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Global Role for ClpP-Containing Proteases in Stationary-Phase Adaptation of *Escherichia coli*

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To elucidate the involvement of proteolysis in the regulation of stationary-phase adaptation, the *clpA*, *clpX*, and *clpP* protease mutants of *Escherichia coli* were subjected to proteome analysis during growth and during carbon starvation. For most of the growth-phase-regulated proteins detected on our gels, the *clpA*, *clpX*, or *clpP* mutant failed to mount the growth-phase regulation found in the wild type. For example, in the *clpP* and *clpA* mutant cultures, the Dps protein, the WrbA protein, and the periplasmic lysine-arginine-ornithine binding protein ArgT did not display the induction typical for late-stationary-phase wild-type cells. On the other hand, in the protease mutants, a number of proteins accumulated to a higher degree than in the wild type, especially in late stationary phase. The proteins affected in this manner include the LeuA, TrxB, GdhA, GlnA, and MetK proteins and alkyl hydroperoxide reductase (AhpC). These proteins may be directly degraded by ClpAP or ClpXP, respectively, or their expression could be modulated by a protease-dependent mechanism. From our data we conclude that the levels of most major growth-phase-regulated proteins in *E. coli* are at some point controlled by the activity of at least one of the ClpP, ClpA, and ClpX proteins. Cultures of the strains lacking functional ClpP or ClpX also displayed a more rapid loss of viability during extended stationary phase than the wild type. Therefore, regulation by proteolysis seems to be more important, especially in resting cells, than previously suspected.

Controlled degradation of cytoplasmic proteins has long been considered essential for survival of bacteria under conditions of severe stress, due to the requirement for efficient removal of misfolded or otherwise damaged proteins (18, 19, 24, 61). The significance of proteolysis has recently been emphasized by the discovery that, in addition to quality control, proteolytic processes participate in a number of regulatory mechanisms. Several bacterial regulatory proteins are subject to conditional proteolysis, thus allowing rapid adjustment of their intracellular levels (20). In *Escherichia coli*, the division regulator SulA and the positive regulator of capsule synthesis RcsA are under proteolytic control (51, 63). Also, transformation competence in *Bacillus subtilis* and cell cycle control in *Caulobacter crescentus* are partly determined by proteolysis (29, 64). Several stress responses are regulated by proteolysis in *B. subtilis* and *E. coli* (12, 16, 17, 34, 35, 42). For example, the heat shock response in *E. coli* is partly controlled by proteolysis of the σ factor σ^{32} (RpoH) (6, 60). Intracellular levels of σ^{s} (RpoS, σ^{38}), the central regulator of the general stress response, are controlled by the protease ClpXP and the recognition factor RssB (4, 52, 53, 71). In addition to roles in quality control and regulation, proteolysis generates amino acids during the initial (stringent) phase of starvation (8). Also, at least two cytoplasmic enzymes are under proteolytic control in *E. coli*, namely cyclopropane fatty acid synthase and homoserine *trans*-succinylase (5, 9, 65).

The Clp proteases (ClpXP and ClpAP) are the best-characterized protease systems described in prokaryotes and have served as model systems for protease structure and function (10, 33, 48, 72). Together with the Lon protease, the Clp proteases are responsible for at least 70% of protein degradation in *E. coli* (46). In *E. coli*, the ClpAP protease was found to be crucial for degradation of starvation proteins, and mutations in *clpP* confer a competitive disadvantage during repeated cycles of starvation and growth (11). The identity of most proteins targeted for degradation by ClpAP, however, remains unknown, and it is unclear how this degradation is directed and controlled. Even in the cases mentioned above, the signals and recognition elements for degradation by Clp proteases are incompletely characterized.

Proteolysis may be the only way of significantly reducing the level of a protein in the absence of growth because of the lack of the dilution of components, as by cell division. Hence, it is expected that regulation by proteolysis is particularly important in nongrowing cells. In the paper presented here, we analyze the impact of mutations in the genes *clpP*, *clpA*, and *clpX* on the regulation of proteins during stationary-phase adaptation in *E. coli*. We observed a strong influence of the protease genotype on proteins regulated by growth phase and identified a number of the proteins affected. Because most of the effects cannot be ascribed to any regulatory system under proteolytic control that is known to date, we initiated a thorough analysis of the mechanisms of protease action in stationary-phase adaptation.

MATERIALS AND METHODS

***E. coli* strains, media, and growth conditions.** The strains used were *E. coli* MC4100 (wild type) (7), RH184 (MC4100 *clpP1*::cat) (47), AM134 (MC4100 *clpX1*::kan) (21), and DT9 (MC4100 *clpA319*::kan) (31). It is likely that ClpX can still be produced in the *clpP1*::cat mutant strain, as it has been shown that *clpX* can be expressed independently of *clpP* (69). All cultures were inoculated from

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2-day-old single colonies on Luria-Bertani (LB) plates into M9 medium (50) amended with trace elements and 0.04% (wt/vol) glucose. Trace elements were added from a 1,000-fold stock solution containing 10 μM $ZnSO_4$, 4 μM $MnCl_2$, 1 μM $CuSO_4$, 1 μM $CoCl_2$, 2 mM H_3BO_4, and 0.1 μM $(NH_4)_6(Mo_7)_{24}$. The cultures were grown aerobically at 37°C with orbital shaking (300 rpm) for 20 h, diluted 1:200 in fresh M9 medium, grown for another 20 h, and finally diluted once again 1:200 in fresh M9 medium. This procedure of double passaging reduces carryover of material from the LB-grown colony and allows increased reproducibility of culture growth. By setting the glucose concentration as low as 0.04%, the stationary phase is defined by carbon limitation (carbon and energy starvation), and density effects are largely excluded. Culture turbidity was measured in an Ultrospec 1000 spectrophotometer (Pharmacia Biotech) at 590 nm. For determination of CFU, drop plate counts (27) were performed on LB plates with incubation at 37°C.

Chemicals and reagents. All reagents used were analytical grade. Chemicals for preparation of samples for proteome analysis were from Amersham Pharmacia Biotech (PlusOne), except for sodium dodecyl sulfate (SDS), which was from Bio-Rad, and DNase I and RNase A (both from bovine pancreas), which were from Boehringer (Mannheim, Germany). Chemicals used for preparation of gels were obtained from Carl Roth (Karlsruhe, Germany).

Sample preparation for two-dimensional (2-D) electrophoretic analysis of proteins. From cultures of all four strains, samples were taken during logarithmic growth (optical density at 590 nm [OD_{590}] of 0.35) and 70 min and 21 h after reaching stationary phase (the onset of the stationary phase was defined as the time when the OD of the culture stopped increasing). Samples of 12 and 10 ml volume were withdrawn from growing and starved populations, respectively, and harvested by centrifugation at 20,000 × *g* for 10 min at 24°C. The pellets were resuspended in 180 μl of SA buffer containing 0.3% (wt/vol) SDS, 4.44 mg of Tris-HCl $liter^{-1}$, 2.56 mg of Tris base $liter^{-1}$, and 20 mM dithiothreitol (DTT) (added freshly). The samples were heated at 100°C for 2 min and subsequently kept at room temperature for 5 min. Then DNase I and RNase A were added at concentrations of 0.064 mg ml^{-1} and 0.09 mg ml^{-1}, respectively, together with 58 mM $MgCl_2$. The mixture was vortexed vigorously, and nuclease digestion was allowed to proceed for 20 min on ice. Subsequently, 675 μl of SB buffer containing 9.5 M urea, 40 g of 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS) $liter^{-1}$, 4.85 g of Tris base $liter^{-1}$, and 20 mM DTT (added freshly) was added to each sample. After vigorous vortexing, the samples were left at room temperature for 10 min and then frozen in aliquots of 200 μl at −80°C. Protein concentrations were estimated by calculating the protein content in samples from the OD_{590}s of the cultures. A culture with an OD_{590} of 1 was determined to contain an average protein concentration of 62.5 μg/ml, as calculated from 16 samples of MC4100 cultures lysed with SA buffer (determination of protein content was made by using the Bio-Rad protein assay with bovine serum albumin as the standard).

Isoelectric focusing. Prior to focusing, samples were thawed, resuspended, left for 10 min at room temperature, and centrifuged at 24°C and 20,000 × *g* for 30 min to remove cell debris and unsolubilized proteins. From the supernatants, aliquots were diluted with at least equal volumes of rehydration buffer (8 M urea, 2% [wt/vol] CHAPS, and 0.5% immobilized pH gradient [IPG] buffer). The IPG strips (18 cm of IPG [pH 4 to 7 or pH 5 to 6]; Pharmacia Biotech) were each loaded with 50 μg of protein concomitant with rehydration. The IPG gels were run in an IPGphor apparatus (Amersham Pharmacia) at 20°C with a maximum of 50 μA per IPG strip. Rehydration of IPG strips was allowed to proceed for 1 h, and the following voltages were applied subsequently: 5.5 h at 30 V, 8 h at 60 V, and 1 h each at 200 and 500 V, after which the voltage was raised to 8,000 V during a 1-h gradient. The final voltage was kept at 8,000 V for 4.5 h, amounting to a total of 40,000 V × h. After focusing, the IPG gels were frozen at −80°C.

Second dimension electrophoresis and documentation. Prior to second dimension electrophoresis, the IPG gels were equilibrated twice for 15 min each in a solution containing 6 M urea, 50 mM Tris-HCl (pH 8.8), 30% (wt/vol) glycerol, 2% SDS, and 64.5 mM DTT (added freshly). After equilibration, the strips were placed on standard continuous Laemmli polyacrylamide gels (12.5% T, 2.6% C; 180 by 180 by 1.5 mm) and sealed with 0.5% agarose dissolved in Laemmli running buffer (37). Gels were run in Bio-Rad Protean II vertical cells in Laemmli buffer with cooling at 12°C for 18 to 19 h. Constant current conditions were set to 5 mA per gel for 1.5 h and subsequently to 14 mA per gel for the remainder of the run.

The gels were then fixed for 30 min in 7.5% (vol/vol) acetic acid and stained with SYPRO Red (Molecular Probes) for 3 h according to the manufacturer's instructions. Glycerol was then added to the staining solution to a final concentration of 6% (wt/vol), and the gels were left immersed with gentle shaking for another 15 min before scanning. Scanning of SYPRO stained gels was performed with a FLA2000G fluorescence scanner (Fuji Photo Film Co., Osaka, Japan) with maximal sensitivity (F1000) at an excitation wavelength of 532 nm and a filter setting of 675 nm. Gels were first scanned at a resolution of 50 μm (for quantitative analysis) and subsequently at 100 μm (as a lower-resolution source of images for figures).

Analysis of 2-D gels. Image files from SYPRO-stained gels were analyzed with the program Image Gauge, version 3.45 (Fuji Photo Film Co.). Ovoid areas (regions of interest) of identical size were used to measure the pixel density of spots, and the patterns of regions of interest created in one gel were transferred to new gels. The adjustment of spot positions was done manually to correct for run-to-run differences and local warping. The averaged intensities of five background areas were subtracted from each spot, and the corrected data were exported into Excel, where the spot intensities were divided by the total intensity in 185 spots. This allowed for the correction of differences in performance of the SYPRO stain and facilitated the comparison between gels. All data presented here were multiplied by a factor of 1,000, consequently representing normalized per mill fractions. For each strain and sampling time, samples from three independent cultures were taken, and each sample was run twice. Hence, the protein composition data consist of average data from 6 gels for every strain and time point. For detection of regulation and of differences between mutant and wild-type data, *t* tests were performed with a level of confidence of 95%.

Identification of proteins by MALDI-TOF (MS). Sample preparation for matrix-assisted laser desorption ionization–time of flight (mass spectrometry) (MALDI-TOF [MS]) was performed according to the method of Shevchenko et al. (58) with slight modifications. Protein spots with 1.5-mm diameters were excised from SYPRO red-stained gels over a UV screen, and the gel pieces were subjected to reduction (100 mM DTT, 56°C for 30 min) and carbamidomethylation (55 mM iodoacetamide for 20 min in the dark). Digestion was performed with trypsin (12.5 ng ml^{-1} in 25 mM NH_4HCO_3) at 37°C for 17 h. Aliquots of the supernatants were then incorporated into a fast evaporation nitrocellulose matrix with α-cyano-4-hydroxy cinnamic acid as the matrix substance. The mass spectrometric measurements were performed on a Reflex MALDI-time of flight mass spectrometer (Bruker Daltonik, Bremen, Germany) equipped with an ion gate and pulsed ion extraction. The acceleration voltage was set to 20 kV, and the reflector voltage was set to 23 kV. The peptide mass fingerprint spectra were matched to the National Center for Biotechnology Information nonredundant database entries by using the program ProFound at http://service.proteometrics.com/prowl-cgi/ProFound.exe. For the initial search, the mass tolerance was set to 50 ppm, one missed cleavage site was tolerated, and the search was restricted to *E. coli* proteins. A size cutoff was set to about 200% of the apparent molecular weight of the protein as estimated from the gels. The proteins were regarded as identified according to the significance criteria of the search program (Z score > 1.645, $P < 0.05$%). A new search was performed with the unmatched peptide masses to identify comigrating proteins.

RESULTS

Growth and survival of strains during starvation. The parental strain (MC4100) and the three *clp* mutant strains displayed identical growth rates and growth yields under the conditions employed, reaching OD_{590}s of 0.45 to 0.5 in the glucose-limited stationary phase, corresponding to 3×10^8 to 5×10^8 CFU ml^{-1}. Overnight, the OD dropped to values between 0.4 and 0.41 in the wild-type and *clpA* and *clpP* mutant cultures and to a value of 0.3 in the *clpX* mutant cultures. Survival after overnight starvation was indistinguishable in the wild type and the *clpA* mutants (87 and 98% of initial colony counts, respectively) and slightly lower in the *clpX* mutant (77%) and the *clpP* mutant (74%) (all data are means of 6 replicates). After another 24 h of starvation (total of 44 h), the wild type and the *clpA* mutant displayed survival rates of 92 and 95%, respectively. At this time, the *clpX* and *clpP* mutants showed a significantly ($P < 0.05$) lower survival rate than the wild type (70 and 41.3% of the initial cell numbers in *clpX* and *clpP* mutants, respectively). After 44 h of starvation, OD values in all four strains were between 0.3 and 0.38.

Proteome analysis of growth phase regulation. The changes in protein composition in the *E. coli* wild type and protease mutants were studied by analyzing SYPRO-stained 2-D gels

TABLE 1. Protease-dependent regulation of growth-phase-regulated proteins

Spot no.	Protein[a]	Description	Ratio relative to log levels after time in stationary phase[b]:								Level during log phase significantly[c]:	
			70 min				21 h					
			WT	*clpP*	*clpA*	*clpX*	WT	*clpP*	*clpA*	*clpX*	Elevated	Reduced
14	GroEL	60-kDa chaperonin	**1.4**	1.1	1.1	1.3	1.4	1.2	1.3	1.0	*clpP*, *clpA*, *clpX*	
94	RbsB	Periplasmic D-ribose binding protein	**1.4**	0.9	1.3	1.1	**2.2**	**4.5**	**2.8**	**3.6**		
101	Dps	DNA protection during starvation	**5.3**	2.5	7.6	2.3	**43.6**	**5.8**	**16.1**	16.5		
107	ArgT	Periplasmic LAO-binding protein[d]	**1.9**	1.6	1.6	1.0	**4.9**	**2.2**	**2.5**	**2.1**		
147	AldA	Aldehyde dehydrogenase A	1.2	1.2	0.8	**1.3**	**1.4**	**2.0**	1.2	**1.9**		
152	WrbA	Trp repressor binding protein	1.3	**4.5**	0.9	0.9	**2.8**	**3.6**	1.5	1.6		*clpP*
15	Tig	Trigger factor	1.0	1.2	0.7	1.1	**0.7**	0.9	**0.8**	**0.8**		
22	GlnA	Glutamine synthetase	1.5	0.9	0.9	0.8	**0.4**	**0.7**	0.7	**0.5**		*clpA*
34	TalB	Transaldolase B	1.0	1.0	1.0	1.0	**0.8**	1.0	1.0	**0.8**		
37	TrxB	Thioredoxin reductase	0.9	1.3	0.9	1.0	**0.7**	0.9	**0.8**	1.0		
55	LeuA	Isopropylmalate synthase	1.0	1.1	0.9	1.6	**0.6**	0.7	0.9	0.9		
108	LeuD	Isopropylmalate isomerase small subunit	0.6	0.9	0.9	**0.4**	**0.2**	**0.3**	**0.3**	**0.4**	*clpA*	
116	AhpC	Alkyl hydroperoxide reductase C22 protein	**0.8**	1.0	1.1	0.9	**0.2**	1.0	**0.3**	**0.6**		
130	RpsF	30S ribosomal protein S6 RS-6C[e]	**0.8**	1.3	1.1	1.0	0.6	0.7	**0.4**	**0.6**		
131	RpsF	30S ribosomal protein S6 RS-6B[e]	**0.5**	0.9	1.0	**0.7**	**0.3**	**0.3**	**0.2**	**0.4**		
148	IlvC	Ketol acid reductoisomerase	1.2	1.1	1.3	**1.4**	**0.6**	0.8	1.0	1.1		*clpA*
168	MetK	*S*-adenosylmethionine synthetase	1.0	0.9	0.9	1.0	**0.8**	0.9	0.9	1.1		
173	GdhA	NADP-specific glutamate dehydrogenase	1.3	0.8	0.9	1.0	**0.3**	0.7	**0.3**	**0.5**		

[a] The spots identified by MALDI (MS) are shown with their protein names according to SWISSPROT. The first 6 proteins are induced during the stationary phase compared to the log phase, and the remaining 12 are repressed in the stationary phase.

[b] Mean spot intensities in the stationary phase were divided by the corresponding mean levels in the log phase (6 parallels each) to give mean ratios, and significant ($P < 0.05$) changes relative to levels in the log phase are highlighted in bold.

[c] Genotypes of strains in which protein levels observed in the log phase were significantly ($P < 0.05$) different from wild-type levels.

[d] LAO, lysine-arginine-ornithine.

[e] The proteins in spots 130 and 131 are two different posttranslationally modified species of the same translation product (54).

(IPG pH of 4 to 7) during growth and starvation in glucose minimal medium (see Materials and Methods). Our gel system allowed analysis of proteins with pIs between 4 and 6.7 and molecular masses between 8 and 90 kDa. The analysis of gels was based on data derived from 6 gels per sample of each strain per time point; samples from three independent cultures were taken and run twice. When comparing mean spot intensities in early or late stationary phase with those during logarithmic growth in the wild-type strain MC4100, significant ($P < 0.05$) induction of a total of 16 proteins could be detected. Significant ($P < 0.05$) reduction of protein amounts could be observed for 23 protein spots. One of these proteins was transiently induced in early stationary phase but reduced during long-term starvation. Hence, the total number of growth-phase-regulated proteins monitored in our study was 38.

The majority of these spots (31 of 38) did not show the wild-type regulation in one or more of the protease mutants (applying the same level of confidence with a P value of <0.05) in early (70 min) or late (21 h) stationary phase. In Table 1, the protein spots for which a reliable identification was obtained by MALDI (MS) are listed. All spots were confirmed to contain only one protein, as judged by searching the unmatched masses. Figure 1 shows the positions of all proteins mentioned in this paper on a 2-D gel stained with SYPRO red. In addition to the identity of proteins, Table 1 also shows a condensed overview of the regulation of the proteins identified. The top 6 rows contain proteins significantly induced during the stationary phase in the wild type, and the remaining 12 rows contain proteins reduced in their amounts under those conditions. The regulation in the four strains is presented as ratios resulting from the division of the mean levels of the proteins in the stationary phase (70 min and 21 h, respectively) by the mean levels of those proteins during logarithmic growth (all means of 6 experiments). Significant changes relative to logarithmic-phase levels (as judged by *t* tests with a level of confidence of 95%) are indicated. To allow a meaningful analysis of strain-dependent growth phase regulation of proteins, Table 1 also contains information on the levels of the proteins during logarithmic growth. Here, the proteins which display significantly different ($P < 0.05$) levels between the wild type and the mutants in the logarithmic phase are indicated by bold type. The majority of the differences in protein composition between the strains were found in the stationary phase, and the differences in protein abundance in the log phase were limited to a very few proteins. For an illustration of the differences between the wild type and the protease mutants, corresponding sections of gels loaded with identical amounts of total protein from growing and starved (21 h) cells of the wild type and the *clpP* mutant are shown in Fig. 2.

Proteins with protease-dependent growth phase regulation. Of the proteins with protease-dependent growth phase regulation, 18 were identified in this study (Table 1). For six of these proteins, the quantification data based on SYPRO-stained gel images are presented in Fig. 3 and 4. In Fig. 3, three growth-phase-regulated proteins with increased levels in the protease mutants compared to levels in wild-type cultures are shown. In wild-type cultures, the levels of the AhpC protein are reduced strongly during stationary-phase adaptation, whereas this reduction is abolished or delayed in the protease mutants studied (Fig. 3A). This effect is most evident in the *clpP* mutant, where levels stayed unchanged during the experiments (Fig. 2C and D, spot 116). Upon prolonged starvation (44 h),

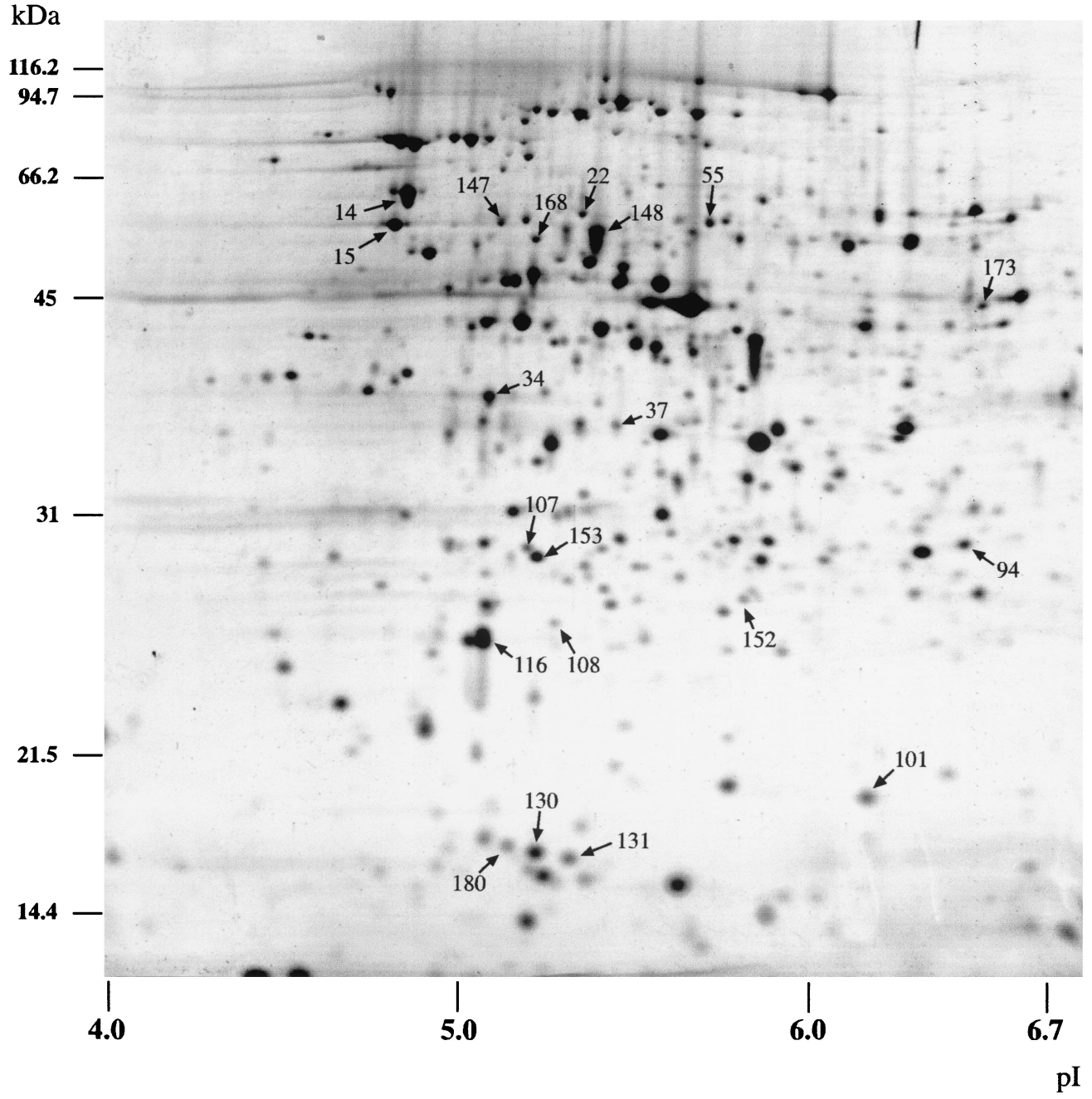


FIG. 1. Positions of all proteins mentioned in this paper on a 2-D gel of a sample of *E. coli* AM134 (MC4100 *clpX1*::kan) stained with SYPRO red. The growth-phase-regulated proteins controlled by ClpP-containing proteases and identified by MALDI (MS) (peptide mass fingerprinting) are listed in Table 1.

amounts of this protein in the *clpP* mutant remain elevated, whereas in the wild type, a further reduction can be observed (data not shown). In mutant cultures lacking a functional ClpA subunit, reduction of the amounts of the AhpC protein in the stationary phase is delayed, but protein amounts are reduced to wild-type levels after overnight starvation. The effects observed in *clpX* mutants are less severe than the effects observed in *clpP* mutants but more pronounced than those seen in the *clpA* strain (Fig. 3A).

The levels of the RbsB protein (spot 94) in the four strains are presented in Fig. 3B. The position of this spot corresponds to that published for the mature periplasmic binding protein of 28.5 kDa (41). In this case, stationary-phase induction is maintained in the *clpA* mutant but amplified after overnight starvation in the *clpP* and *clpX* mutant cultures relative to wild-type regulation. The levels of the LeuA protein (Fig. 3C, spot 55) are reduced slightly but significantly during the stationary phase in the wild type, whereas this reduction is largely abol-

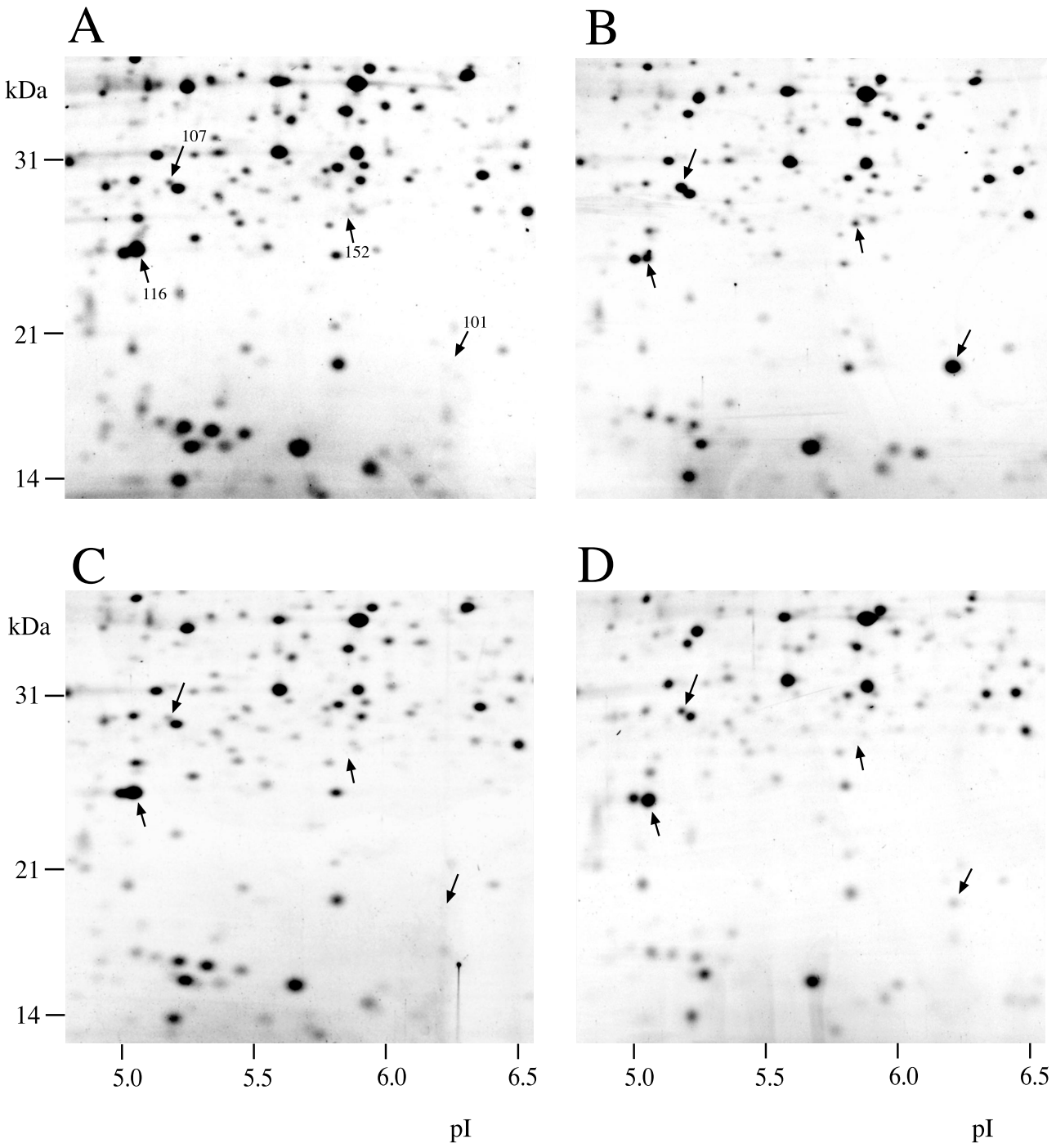
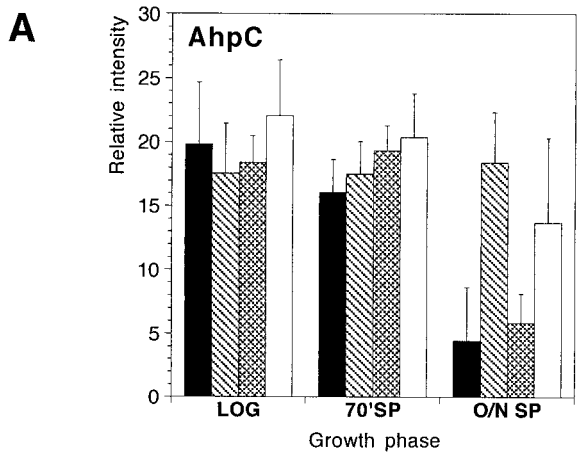
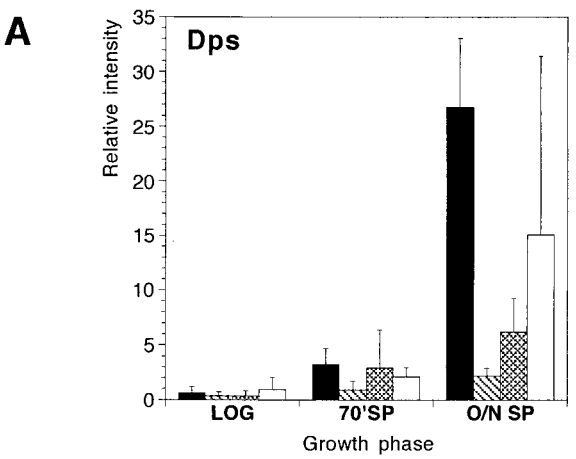


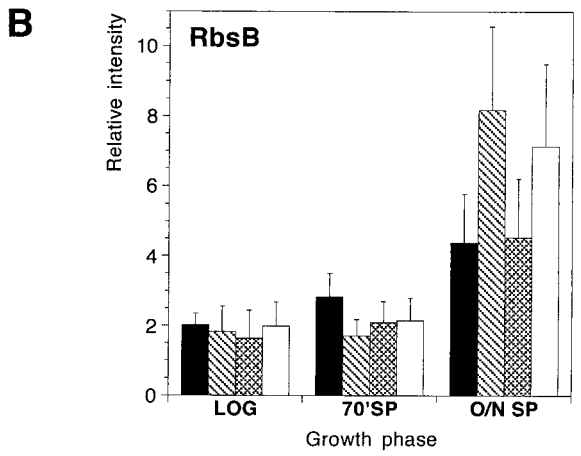
FIG. 2. Growth phase regulation of proteins in wild-type and *clpP E. coli*. Corresponding regions of gels obtained from logarithmically growing (A and C) and starved (for 21 h) (B and D) samples of the wild type (A and B) and the *clpP* mutant (C and D). In panel A, the positions of relevant spots are indicated with the identification numbers used in Fig. 1, Table 1, and the text.

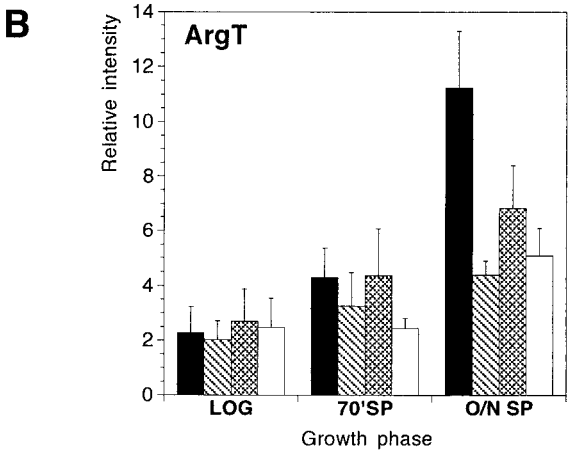
ished in all three of the mutants tested. The differences between the wild type and mutants observed for the levels of RbsB and LeuA proteins after 21 h of starvation are maintained after a further 23 h (44 h) (data not shown).

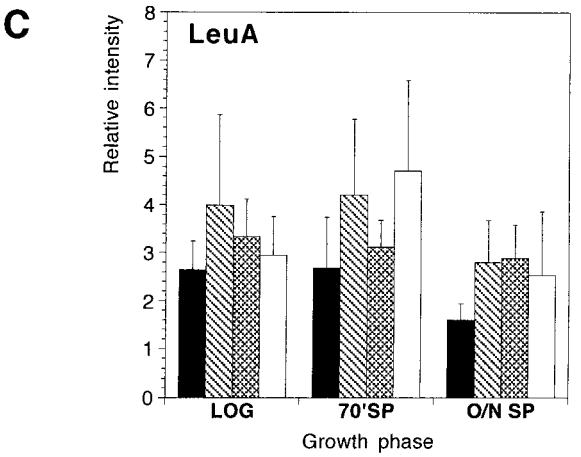
In addition to the data shown in Fig. 3, several other growth-phase-regulated proteins display elevated levels in one or two of the mutants compared to the levels in the wild type. Of these proteins, the GroEL, AldA, Tig, MetK, TalB, TrxB, GlnA, GdhA, LeuD, and RpsF proteins were identified (Table 1). GroEL levels are elevated in the *clpP*, *clpA*, and *clpX* mutants in the logarithmic phase such that further induction is not observed during starvation. The reduction of amounts of the trigger factor protein Tig (spot 15) and of the GlnA protein (spot 22) in the late stationary phase is less pronounced in the











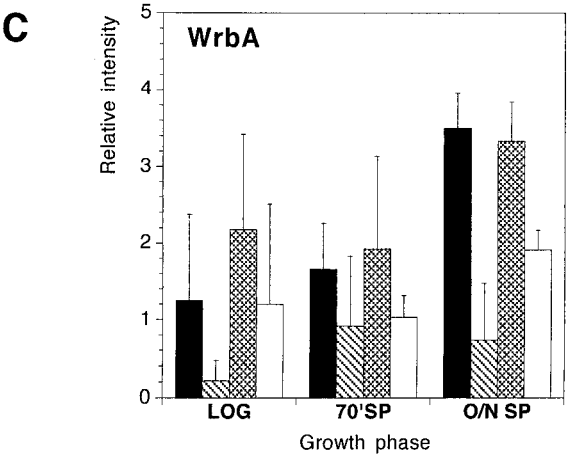


FIG. 3. Levels of proteins displaying increased amounts in one or more of the protease mutants compared to the wild type. Relative protein levels were determined from SYPRO-stained 2-D gels as described in Materials and Methods. Data are shown from the AhpC protein (spot 116) (A), the RbsB protein (spot 94) (B), and the LeuA protein (spot 55) (C). Wild-type protein levels are shown as filled black bars, data from *clpP* mutant cultures are presented as hatched bars, data from *clpA* cultures are presented as double-hatched bars, and data from *clpX* cultures are presented as open (white) bars. SP, stationary phase; 70′SP, 70 min after reaching the stationary phase; O/N, overnight.

FIG. 4. Levels of proteins displaying reduced amounts in one or more of the protease mutants compared to the wild type. Relative protein levels were determined from SYPRO-stained 2-D gels as described in Materials and Methods. Data are shown for the Dps protein (spot 101) (A), the ArgT protein (spot 107) (B), and the WrbA protein (spot 152) (C). Wild-type protein levels are shown as filled black bars, data from *clpP* mutant cultures are presented as hatched bars, data from *clpA* cultures are presented as double hatched bars, and data from *clpX* cultures are presented as open (white) bars. SP, stationary phase; 70′SP, 70 min after reaching the stationary phase; O/N, overnight.

protease mutants than in the wild type (Table 1). With regard to the GlnA protein, several posttranslational modifications have been reported. While adenylylation of GlnA leads to inhibition of its enzymatic activity (56), oxidation and carbonylation have been connected with the stability of the enzyme and with processes of cellular stress and aging (14, 15, 40). From our MALDI (MS) data, we cannot determine whether the protein in spot 22 represents a modified GlnA protein.

The LeuD (spot 108) and RpsF (spots 130 and 131) proteins are reduced significantly after 70 min of starvation in the wild type but are maintained at elevated levels in the early stationary phase in the *clpP* and *clpA* mutants. After overnight starvation, however, no significant differences between protein levels in the wild-type and mutant cultures can be detected. Hence, the effects of mutations in the *clp* genes on the levels of these proteins are transient and result merely in a delay in the reduction of protein amounts during the stationary phase. Concerning the RpsF proteins, it may be pointed out that three protein spots on our 2-D gels were identified as derivatives of the ribosomal protein RS-6 (Fig. 1, spots 130, 131, and 180). It is well documented in the literature that the ribosomal protein RS-6 occurs in several posttranslational modifications of *E. coli*, which differ only in the length of the glutamic acid chain added at the carboxy terminus (30, 54). These modifications can be separated on 2-D gels, and the positions of spots 130 and 131 observed on our gels are in good agreement with the positions published for RS-6C and RS-6B, respectively (54). In fact, the peptide mass lists obtained from two separate gels for spot 131 contain masses of 2,470.89 and 2,470.95 Da, respectively. This corresponds to the theoretical mass (2,470.97 Da) of the C-terminal peptide of the ribosomal protein modified with a single glutamic acid residue (RS-6B) as created by trypsin digestion with two missed cleavages (ERRDDFANET ADDAEAGDSEEE). Hence, it is plausible that the protein spots 131 and 130 correspond to the first and second glutamylation products RS-6B and RS-6C, respectively, and that spot 180 represents a further glutamylation product which has hitherto not been detected on 2-D gels.

Figure 4 shows three sets of data from proteins which are induced in wild-type *E. coli* during the stationary phase but display reduced induction (due to reduced levels) in the stationary phase in some of the protease mutants. The Dps protein (Fig. 4A) is strongly induced in the wild type after 70 min of starvation and further induced in late stationary phase. This induction is severely reduced in the *clpP* mutant (Fig. 2 and 4A) and also significantly reduced in the *clpA* mutant, leading to lowered levels of this protein in these mutants in the stationary phase. After 44 h, these differences in protein levels are maintained (data not shown). In the *clpX* mutant, the protein levels after overnight starvation were observed to be highly variable from gel to gel (Fig. 4A). Due to the high standard deviation, no significant difference can be detected between the levels of Dps protein in the *clpX* mutant and the wild type.

Similar to the observations for Dps, the ArgT protein (Fig. 2 and 4B, spot 107) shows a mode of regulation dependent on ClpAP (Table 1). In addition, however, the ArgT protein is clearly also dependent on the ClpX subunit for full induction during the stationary phase. The ArgT protein occurs as the cytoplasmic precursor protein of 28 kDa (pI, 5.62) and as the mature periplasmic protein of 25.8 kDa (pI, 5.22). The material detected at the position of spot 107 on our gels probably constitutes the processed (mature) periplasmic protein because no fragments of the signal sequence have been detected in the MALDI (MS) spectra. Also, the position on our gels matches perfectly with the position calculated (see above) and published for the mature form of ArgT (SWISSPROT [http://www.expasy.ch/cgi-bin/nice2dpage.pl?P09551]) (41).

Figure 4C shows the regulation of the amounts of the WrbA protein (spot 152) during logarithmic growth and carbon starvation. Considerable induction of WrbA can be observed in the wild-type and *clpP* strains, but protein levels are generally lower in the *clpP* mutant than in the wild type (Table 1 and Fig. 2). Hence, in the *clpA* and *clpP* mutants, the induction pattern is maintained, but the absolute levels of the protein are affected. Upon starvation for 44 h, the WrbA protein was further induced in the wild type while the differences between the wild-type levels and those of the *clpA* and *clpP* mutants were preserved (data not shown).

It was further tested whether protease mutants accumulate increased amounts of aggregated proteins in inclusion bodies (57, 62) which may not enter a 2-D gel under our standard conditions. For this purpose, aggregated proteins were prepared according to the method of Tomoyasu et al. (62) from wild-type and *clpP* mutant cultures after 21 and 44 h of starvation. After extraction, the samples resulting from 125 μg of total protein (extracts from 8 ml of culture each) were treated as described for all other proteome samples (including the boiling and nuclease treatment steps; see Materials and Methods) and run under conditions identical to those for all other samples. Very few faint spots could be detected by SYPRO staining, although the starting material exceeded the normally applied material 2.5-fold (125 instead of 50 μg of total protein). The spots were identical in intensity in gels from samples of *clpP* and wild-type cultures both after 21 and 44 h (data not shown). Hence, the data presented here are not complicated significantly by the differential loss of soluble protein due to inclusion body formation.

DISCUSSION

In recent years, the relevance of controlled proteolysis for gene regulation and stress survival of bacteria has been increasingly recognized. Among others, several global regulatory proteins have been shown to be under proteolytic control (20). With *E. coli,* peptidase mutants have been shown to be essential for the viability of carbon-starved cultures (55), and the degradation of several starvation proteins was shown to be dependent on ClpAP (11). Recently, it was shown that several enzymes are degraded rapidly in stationary-phase *E. coli* due to ATP-dependent proteolysis while the same enzymes are stable during growth (49). Those findings underscore the significance of proteolysis in growth phase regulation and confirm some of the results obtained in our study.

In the study described here, three *E. coli* protease mutants (*clpP*, *clpA*, and *clpX*) were investigated regarding their effects on viability and growth phase regulation of proteins. The decreased viability of the *clpP* mutant after prolonged (44 h) starvation points towards a significant role for ClpP-containing proteases during extended stationary phase. The effect of the *clpX* mutation on survival is less severe but also detectable. In

cultures starved for up to 21 h, however, none of the mutant strains employed displayed a significant loss of viability (compared to the wild type) ($P < 0.05$). Hence, the proteome data presented here (taken at times up to 21 h) are not influenced to any major degree by the proteins of nonviable cells.

Clp proteases play a major role in stationary-phase regulation. As a result of our proteomic approach, proteins were identified whose amounts are significantly regulated in wild-type *E. coli* during the growth phases studied. The regulation of these proteins was then compared with the data obtained with the protease mutants. The overall result of our study is that proteolysis by ClpP-containing proteases is far more important for regulation in *E. coli* than previously assumed. In particular, the regulation of all of the 16 proteins that displayed significant induction in early or late stationary phase (and of 20 of the 23 proteins that showed reduction) was impaired in at least one of the mutants studied (data not shown). Stationary-phase regulation of some proteins is clearly dependent on a particular protease, such as the induction of Dps (dependent on ClpAP) and the reduction of TrxB and AhpC (dependent on ClpXP). Other proteins are regulated in a way that is dependent on ClpP but not on ClpA or ClpX. This type of dependence on ClpP alone was observed for the GdhA and AldA proteins and can be explained by a mechanism requiring either ClpAP or ClpXP. Other proteins (such as LeuA, ArgT, and MetK) are only regulated normally if both ClpAP and ClpXP are present in the cell. For the interpretation of some of the more complex data (for example, spot 15, the Tig protein), it may be kept in mind that the ATPase subunits of the proteases can act as chaperones independently of the proteolytic core (38, 43, 66).

Growth-phase-regulated proteins that accumulate at higher levels in *clp* mutants: putative protease substrates. We have shown that several proteins accumulate at higher levels than those of the wild type in some of the protease mutants and propose that these proteins are substrates of the respective ClpP-containing proteases. The normalized amounts of most of these proteins are reduced during starvation adaptation in the wild type (AhpC, LeuA, Tig, MetK, TalB, TrxB, GlnA, GdhA, LeuD, and the RpsF proteins RS-6B and RS-6C). Only three of the proteins accumulating in one or more protease mutants are stationary-phase-induced in the wild type (GroEL, AldA, and RbsB). The effect of the protease genotype on GroEL levels is probably a consequence of a moderate induction of the heat shock response due to the increased accumulation of misfolded proteins in the absence of functional ClpP-containing proteases.

The induction of the RbsB protein was amplified in the protease mutants compared to the induction in the wild type (Fig. 3B). This protein has been described as induced as part of the physiological short-term adaptation to glucose limitation (67). The results described in this study, however, ought to be seen in the light of the fact that the wild-type laboratory strain MC4100 employed in this study carries a mutation in the *rbsR* repressor gene. Hence, the data presented here may be considered an opportunity to study the control of a periplasmic binding protein in the absence of transcriptional regulation. The increased induction of RbsB in the *clpP* and *clpX* mutant backgrounds indicates that proteolysis in the cytoplasm contributes to the control of the final levels of a periplasmic ribose binding protein. The precursor of the binding protein could be subject to proteolytic control either during or right after its synthesis.

The AhpC, LeuA, LeuD, TalB, TrxB, Tig, and MetK proteins have not been previously observed as substrates of any *E. coli* proteases. On the other hand, the turnover of GlnA after oxidative inactivation (40) and the degradation of ribosomal proteins due to the stringent response or in response to starvation in general (8, 36) have been reported. The degradation of GdhA, GlnA, and several other enzymes by ATP-dependent proteases in the stationary phase has recently been shown (49). Extensive analysis of sequence similarities between published ClpAP substrates (RepA, ClpA, MazE, and MetA) (1, 5, 22, 28) and those proteins proposed to be ClpAP substrates based on our results (AhpC, AldA, LeuA, LeuD, GdhA, GlnA, TalB, TrxB, Tig, MetK, and RpsF) was performed with two different programs. The TMSA (http://cbcsrv.watson.ibm.com/) and CLUSTALW (www.ebi.ac.uk/clustalw/) programs were used to search for sequence elements based on either identity or similarity (chemical or structural). In the comparisons of any pairs or groups of published and potential substrates, no statistically significant common sequence elements could be detected in proteins that might be recognized by ClpAP. Hence, there are no clues from the primary structure of the proteins as to how ClpAP might be able to bind and degrade such a diverse spectrum of polypeptides. However, even if all of those proteins are degraded by ClpAP, initial recognition could be different for different substrates, since various recognition or targeting factors could be involved (see below).

The mechanisms of substrate recognition by ClpAP (and by *E. coli* proteases in general) are still largely unknown. While eukaryotic cells possess a general pathway for marking proteins for degradation by means of ubiquitination (26), so far no analogous general tagging system for proteins could be found in bacteria. The only covalent modification system directing proteins for degradation in *Escherichia coli* is the SsrA/SspB system. This system adds a polypeptide tag consisting of 11 amino acid residues to incompletely translated polypeptides. The tagged translation product is then released from the ribosome and degraded rapidly, thus ensuring both the quality of the translation and the functionality of the ribosomes (23, 32, 39). Recently, a role for polyphosphate in the proteolytic degradation of several ribosomal proteins during amino acid starvation in *E. coli* has been established: polyphosphate appears to form complexes with the Lon protease and the substrates, promoting degradation of a set of ribosomal proteins (36). In another recent paper, the ClpS protein was described as acting as the substrate modulator of the ClpAP protease by diverting the specificity of the protease away from SsrA-tagged proteins and towards aggregated proteins (13). Additional factors mediating between proteases and their substrates will probably be identified in the near future. These may be adapter proteins, such as RssB, SspB, and ClpS, or other molecules or metabolites, such as polyphosphate (18, 36, 39, 52, 53). It is also possible that oxidation reactions which have been observed to be highly differential in the stationary phase might serve in marking proteins for degradation, as observed for GlnA (14, 15, 40, 59). Alternatively, ligands such as NADPH might destabilize specific proteins, as shown for GdhA in vitro (49).

Growth-phase-regulated proteins with reduced levels in *clp* mutants: Clp proteins can positively contribute to stationary-phase induction. Decreased protein levels in protease mutants in comparison to wild-type levels are indicative of indirect effects of proteolysis. These may be due to lowered rates of synthesis or reduced stability of the protein in the mutant. Alternative explanations include the truncation or modification of the protein in the mutant strain due to the action of an otherwise inactive enzyme, leading to a change in the position of the protein spot. On our 2-D gels, however, we have not detected any changes in the relevant regions of the gels hinting at any genotype-dependent modification or truncation. Also, in the literature available, there are no indications of modifications of any of the proteins identified other than the processing of the signal peptides from the RbsB and ArgT precursors (as discussed in Results). It may be stressed here that spot 153 directly adjacent to ArgT (Fig. 1) was identified to be a different protein (FliY). Hence, it can probably be excluded that the intensities of the spots in question are misleading due to post-translational modifications. In addition, we are also confident that the data obtained for protein levels in the protease mutants are not affected by inclusion body formation (see Results).

Several proteins identified here display levels in some protease mutants that are significantly reduced compared to those in the wild type, either during growth (GlnA and IlvC) or during starvation (Dps and ArgT). One protein (WrbA) shows generally reduced levels in *clpP* mutants, irrespective of the growth phase. Due to the reduced levels of the GlnA and IlvC proteins during growth in the *clpA* mutant investigated, the repression of these proteins during starvation is abolished in these mutants (Table 1). For GlnA, the situation is complicated by GlnA being a potential substrate of the Clp proteases in the stationary phase (see above).

Stationary-phase induction of the WrbA and Dps proteins has been reported to be dependent on RpoS (3, 44, 68). Considering the fact that RpoS is under proteolytic control of ClpXP (4, 70), it is expected that *clpP* or *clpX* mutants should exhibit elevated levels of RpoS-controlled proteins (particularly during growth) because of accumulating amounts of RpoS in these protease mutants. The reduced levels of WrbA and Dps proteins in *clpP* and *clpX* strains can consequently not be due to effects on RpoS levels in these mutants and therefore must reflect a hitherto undetected level of additional control. Generally, only a few proteins (and no RpoS-controlled proteins) could be detected in increased amounts in *clpP* and *clpX* mutants during growth. Hence, an increased amount of RpoS alone does not lead to a significant change in global protein composition in growing cells.

The Dps protein (PexB, spot 101) has been described as one of the major starvation proteins in *E. coli*, and it has important roles in the protection of DNA during the stationary phase and during oxidative stress in this organism (2, 44, 45). The expression of this protein is governed by RpoS and integration host factor during starvation and by OxyR during oxidative stress (3). The drastic influence of the protease genotype on the levels of Dps in the stationary phase (Fig. 2 and 4A) indicates that the presence of the ClpAP protease is required for full induction of this protective protein. To date, this effect cannot be ascribed to any known regulatory mechanism because none of the regulators mentioned above are known to be under the control of the ClpAP protease. Similarly, the involvement of ClpP-containing proteases in the induction of ArgT and WrbA proteins cannot be ascribed to any known mechanism. While the function of the ArgT protein is established as a periplasmic binding protein for several amino acids, the function of the WrbA protein is not clear. Initially (based on the formation of complexes between the protein and DNA containing a TrpR-specific operator), this protein was suggested to be involved in blocking transcription of *trp* genes in the stationary phase (68), and more recently, it was proposed to be a member of a new family of flavodoxin-like proteins (25).

Conclusions. This is the first published account of the influence of proteases on the global regulation of growth phase transitions in *E. coli* that includes the identification of the proteins affected. Taken together, we have established that protease-dependent processes govern the growth phase regulation of many proteins in *E. coli*. A number of proteins (AhpC, AldA, GdhA, GlnA, LeuA, LeuD, MetK, TalB, TrxB, RS-6B, and RS-6C) were shown here to accumulate in protease mutants in the stationary phase. It is hypothesized that these proteins are degraded by the respective Clp protease(s) in the stationary phase. Further experiments are required to confirm this hypothesis and to determine the role of degradation of these proteins in the stationary phase. In addition to the direct effects of Clp proteases, we have shown that ClpP-containing proteases can positively control the levels of several stationary-phase-induced proteins. In *clpP* mutants, the ArgT, WrbA, and Dps proteins fail to be induced to wild-type levels in the late stationary phase. Because Dps is of considerable importance for stress survival, it will be interesting to elucidate the mechanism(s) by which ClpP-containing proteases control the regulation of this protein. Investigations in our laboratory are currently addressing this issue.

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