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Heat-Shock and Stringent Responses Have Overlapping Protease Activity in *Escherichia coli*

Implications for Heterologous Protein Yield

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Abstract

The cellular response of a heat-shocked controlled chemostat of *Escherichia coli* JM105 [pSH101] was characterized and compared to that of a similar culture induced by isopropyl-β-D-thiogalactopyranoside (IPTG). The proteases elicited by the IPTG pulse were previously shown to be upregulated by the stringent stress response and were shown here to be upregulated by heat shock, although to a lesser extent. Owing to the apparent overlap between these responses, a relaxed mutant (*rel*$^-$, devoid of the stringent response; JM109) was examined for its response to both a chemically imposed stringent response and to IPTG induction in controlled chemostats. There was no significant upregulation of protease activity under either imposed stress. More important, a nine-fold increase of chloramphenicol acetyltransferase (CAT) activity was found for the IPTG-induced relaxed mutant culture. Additionally, the responses from heat shock and IPTG induction were examined in batch cultures. The culture that was simultaneously IPTG-induced and heat-shocked was observed to have the highest CAT activity as well as the most rapid loss in activity after a maximum. Control experiments indicated that the heat shock did not affect loss of CAT activity; instead, the loss of activity correlated with the amount of CAT synthesized. Furthermore, an increase in CAT expression was found during heat shock. Results indicated that heat shock and, alternatively, the use of stringent response-mutant hosts could both be used to facilitate increased recombinant protein yields in the *E. coli* expression system.

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Index Entries: Recombinant; *Escherichia coli*; heat-shock response; stringent response; protein yield.

Introduction

The availability of strong promoters (native and synthetic) has led to extremely high levels of recombinant protein accumulation in *Escherichia coli (1,2)*. However, rapid accumulation of protein, in either aggregated or soluble form, is uniformly not observed. In particular, when a recombinant protein has an amino acid composition significantly different from that of the "average" *E. coli* protein, yields can be extremely low and can be a function of how and at what point the culture was induced, also known as the induction policy *(3)*. We hypothesized that rapid induction of a recombinant protein with an unusual amino acid composition can cause a temporary shortage of intracellular amino acids, particularly those in abundance in the recombinant protein. In this way, the induction of recombinant proteins could cause a stringent response in *E. coli* owing to the imbalance in amino acid uptake into the elongating polypeptide relative to the endogenous amino acid synthesis rate. The depletion of an intracellular amino acid pool would result in a change in the aminoacylated-tRNA to uncharged tRNA ratio, and induction of the classical stringent response *(4,5)*. Coincidentally, several heat-shock proteins are observed to increase during a stringent response *(6)*. At least two heat-shock proteins are proteases (La and Clp) *(7)*, so that the overexpression of a recombinant protein could induce, albeit indirectly, elevated protease activity. La and Clp proteases have broad specificity and have been implicated in the degradation of abnormal proteins *(7–9)*, which could result in the recombinant protein being degraded due to its own overexpression.

In the present study, continuous cultures were used to investigate the overlapping relationship among the heat-shock and the stringent stress responses, and the response observed during the overexpression of recombinant proteins. In particular, the induction of protease activity owing to a heat shock and IPTG induction was addressed. Additionally, a rel^- mutant (also referred to as a "relaxed" mutant), which is devoid of the classical stringent response, was compared to the rel^+ parent strain.

Chloramphenicol acetyltransferase (CAT) was the model recombinant protein and was induced by IPTG addition, which induced the *trc* promoter, a hybrid promoter derived from the *tac* and *trp* promoters. The plasmid containing the CAT gene, [pSH101], has been described previously *(10)*. CAT was selected as the recombinant protein because it is relatively insensitive to proteolysis. CAT contains three times more phenylalanine than the "average" *E. coli* protein; thus, a rapid increase in CAT synthesis might preferentially deplete the aromatic amino acids (phenylalanine in particular), such that a stringent response is elicited *(3,11,12)*. Although CAT is not directly degraded by the quantified proteases, the upregulation of any protease diverts amino acid resources that could be used for recombinant

protein synthesis, and thus the upregulation of any protease is an indicator of stress. Since heat-shock proteins accompany the induction of the stringent response, the effect of a heat shock on recombinant protein production was examined with and without IPTG induction. The present study demonstrates the degree of overlap between the stress responses with regard to protease activity and also demonstrates that significantly more CAT can be produced by using relaxed mutants and heat shock. The implications of the stress response overlap are discussed.

Materials and Methods

Cell Lines

E. coli JM105 [pSH101] was used for the heat-shock experiments with the genotype *thi rpsL endA1 sbcB15 hsdR4* Δ(*lac-proAB*) F' [*traD36 lacI*^{q} Δ(*lacZ*)ΔM15 *proAB*$^-$] λ$^-$ (American Type Culture Collection, ATCC no. 47016), which is a *lacI*^{q} and *rel*$^+$ strain, purchased from Pharmacia (Piscataway, NJ). The plasmid-bearing cells are designated *E. coli* JM105 [pSH101]. *E. coli* JM109 *thi endA1 gyrA96 hsdR17 supE44 relA1* Δ(*lac-proAB*) F' [*traD36 lacI*^{q} Δ(*lacZ*)ΔM15 *proAB*$^+$] λ$^-$ (ATCC no. 53323) was the relaxed mutant strain used for the stringent response and IPTG-induction experiments, which was purchased from Pharmacia. *E. coli* JM109 is *lacI*^{q} and *rel*$^-$. The plasmid-bearing cells are designated *E. coli* JM109 [pSH101]. *E. coli* JM109 was derived from *E. coli* JM105. The JM105 strain contains two mutations not found in JM109. These mutations are the *sbcB* gene for endonuclease I and *rpsL* gene for a small ribosomal protein that confers streptomycin resistance *(13,14)*. These mutations are not associated with the stringent or heat-shock responses. The JM109 strain contains two mutations not found in JM105. These mutation are the *supE* gene for the amber UAG mutation suppressor and *gyrA* gene for DNA gyrase *(13)*. These mutations are also not associated with the heat-shock or stringent responses. The *relA1* mutation is the only significant difference between the JM105 and JM109 strains, as related to these studies.

Culture Conditions and Bioreactor System

Continuous fermentations were run in a MultiGen (New Brunswick Scientific, Edison, NJ) bioreactor (2 L) with a 1-L working volume and a dilution rate of 0.35 h^{-1}. M9 minimal medium (4 g/L glucose) was used and prepared as described by Rodriguez and Tait *(15)*, except that casamino acids were omitted, and 25 µg/mL of streptomycin (Sigma, St. Louis, MO), 40 µg/mL of ampicillin (Sigma), and 1 µg/mL of thiamine (Sigma) were added. Thiamine is required by *E. coli* JM105 and JM109 for growth in M9 minimal medium, and ampicillin ensures the maintenance of the plasmid pSH101. The temperature was controlled at 37 ± 0.5°C and pH was maintained at 7.0 ± 0.3. The dissolved oxygen concentration was maintained above 25% air saturation. Shake flasks containing M9 minimal medium

were inoculated with a 2% v/v Luria broth (LB) culture, grown overnight at 37°C and at 200 rpm. These M9 minimal medium cultures grew for 12 h, at which time they were used to inoculate the bioreactor (0.3% v/v).

Heat-Shocked Chemostat

A chemostat of *E. coli* JM105 [pSH101] with a dilution rate of 0.35 h^{-1} was heat-shocked by raising the bioreactor temperature from 37 to 42°C in 13 min with the aid of a heating jacket. The chemostat was maintained at 42 ± 0.2°C for 2 h and returned to 37°C in 5 min by evapocooling the bioreactor's exterior. Two heat shocks were applied, 48 h apart. The temperature 42°C is a standard heat-shock temperature for *E. coli (16)*. In a similarly heat-shocked chemostat with *E. coli* JM105 expressing CAT, *GroEL* was upregulated, demonstrating induction of the classical response *(17)*.

Heat-Shocked Shake Flasks

Four shake flasks were used to examine the effects of a heat shock and simultaneous recombinant protein overexpression. Cells in two shake flask cultures, growing exponentially, were heat-shocked by transferring the flasks from a 30°C shaker incubator to a 42°C shaker water bath for 2 h. Heat-shock experiments were maintained at 42°C for only 2 h because of limited cell survival and rapid cellular metabolic changes *(16,18)*. One of these flasks was simultaneously induced with 5 m*M* IPTG. Two additional flasks were grown at 30°C as controls, and one was induced with 5 m*M* IPTG.

Stringent-Response Chemostat

A chemostat of *E. coli* JM109 [pSH101] with a dilution rate of 0.35 h^{-1} was chemically induced to produce a stringent response by the pulse addition of serine hydroxamate (100 mg/L) and 3 d later was induced for CAT expression by a pulse addition of IPTG (5 m*M*) *(19)*. Pulse additions are additions only to the bioreactor and not to the nutrient feed; therefore, the addition almost completely washes out of the bioreactor in three residence times (8.5 h). Calculated bioreactor concentrations with time are given in the Results section. In each case, the induction was initiated after steady state was reached. Serine hydroxamate inhibits the aminoacylation of seryl-tRNA without inhibiting serine synthesis or ppGpp (guanosine 5'-diphosphate 3'-diphosphate) decay *(4,20)*. Adenine (40 mg/L) was added concurrently with the serine hydroxamate, in order to eliminate potential serine deficiencies in nucleotide synthesis *(20)*.

Analysis of Culture Samples

Specific CAT activity, protease activity, cell density (optical density [OD]), and the glucose concentration were monitored. Glucose concentrations were determined from cell-free supernatants on a YSI Model 27 (YSI Incorporated, Yellow Springs, OH) glucose analyzer *(10)*. Cell growth was monitored on-line by optical density (at 600 nm) with a Spectrophotometer 21 (Milton Roy) and recorded using Labtech Notebook (Laboratory Technologies, Wilmington, MA) for the chemostat cultures. A flowcell

cuvet with a 1-mm pathlength was used. A small bubble trap was necessary to avoid erroneous data owing to bubble entrainment *(19)*. For the shake flask studies, cell density was measured off-line by optical density (at 600 nm) and diluted to maintain linearity.

Cell extract for the CAT and protease activity assays was prepared according to Rodriguez and Tait *(15)*, except 15 mL of fermentation broth were used for each sample, and after centrifugation and washing the final volume was 0.5 mL. Samples were sonicated in TDTT (50 m*M* Tris-HCl; 30 µ*M* dithiothreitol [DTT], pH 7.8) buffer on ice for 80 s with a 30% pulsed duty cycle using a Sonifier Cell Disrupter 350 with a microtip (Branson Ultrasonic Corporation, Danbury, CT). The supernatant was divided and stored at –20°C until assayed.

The CAT activity assay was performed as described by Rodriguez and Tait *(15)*. Cell extract samples were diluted up to one-twentieth with TDTT to maintain linearity. Total cellular protein was measured by a protein assay dye reagent concentrate (Bio-Rad, Hercules, CA) with bovine serum albumin as a standard *(15)*. Specific CAT activity is reported as nmol CAT/min · mg total protein and is the average of duplicates or triplicates. The specific CAT activity values are reproducible with an accuracy of ±5%.

The cell extract just described was also used for the protease activity assay. The sodium dodecyl sulfate-gelatin polyacrylamide gel electrophoresis (SDS-GPAGE) technique, which differentiates proteases by molecular weight and activity, was described previously *(21)*. Briefly, SDS-GPAGE sample preparations do not contain the normal concentration of DTT or β-mercaptoethanol (β-MeOH). Purified CAT, gelatin, and glycerol were obtained from Sigma. Tris base, acrylamide, *N*,*N*′-bis-acrylylcystamine, sodium dodecyl sulfate, ammonium persulfate, glycine, molecular weight markers, bromophenol blue, and *N*,*N*,*N*′,*N*′-tetramethyl ethylenediamine were obtained from Bio-Rad. Preparations of the samples, molecular weight markers, and purified CAT were incubated at 37°C for 20 min, instead of the usual 95°C for 5 min. Tris-HCl, gelatin, and glycine stock solutions were autoclaved in order to reduce the probability of bacterial protease contamination *(21)*. The SDS-GPAGE gels were washed with 2.5% (v/v) Triton X-100 and incubated for 24 h at 37°C in the incubation buffer. The SDS-GPAGE gels were stained with 0.2% amido black *(21)*. Based on duplicate and triplicate loadings, the SDS-GPAGE protease activity values are reproducible to ±10%.

Results

Continuous Culture: Mild Heat Shock

For mild heat shock in continuous culture, a chemostat of *E. coli* JM105 [pSH101] with a dilution rate of 0.35 h^{-1} was heat-shocked twice by manipulating the bioreactor temperature as shown in Fig. 1A. Specific CAT activity, protease activity, and glucose concentration were determined every 20–45 min during the 2-h heat shock, and cell density was measured on-line every 6 min (*see* Fig. 1B,D).

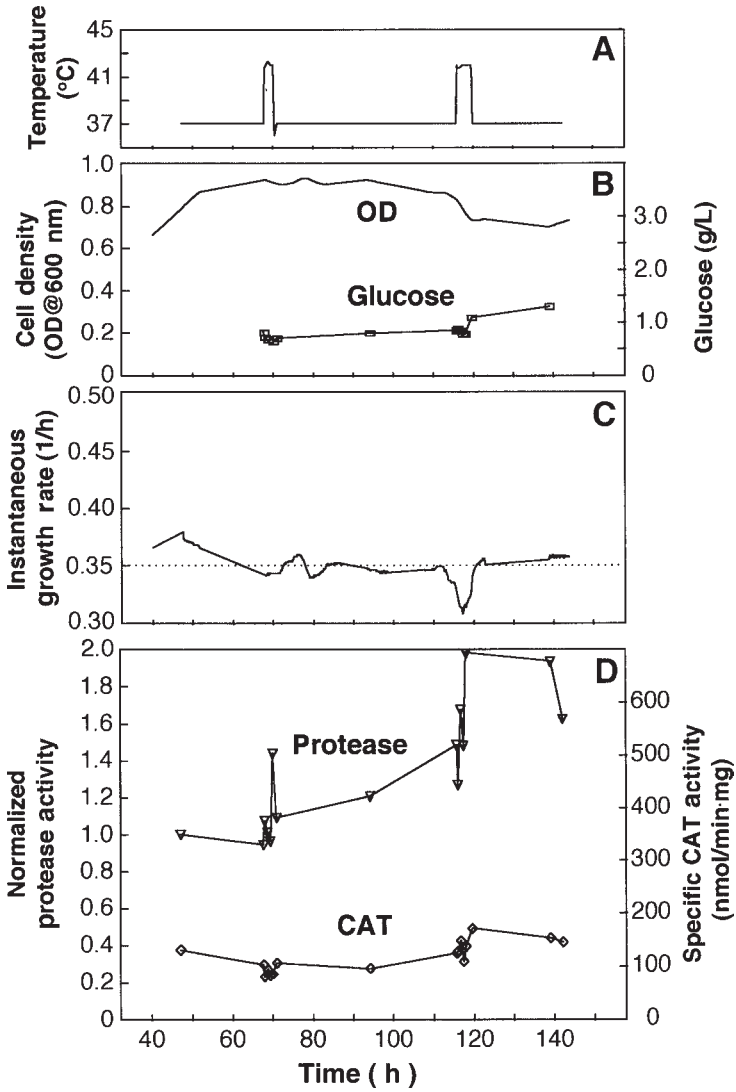

Fig. 1. Temperature profile **(A)**, cell density and glucose concentration **(B)**, instantaneous growth rate **(C)**, and specific CAT activity **(D)** vs time for *E. coli* JM105 [pSH101] in a chemostat containing M9 minimal medium. Two heat pulses were applied at 69 and 116 h.

During the first heat shock, the OD, glucose concentration, and instantaneous growth rate remained relatively constant (*see* Fig. 1B,C). The specific CAT activity was relatively constant during the first heat shock, as expected since IPTG was not added to induce CAT overexpression. The protease activity, as measured by the intensity of the 34- and 39-kDa proteases on SDS-GPAGE gels, increased by 25% following the first heat shock and remained elevated for 46 h after the heat shock was removed. As stated in the Materials and Methods section, these protease activity values are

from duplicate samples and accurate to ±10%. A sustained 30% increase in 34- and 39-kDa protease activity was noted by Holoman *(17)* for a similar heat-shocked chemostat. Additionally, a 66-kDa protease was detected during the heat shock that faded when the temperature was returned to 37°C (data not shown). This 66-kDa protease was not quantified because there was no pre-heat-shock activity with which to normalize the heat-shock protease activity.

For the second heat shock (at 116 h), the glucose concentration dropped slightly initially, and rose after the temperature was returned to 37°C. Likewise, the OD dropped as a result of the second heat shock, and it remained depressed until the end of the fermentation. The initially reduced cell density, decreased glucose concentration, and continuous addition of 4 g/L of glucose to the chemostat indicated that the cells were consuming more glucose per cell during the heat shock compared to before and after the heat shock by about 30%. The instantaneous growth rate decreased during the second heat shock and recovered when the bioreactor was cooled back to 37°C. The specific CAT activity remained constant at background levels during the second heat shock as expected, since no inducer was added. The protease activity during the second heat shock increased by an additional 30%, compared to the samples taken just prior to the second heat shock. The protease activity during the second heat shock was approximately double that of the samples taken prior to any heat shock. Again, the 66-kDa protease was also detected during the second heat shock and faded when the growth temperature was returned to 37°C (data not shown). The 66-kDa protease data are not included in Fig. 1D because the protease was not observed prior to the heat shock, and therefore could not be normalized. By contrast, the 34- and 39-kDa proteases remained elevated even after the growth temperature was returned to 37°C.

Stringent-Response and IPTG-Induction Chemostats

The mild heat-shock chemostat experiments demonstrated increased protease activity (34 and 39 kDa) for both heat shocks, in addition to a decreased growth rate and increased glucose consumption rate per cell for the second heat shock, an indication of cell stress. Interestingly, these are the same proteases observed to be upregulated during CAT overexpression, and a chemically imposed stringent response *(17,19)*. Using a relaxed strain (JM109), devoid of the classical stringent response, but otherwise quite similar to the "normal" strain (JM105), experiments were run under stringent-stress and recombinant protein overexpression stress conditions to examine the responses with respect to protease activity, cell density, glucose consumption, and CAT activity, as well as to determine whether an overlap exists. Figure 2 depicts the OD, glucose, specific CAT activity, protease activity, serine hydroxamate, and IPTG concentrations for a chemostat of *E. coli* JM109 [pSH101], a relaxed mutant, with a dilution rate of 0.35 h^{-1} (Fig. 2, solid lines). Serine hydroxamate (100 mg/L) was pulse added to the bioreactor as indicated. After an elapsed time of 72 h and reestablished

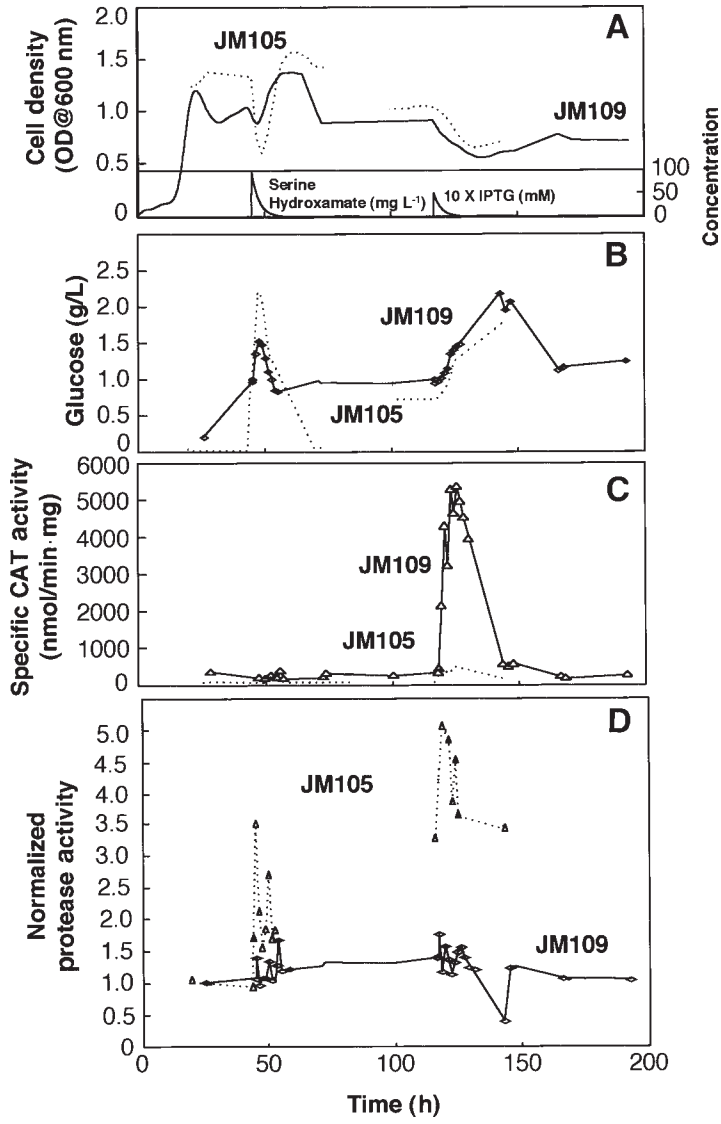

Fig. 2. Cell density, predicted serine hydroxamate concentration, and predicted IPTG concentration **(A)**, glucose concentration **(B)**, specific CAT activity **(C)**, normalized (34 and 39 kDa) protease activity **(D)** vs time for *E. coli* JM109 [pSH101] in a chemostat containing M9 minimal medium, given as solid lines. Dashed lines indicate data from *E. coli* JM105 [pSH101] serine hydroxamate addition and IPTG addition. *E. coli* JM105 data were aligned to the time of the initial stress *(19)*. Serine hydroxamate pulse was added at 45 h and IPTG pulse was added at 117 h.

steady state, the chemostat was pulse induced with 5 m*M* IPTG. Dashed lines in Fig. 2 depict *E. coli* JM105 [pSH101] data from previous work *(19)*. The first data set is from a chemically imposed stringent response (addition of serine hydroxamate). The second data set is from a second stress induction

with IPTG. The previous data has been aligned to the initial time of the imposed stress *(19)*. For the serine hydroxamate addition to JM109, the glucose concentration increased slightly, the OD dropped slightly, and the specific CAT activity was relatively constant at background levels. The protease activity increased 1.5 times for the JM109 culture and remained slightly elevated (similar to the heat-shock chemostats of JM105). For JM105, quite clear perturbations were observed in the same direction as for the JM109 culture, but to a much greater extent. For the cell density, the JM105 response was twofold more reduced, indicating partial cell washout. The chemically imposed stringent response resulted in a 3.5-fold increase in the protease activity. Finally, the observed perturbation in glucose was four times greater for JM105 than for JM109. The cells were not consuming the glucose (4 g/L) supplied by the feed medium (0.35 h^{-1}) at the same rate as prior to the stress; therefore, glucose accumulated in the bioreactor.

For the IPTG induction, the glucose concentration in the bioreactor of the JM109 culture dramatically increased and did not return to basal levels until nearly 60 h later, long after the IPTG had been diluted out of the bioreactor (IPTG washout took only 8.5 h). Additionally, the OD dropped and recovered after 60 h. The IPTG-induced culture produced significant amounts of specific CAT activity. Again, the overlaid results from JM105 showed that the OD and glucose concentration dynamics were similar *(19)*. Notably, the JM105 (*rel*$^+$) and JM109 (*rel*$^-$) cultures recovered similarly with respect to cell density and glucose concentration; however, the JM109 culture lagged by approx 2 h for the cell density recovery. Interestingly and more important, the maximum specific CAT activity obtained for *E. coli* JM109 [pSH101] was roughly *ninefold* higher than that observed for the IPTG-induced *E. coli* JM105 [pSH101] chemostat. For JM109, the protease activity increased insignificantly compared to preinduction or the chemically imposed stringent response, whereas the protease activity of the JM105 cells increased more than fivefold compared to the uninduced cells owing to IPTG induction. The JM105 cells maintained elevated (threefold) 34- and 39-kDa protease activity for 40 h after the IPTG had washed out of the bioreactor *(19)*.

Coincident Heat Shock and IPTG Induction in Shake Flasks

Earlier experiments of IPTG induction in shake flasks conducted in rich media have demonstrated that the presence or absence of CAT was not responsible for the upregulation of the 34- and 39-kDa proteases, but the expression rate relative to the availability of nutrients to synthesize the recombinant protein was significant *(19)*. Also, the 34- and 39-kDa proteases shown here have previously been shown not to degrade CAT; however, another protease (approx 10 kDa) with CAT specificity was found *(17)*. The response of this 10-kDa protease to heat shock has not been determined. Since the mild heat shock amplified 34- and 39-kDa protease activity and did not reduce the specific CAT activity from background levels to zero (Fig. 1D), we investigated inducing the heat-shock response while also

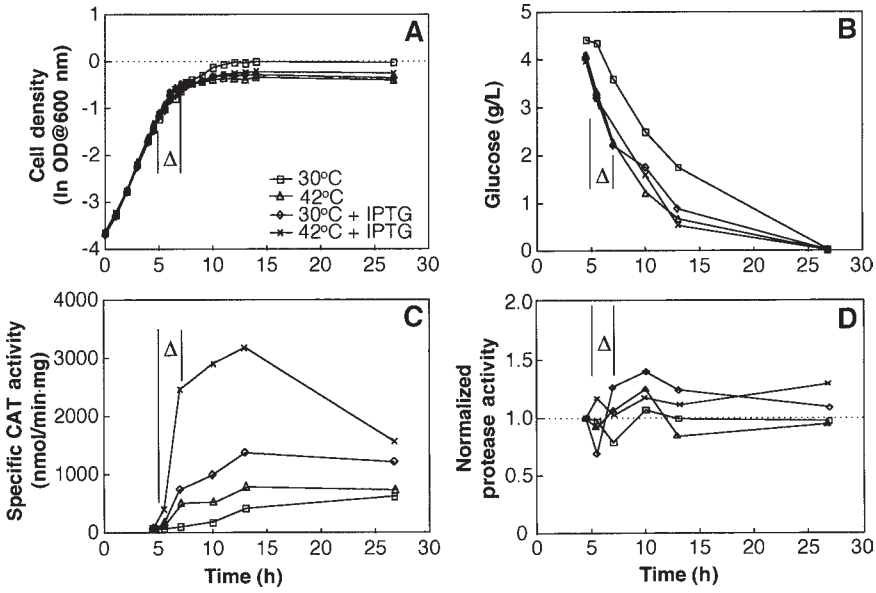

Fig. 3. Cell density **(A)**, glucose concentration **(B)**, specific CAT activity **(C)**, and normalized (34 and 39 kDa) protease activity **(D)** vs time for *E. coli* JM105 [pSH101] in shake flasks containing M9 minimal medium. Induction was at 5 h, and the heat shock was from 5 to 7 h (as indicted by Δ).

inducing recombinant protein expression in the *rel*$^+$ parent strain *E. coli* JM105. The coincident heat-shock and IPTG-induction studies were conducted to determine the effects of elevated temperature on protein overexpression. That is, there may be benefits from some of the heat-shock proteins (chaperones, transcription factors, and so forth) on CAT yield that outweigh any negative effects of the upregulation of the 34- and 39-kDa proteases and expression of the 66-kDa protease.

Shake flask experiments were heat-shocked during the midexponential growth phase (at 5 h) for 2 h. Figure 3A,B gives the growth curves and glucose concentrations. The cells grown at 30°C and induced had a slightly higher cell mass yield compared to the other "stressed" cultures, which in turn were roughly equivalent in terms of growth rate, glucose uptake, and cell yield. Note, that the heat shock in this case was more severe than in the chemostat experiments described previously. Here, the cells were initially grown at 30°C, and two of the cultures were shifted to 42°C for the heat shock. Figure 3C depicts the specific CAT activity of the four parallel cultures with time. The uninduced heat-shock culture (denoted 42°C) had a higher specific CAT activity than that of the uninduced (control) 30°C culture. The induced heat-shock culture (denoted 42°C + IPTG) accumulated specific CAT activity during the heat shock at nearly threefold the rate observed for the induced 30°C culture. Interestingly, the specific rates of CAT production were roughly equivalent when the temperature was returned to 30°C (at 7 h) for these two induced cultures.

Figure 3D gives the 34- and 39-kDa protease activities for the coincident heat shock IPTG induction experiments. Interestingly, only slight increases in protease activity were observed, with both of the induced cultures having the highest protease activities. The increases were roughly similar to those observed for the heat-shocked chemostat experiments. The only culture to experience a significant decrease in the specific CAT activity over time (hypothesized owing to proteolysis of the CAT) was the induced heat-shocked (42°C + IPTG) culture, and this occurred after a pronounced maximum. For the heat-shock, IPTG-induced culture, the 34- and 39-kDa proteases were still elevated slightly. In addition to the 34- and 39-kDa proteases that were quantified, two proteases with approximate molecular weights of 20 and 55 kDa were detected and had similar activities for all four conditions at all of the time points. Also, the 66-kDa protease was detected and faded as described for the chemostat heat-shock experiments, but with less intensity (gels not shown).

Discussion

Continuous Culture: Mild Heat Shock

For mild heat shock in continuous culture, the behavior of the OD and glucose concentration was quite similar to the IPTG–pulse induction chemostat described by Harcum and Bentley *(19)*, with respect to the first stress not significantly affecting the OD and glucose concentration, but the second stress causing the OD and glucose concentrations to change. The 34- and 39-kDa proteases were also observed to remain elevated for the recombinant protein overexpression chemostats long after the stress was removed, over 24 h *(19)*. Holoman *(17)* also noted elevated 34- and 39-kDa protease activity up to 6 h after the heat shock was removed. Based on the response of the proteases and changes observed in OD and glucose concentration, it would seem that the overexpression of a recombinant protein induces a stress response that is overlapping but not identical to either the stringent or heat-shock response. Also, the appearance of the 66-kDa proteases during the heat shock and the 80-kDa proteases previously observed during the stringent response *(19)*, respectively, indicate that many as-yet unknown cellular mechanisms may be involved in the stress responses.

The appearance of the 66-kDa protease in the heat-shocked chemostat culture suggested that other as-yet undetected proteases might be elevated by cellular stress and may play a significant role in recombinant protein degradation. Interestingly, the observed elevated proteases (34 and 39 kDa) were the same proteases observed to increase during the stringent response and during recombinant protein overexpression. However, the magnitude of the increase in 34- and 39-kDa protease activities in the present heat-shock experiments was not as great as with the IPTG-induction or the stringent-response experiments *(19)*.

Heat-Shock and Induction Effects in Shake Flasks

In rich-media IPTG-induction experiments, the 34- and 39-kDa protease activity, in addition to a 26-kDa protease, were upregulated owing to recombinant protein overexpression. In the nutrient-rich media (LB and M9 minimal media with casamino acids), the proteases were upregulated less than the cells grown in M9 minimal medium *(19)*. Thus, the difference in protease upregulation between the chemostat heat-shock and the batch heat-shock experiments was not due solely to the switch from chemostat to batch.

The accumulation of CAT in the shake flasks should have been constant unless inclusion body formation, ablative translation, or proteolytic degradation occurred. Ramírez and Bentley *(3)* demonstrated that inclusion bodies were not formed, rather that CAT was degraded. Ablative translation is not likely owing to the continued CAT expression and cell growth observed during the heat shock. Thus, in the present experiments, the proteolytic degradation of CAT was likely responsible for the reduction in the specific CAT activity. The specific CAT activity and 34-/39-kDa protease activity both increased for the induced cultures, but only slightly increased for the uninduced heat-shock culture.

The *trc* promoter is a leaky promoter; therefore, in batch cultures, the specific CAT activity should increase at a constant rate unless the CAT is being degraded. The control (30°C) culture accumulated CAT for the entire 24 h. The uninduced heat-shocked (42°C) culture accumulated CAT between 7 and 13 h at the same rate as the control culture; however, between 13 and 24 h the uninduced heat-shocked culture did not accumulate CAT. Either the heat shock had a delayed effect and slowed the synthesis rate, or the heat shock increased the degradation rate of CAT. Slowed synthesis or increased degradation rates of CAT both attest that long culture times are not beneficial for recombinant product formation. Other proteases were detected, and possibly undetected proteases (by the SDS-GPAGE method) were upregulated, likely causing the observed decrease in CAT accumulation.

For the heat-shocked, IPTG-induction experiments, the high accumulation of observed specific CAT activity for the 42°C cultures was most likely the result of increased transcription or translation rates due to the increased temperature. One possible molecular explanation would be increased *GroEL* or other chaperone protein during heat shock *(16,17)*. The similarity in the CAT accumulation rates (slope of the CAT activity curve) following the return to 30°C supports this heat-shock plus induced hypothesis. Note, however, the decrease in specific CAT activity in the most stressed culture was most probably due to proteolysis induced by recombinant protein overexpression. Increased induction temperatures could be used to improve the yield of a recombinant protein, as long as the product concentration is carefully monitored, such that the optimal yield is harvested.

Relaxed Mutant Stringent Response and IPTG-Induction Continuous Culture

In the heat-shock experiments, the 34-/39-kDa protease activities were amplified significantly (two times) with little effect on the background-specific CAT activity. Since the 34- and 39-kDa proteases are not CAT specific *(17)*, the upregulation of these proteases indicates stress and nutrient depletion and should not result in CAT degradation. Apparently other proteases have specificity to CAT, and are upregulated similar to the 34- and 39-kDa proteases, when CAT is being overexpressed, but not during a heat shock. Consequently, our attention turned to the overlap in the stringent stress response and overexpression. The results of similar stresses in JM105 and JM109 are therefore compared.

Figure 2 shows the culture parameters for JM109 and JM105 during a stringent response and IPTG induction. The JM105 data are from Harcum and Bentley *(19)* in which the JM105 was cultured at the same dilution rate (0.35 h^{-1}) and the stresses (serine hydroxamate and IPTG additions) were the same. The cellular response observed for the IPTG induction was quite similar to the observed response of glucose and OD for the second IPTG pulse induction of *E. coli* JM105 [pSH101] *(19)*. The protease activity for induced *E. coli* JM105 [pSH101] remained elevated for 48 h, whereas the protease activity due to IPTG induction in *E. coli* JM109 [pSH101] was never significantly amplified and returned to background levels soon after the IPTG washout of the chemostat. The slight (25%) increase in protease activity noted between the stringent response and IPTG induction (in JM109) could be the result of an adaptation mechanism similar to that commonly observed for the heat-shock response; that is, cells previously exposed to a heat shock have higher survival rates than cells not previously exposed to a heat shock *(22)*. Indeed, the heat-shock JM105 experiments suggest an adaptation mechanism expressed as a sustained increase in protease activity (25%).

Unlike the response noted for *E. coli* JM105 [pSH101] during the serine hydroxamate pulse addition, *E. coli* JM109 [pSH101] was not as stressed, as indicated by the OD and glucose changes relative to the changes observed for JM105 (Fig. 2A,B). However, even though these cells are *rel*$^-$ mutants and cannot trigger the stringent response *(4)*, the cells did undergo a slower, but significant change as a result of the aminoacylated-tRNA (specifically seryl-tRNA) shortage. The slow return to steady-state behavior is typical for *rel*$^-$ mutants *(4)*. There was a slight increase in protease activity (34 and 39 kDa) and an oscillatory response, particularly for the glucose concentration. The specific CAT activity was relatively constant at background levels. The behavior of *E. coli* JM105 [pSH101] was dramatically different from that observed for *E. coli* JM109 [pSH101] under similar conditions *(19)*. For IPTG-induced *E. coli* JM105 [pSH101], the protease activity increased to five times that of the background levels. For JM109, increased protease activity was noted but was similar to the heat-shock chemostat values. Note also

that *E. coli* JM105 [pSH101] experienced a 3.5-fold increase in protease activity due to the addition of serine hydroxamate. In both cultures, the specific CAT activity decreased rapidly after the maximum, owing to IPTG washout and/or proteolytic activity.

The continuous culture results demonstrate that the 34- and 39-kDa proteases were amplified significantly during overexpression of CAT and during the stringent-stress response. Furthermore, the mutant (*rel*$^-$) results corroborate our hypothesis that these proteases were strongly influenced by nutrient (amino acid) perturbations. It is particularly noteworthy that the JM109 culture resulted in nearly nine times more specific CAT activity than JM105, which was more sensitive to an applied stress. Although the large increase in 34-/39-kDa protease activity in the IPTG-induced JM105 culture was coincident with very low CAT activity, and a very high CAT activity was found in the JM109 culture that had a minimal increase in protease activity, it is premature to suggest a direct linkage. For example, the heat-shock response chemostat experiments showed an increase in 34- and 39-kDa protease activity, without a dramatic decrease in CAT activity. That is, during the heat shock, a twofold increase in protease activity was observed with no significant decrease in specific CAT activity. However, when cultures were heat-shocked and IPTG induced, CAT was degraded by proteases yet unidentified. Thus, it remains unclear at this point whether the 34-/39-kDa protease amplification was directly linked to CAT degradation. These results do suggest, however, that for chloramphenicol acetyltransferase, an increase in temperature might not adversely affect yield, but instead might increase yield.

Conclusion

The relaxed mutant was not adversely affected by the addition of serine hydroxamate used to inhibit aminoacylation of tRNA. This was unlike the response observed by *E. coli* JM105 [pSH101]. However, IPTG induction for both cultures resulted in a decreased growth rate in conjunction with CAT production and increased glucose concentrations. Moreover, the same proteases were found to be amplified. This implies that the stress resulting from IPTG induction is related to, but not identical to, a stringent response. And, of the three stresses examined, the heat-shock response seems to be less related to the stringent and overexpression stress responses, than the stringent and overexpression stress responses are to each other. The cellular mechanisms that recognize amino acid deficiencies are probably related, such that coordinated nutrient feeding could reduce the enhancement of protease activity during an IPTG-induction stress for *rel*$^+$ cells. Ramírez and Bentley *(3)* demonstrated that the controlled feeding of phenylalanine to batch cultures improved the CAT yield twofold, reduced heat-shock protein levels, and reduced the 34- and 39-kDa protease activity. Yet, CAT was still degraded, indicating an additional nutrient stress. Interestingly, the heat-shocked IPTG-induced JM105 culture had

relatively constant 34- and 39-kDa protease activity, and the highest CAT activity. Unfortunately, the CAT activity was less stable for the heat-shock-induced culture, but an early harvest could be used to maximize the CAT yield. Finally, most relevant to the production of recombinant proteins, the relaxed mutant had a *ninefold* higher CAT activity compared to the parent strain.

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