the cell. For instance, Bond et al. (7) demonstrated that the *B. subtilis* aspartate transcarbamoylase (PyrB), catalyzing the first committed step of pyrimidine biosynthesis, was rapidly turned over with a half-life of 1.5 h in exponentially growing cells with ammonium as the nitrogen source, whereas supplementation with certain amino acids blocked this turnover.

In this study we provided evidence for the influence of ClpCP on the stability of PyrB. Strikingly, several other first-committed-step enzymes, namely, those for biosynthesis of aromatic and branched-chain amino acids, glutamate, cell wall precursors, purines, and pyrimidines, were shown to be Clp substrate candidates. We demonstrated in a previous study (26) that MurAA is a ClpCP substrate. Using the same immunoprecipitation approach we now also define GlmS, IlvB, PurF, and PyrB as ClpCP substrates. On the one hand, it will be interesting to see whether the other first-committed-step enzymes (AroA, CarB, GltA, PycA) will react similarly and can be added to the list of Clp substrates once antisera are available. On the other hand, it will be necessary to specifically test the degradation profiles of substrate candidates further down the biosynthetic pathways. For example, IlvC and IlvD act at the second and third step, respectively, of branched-chain amino acid biosynthesis; LeuC and LeuD form an enzyme complex for the second reaction with respect to leucine, and MetE and ThrC catalyze the final steps of methionine and threonine biosynthesis, respectively. Apart from PurF three other purine biosynthetic enzymes catalyzing the fourth step (PurQ, PurL, and PurS) and one catalyzing the eighth step (PurB) show up as Clp substrates. Indeed, it seems that these enzymes were degraded with different kinetics; enzymes catalyzing the last steps of the purine biosynthetic pathway were proteolyzed more slowly than the initial and intermediate enzymes. Determining the half-lives of these proteins in the wild-type and *clp* mutant cells will help to define whether degradation of first-committed-step enzymes constitutes a special case. Furthermore, the newly described Clp substrates may represent just the tip of the iceberg, because not all the cells of a population will behave in similar ways.

Moreover, adaptor proteins are known in many cases to be present to assist the Clp machine in the degradation process, which adds another level of regulation to proteolysis (10, 16, 22–24, 43, 52). In any case, Clp-dependent proteolysis has a decisive impact on central metabolic pathways in *B. subtilis*. Our data imply that Clp proteases down-regulate biosynthetic pathways for cell wall precursors, amino acids, and nucleotides upon entry into a nongrowing state, thus contributing to an economical adaptive redistribution of cellular resources under these limiting conditions.

Although our data increase the number of recognized ClpCP substrates, we have not been able to propose common sequence motifs by which these proteins are recognized. It is conceivable that the characteristics which render a protein a Clp substrate lie beyond the primary amino acid sequence; it may be instead a structural feature of some other sort. Appar-

ently, nonfunctional, unprotected, or "unemployed" proteins are recognized and degraded by Clp proteases.

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