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Serine Hydroxamate and the Transcriptome of High Cell Density Recombinant *Escherichia coli* MG1655

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Abstract For more than 30 years, serine hydroxamate has been used to chemically stimulate a stringent response in *Escherichia coli* and other bacteria. These studies have elucidated numerous characteristics of the classical stringent response beyond the simple cellular response to an amino acid shortage, including phospholipid synthesis and protease upregulation. In this study, the effects of a serine hydroxamate addition on high-cell-density recombinant *E. coli* were examined and compared to the effects of recombinant protein production to determine overlaps, as recombinant protein production stress has often been attributed to amino acid shortages. Both the transcriptome and growth characteristics were evaluated and compared. The serine hydroxamate addition profoundly decreased the culture growth rate, whereas recombinant protein production did not. Conversely, the transcriptome profile of the recombinant *E. coli* cultures were relatively unaffected by the serine hydroxamate addition, yet recombinant protein production dramatically changed the transcriptome profile. A subset of the classical stringent response genes were effected by the serine hydroxamate addition, whereas recombinant protein production regulated numerous classical stringent response genes but not all. The genes that were regulated by the serine hydroxamate addition include numerous fatty acid synthesis genes, in agreement with altered phospholipids synthesis reports. These results indicate that recombinant protein production and the stringent response have many overlapping responses but are far from identical.

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Introduction

The stringent response is a global bacterial stress response to nutritional stress. The stringent response is induced specifically by a high uncharged transfer ribonucleic acid (tRNA) to aminoacylated tRNA ratio, caused by intracellular amino acid limitations (or shortages) due to protein synthesis demands [1–4]. This response can be caused either by limiting the availability of amino acids or by limiting the ability to aminoacylate tRNA, even in the presence of abundant cognate amino acids. Recent work has related the regulatory effects of ppGpp and pppGpp (guanosine 3′,5′-*bis* (diphosphate) and guanosine 5′-triphosphate 3′-diphosphate, respectively), collectively known as (p)ppGpp, to nutritional stress [1]. There are still conflicting hypotheses regarding the regulatory effects of the (p)ppGpp concentration in the stringent response; however, relaxed mutants (*relA*$^-$) have been used widely to better understand the importance of this cellular response [1, 5–7]. Specifically, the normal *relA* gene encodes the reversible enzyme ppGpp synthetase I that catalyzes the synthesis of both ppGpp and pppGpp from adenosine triphosphate (ATP) and either guanosine diphosphate or guanosine triphosphate (GTP), respectively [8]. The rapid onset of the stringent response has been correlated with the accumulation of ppGpp [9].

L-Serine hydroxamate (serine hydroxamate) is an analogue for L-serine. Serine hydroxamate inhibits *Escherichia coli* cell growth by binding seryl-tRNA synthetase [10]. Serine hydroxamate has been used widely to mediate the stringent response in *E. coli* and other bacteria [7, 11] and with low-cell-density recombinant *E. coli* cultures to compare the behavior of the cells to a stringent response and recombinant protein production [12]. A relaxed mutant (*relA*$^-$) was observed to have improved recombinant protein production levels due to isopropyl-1-thio-β-D-galactopyranoside (IPTG) induction compared to the *relA* normal strain. Additionally, it was observed that proteases were not upregulated in the relaxed mutant due to a serine hydroxamate addition, whereas the parental strain was observed to have upregulated protease activity [12].

It was hypothesized that recombinant protein production leads to a stringent response due to the high amino acid synthesis demands related to recombinant protein synthesis. A comparison of the transcriptomes during recombinant protein production and a chemical imposed stringent response would assist with determining what portion of the "metabolic burden" associated with recombinant protein production is due to amino acid shortages. In this study, the transcriptome profiles of recombinant *E. coli* were examined and compared for the three culture conditions: (1) normal growth, no external stress, (2) L-serine hydroxamate addition (to mediate a stringent response), and (3) IPTG induction to produce the recombinant protein chloramphenicol acetyltransferase (CAT). The transcriptome profiles from these three conditions were analyzed using Affymetrix Anti-sense *E. coli* GeneChip® microarrays.

Materials and Methods

E. coli MG1655 [pPROEx-CAT] was used for all studies. The plasmid [pPROEx-CAT] contained the *trc* promoter to control CAT expression, the pBR322 origin of replication, ampicillin resistance, and the *lacI* gene (Invitrogen). One milliliter of frozen cells was

inoculated into 500 mL shake flasks with 100 mL Luria–Bertani (LB) medium with (0.5% *w*/*v*) glucose and 40 mg/L ampicillin. The LB medium was prepared as previously described [13]. The cultures were incubated overnight at 37°C and agitated by shaking at 200 rpm. A 4-L fed-batch fermenter (Phillips Petroleum), with a 50% working volume, was inoculated with the overnight culture. Cells were initially cultured in the LB medium with 5 g/L glucose in the fermenter. Once glucose was consumed, a feed medium was used. The feed medium contained 500 g/L glucose and 70 g/L $MgSO_4 \cdot 7H_2O$. The glucose concentration was maintained at approximately 1 g/L. The glucose concentration was measured by a blood glucose meter (One Touch Profile). Sterile antifoam (0.1% *v*/*v*) was added to the fed-batch fermenter. Air or oxygen was sparged continuously into the fermenter at approximately 1,800 mL/min. Cultures were agitated at approximately 500 rpm. The dissolved oxygen (DO) level was measured by a DO probe (Cole Parmer). The DO level was controlled at 60% saturation by adjusting agitation speed and air/oxygen flow rate. Sterile 5 M NaOH was used to control pH online to 7.0±0.2. The optical density (OD) was measured with a Beckman DU® 640 spectrophotometer at 595 nm. L-Serine hydroxamate was added to the *E. coli* cultures at 100 mg/L. To avoid complications due to the effects of serine starvation on the supply of purines, adenine was added to the medium at 40 mg/L [10]. Serine hydroxamate was added to the *E. coli* cultures when the cell density reached approximately 70% of the fermenter's maximum level (approximately 11.5 OD). Recombinant protein expression was induced with 5 mM IPTG at approximately the same OD. The control fermentations were run similarly, except without either stress. For the serine hydroxamate fermentations, cells were harvested immediately prior to the serine hydroxamate addition (time S0) and 1-h after serine hydroxamate addition (time S1). For the IPTG-induced fermentations, cells were harvested immediately prior to the IPTG addition (time S0) and 1-h postinduction (time S1). For the uninduced (unstressed) fermentations, cells were harvested at time S0 and time S1, for which the timing was synchronized with the serine hydroxamate and IPTG fermentations based on OD for time S0 and time for time S1. All fermentation experiments were conducted in duplicate.

The specific CAT activity and cell harvest procedures have been described previously [14]. Each fermentation condition was repeated (two biological replicates). RNA from each biological replicates was purified and processed independently. Three biological replicates were obtained for the control condition (time S0), since prior the serine hydroxamate or IPTG addition, all fermentations were replicates. Prior to deoxyribonucleic acid (DNA) microarray hybridization, where only two biological replicates existed, one of the processed samples was divided into two technical replicates, resulting in three separate hybridized chips. All time S1 samples contained three technical replicates from two biological duplicates, whereas the control time S0 measurement contained three biological replicates. The variance between biological replicates and between technical replicates was similar; therefore, gene expression levels were weighted equally. The control prestress samples corresponded to zero time with respect to the stresses (serine hydroxamate or IPTG). The control 1-h samples corresponded to 1-h after the control samples for the unstressed cultures. The serine hydroxamate 1-h samples corresponded to the 1-h after the serine hydroxamate addition and will be referred to as the serine hydroxamate cultures. The IPTG 1-h samples corresponded to 1 h after the IPTG addition and will be referred to as the IPTG-induced cultures.

The total RNA isolation, complementary DNA synthesis, fragmentation, labeling, array hybridization, and scanning methods have been described previously [14]. Briefly, Microarray suit 5.0 (Affymetrix) was used to average the scans and process the data, and further data analysis was conducted using Affymetrix® Data Mining Tool (DMT 3.0).

GeneSpring® software was used to analyze the raw data. The raw intensities (per gene) were normalized to the median array intensity and set to a constant value (150). This effectively normalized the gene intensities to the total RNA loaded on the array. Welch analysis of variance (ANOVA) tests with Benjamini and Hochberg false discovery rate corrections (parametric tests assumes equal variances) were used for the multiple comparisons with a cutoff value of $p \leq 0.05$. Tukey post-hoc analysis was also used. Welch t tests with a Benjamini and Hochberg false discovery rate correction ($p \leq 0.05$) were used for all pairwise comparisons only examining the regulated genes as determined by the ANOVA analysis. The raw data intensities for all DNA microarrays discussed may be obtained from the University of Wisconsin *E. coli* Genome Project via "A Systematic Annotation Package for Community Analysis of Genomes" as a guest (password: guest; https://asap.ahabs.wisc.edu/annotation/php/logon.php) [15].

Fatty acid extracts were prepared by methanolysis and analyzed by the Clemson Multi-User Analytical Facility using a Hewlett-Packard 5890A gas chromatograph with a HP 5971A mass-selective and flame ionization detectors, autosampler, with HP 3365 Series II ChemStation software according to the instructions of the Microbial Identification System (MIDI; Microbial ID). Polar lipids were extracted according to Golden and Powell [16] and identified using Micromass capillary LC interfaced with an ESI Q-Tof microT MS (both from Waters, USA) housed in the Clemson University Genomics Institute, Clemson University [16].

Results and Discussion

Previous research has indicated that serine hydroxamate stimulated a stringent response by blocking the incorporation of serine into proteins, specifically by blocking the charging of seryl-tRNA [10, 11], essentially impeding protein synthesis. Additionally, it has been proposed that recombinant protein production can stimulate the stringent response due to transient intracellular amino acid depletions [12]. The effects of serine hydroxamate and IPTG-induced recombinant protein production on high-cell-density recombinant cultures were investigated to identify genes affected by both the classical stringent response and recombinant protein production. Recombinant *E. coli* MG1655 [pPROEx-CAT] were cultured in parallel fed-batch fermenters. The cultures were exposed to serine hydroxamate or IPTG at synchronized cell densities with control cultures. Figure 1 shows the growth curves for the serine hydroxamate, IPTG-induced, and control fermentations with the samples' time indicated by the vertical lines relative to the synchronization time. The growth curves are shown on linear y-scales, such that at the high cell densities, the significant cell density differences between the control, IPTG-induced, and serine hydroxamate cultures can be observed. The specific CAT activities and glucose concentrations for the three culture conditions are also shown in Fig. 1. The growth characteristics prior to the serine hydroxamate or IPTG additions were the same for all the fermentation conditions. The preaddition-specific growth rates were 0.4 h^{-1}, which were fairly typical for fed-batch cultures [17]. The maximum cell density reached by the control cultures was 16.4 OD_{595}. The maximum cell density reached by the serine hydroxamate and IPTG cultures were 14.3 OD_{595} and 17.1 OD_{595}, respectively. Glucose was maintained at approximately 1 g/L for all the cultures by periodic feeding of the concentrated feed. The glucose profiles were not significantly different between the culture conditions; however, the glucose consumption for serine hydroxamate and IPTG-induced cultures was significantly higher than the control cultures. Specifically, the control cultures consumed 46.5 g glucose,

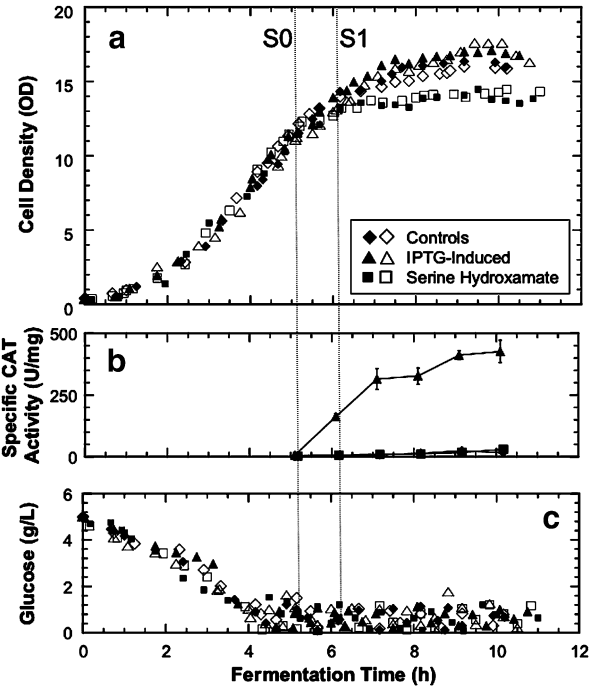

Fig. 1 Growth curves (**a**) for replicate recombinant *E. coli* MG1655 [pPROEx-CAT] cultures in fed-batch fermenters for the control (*solid* and *hollow diamonds*), IPTG-induced (*solid* and *hollow triangles*), and L-serine hydroxamate addition (*solid* and *hollow squares*) conditions. The *dashed lines* (S0 and S1) indicate the sample time relative to the stresses (IPTG-induction or L-serine hydroxamate addition) and the sample time 1-h poststress, respectively. (**b**) shows the average CAT expression for each of the three conditions and (**c**) shows the glucose concentrations

whereas both the serine hydroxamate and IPTG cultures consumed 56 g glucose during the course of the fermentations.

The serine hydroxamate addition stressed the high-cell-density recombinant cultures as evidenced by the dramatic decrease in the growth rate compared to the control recombinant and IPTG-induced recombinant cultures (Fig. 1a). Additionally, the higher glucose consumption of the serine hydroxamate culture matched the level observed for the IPTG-induced recombinant cultures that were overexpressing a recombinant protein compared to the control recombinant cultures that were not. This is interesting, since recombinant protein production is known to cause a metabolic burden on the cells [18], yet the recombinant serine hydroxamate addition cultures were utilizing glucose at very high levels but not expressing any recombinant protein or growing well (Fig. 1b). The serine hydroxamate addition alone reduced growth rate and cell yield from glucose. Previous serine hydroxamate addition cultures were conducted as batch studies, so the decrease in cell yield from glucose was not observable.

To determine the effect of serine hydroxamate on the transcriptome of recombinant cells and compare these results to recombinant protein production, samples from the synchronized cultures were obtained. The ANOVA and Tukey post-hoc analysis of the control and serine hydroxamate cultures identified 1,128 genes with differential expression between one of the three pairs. The Welch *t* test analysis between the control sample time 1 and serine hydroxamate sample time 1 identified 724 genes with differential expression. Ninety of these genes had a greater than twofold difference between the control sample time 0 and control sample time 1 conditions, so these genes were not considered in the screening phase, as these genes appear to be more sensitive to the media composition than serine hydroxamate. These remaining 631 genes were used to determine to what extent the serine hydroxamate addition and recombinant protein production elicited classical stress responses at the transcriptome level.

Most of these 631 genes regulated by the serine hydroxamate addition were also significantly regulated by IPTG induction of the recombinant protein CAT (487 genes); however, the direction of the regulation was not always the same. The effects of recombinant protein production on the transcriptome have been presented elsewhere [14], so these effects will only be discussed in this study, as related to the serine hydroxamate response and the stringent response. A list of these 631 serine hydroxamate-sensitive genes with fold changes relative to the control sample time 1 cultures for both the serine hydroxamate and IPTG additions for genes with $p \leq 0.05$ can be found in the Online Supplementary Materials (http://www.eng.clemson.edu/bio/people/harcum.html). Of the 631 regulated genes, 223 of these genes had twofold or greater change and these 223 genes with fold changes are provided in the Appendix for both the serine hydroxamate and IPTG-induced cultures.

To determine the extent of the classical stringent response induced by the serine hydroxamate addition, the genes identified by Cashel et al. [1] as stringent response genes were examined for the serine hydroxamate cultures. Cashel et al. [1] noted that 47 genes or proteins were normally upregulated due to the stringent response and 31 genes or proteins were normally downregulated, excluding the ribosomal RNA (rRNA)-related genes [1], where many of these genes and protein were identified using mutants, whereas in the studies presented here, the parental strain MG1655 was the host organism. For serine hydroxamate-treated cultures, 18 of the 47 normally upregulated genes were regulated, and 14 of these genes were detected as upregulated. The four downregulated genes were *metCFK* and *thrA*. Table 1 lists all of the classical stringent response genes with fold changes for the serine hydroxamate and IPTG-induced cultures relative to the control 1-h samples ($p \leq 0.05$). For the genes normally observed to be downregulated by the stringent response, the serine hydroxamate treatment only appears to have regulated *valS*; however, the *valS* gene was observed to be upregulated 1.6-fold. In comparison, recombinant protein production (IPTG cultures) significantly regulated 36 of the 47 normally upregulated classical stringent response genes; however, many of these genes were observed to be downregulated. For the genes normally downregulated by the classical stringent response, 30 of the 31 genes were regulated by recombinant protein induction, where only one of these genes was observed to be upregulated (*pyrI*) [14].

Based on the behavior of the classical stringent response genes, in this study, it would seem that serine hydroxamate mediated a mild stringent response in the high-cell-density recombinant cultures, although severely affected the cells ability to grow. In previous studies where serine hydroxamate has been used to mediate the classical stringent response in recombinant *E. coli*, the cell densities were much lower (1 OD compared to 11 OD); thus, the ratio of serine hydroxamate to cells in previous studies have always been much higher [7, 12]. The higher cell densities of this study might have dissipated the negative impact of serine hydroxamate, resulting in a stringent response that was attenuated at the transcriptome level. Since serine hydroxamate inhibits protein synthesis indirectly, it could also be that the high cell density cultures are more sensitive to disturbance in protein synthesis, and without protein synthesis, transcriptome changes have a self-limiting range of effect.

Since the serine hydroxamate addition caused a dramatic decrease in the culture growth rate, the genes associated with the classical stationary phase response were examined. The classical stationary phase response contains 74 genes, which, as proteins or genes, have been observed to be upregulated as the cells enter the stationary phase [19, 20]. For the serine hydroxamate cultures, 12 of the 74 classical stationary phase genes were significantly regulated; however, only six of these genes were upregulated in the presence of serine hydroxamate (*clpA*, *dacC*, *himD*, *osmB*, *proP*, and *treA*). The serine hydroxamate addition did not mediate a full classical stationary phase response, even though the growth rates

Table 1 Gene expression fold changes for the serine hydroxamate addition (SH) and recombinant protein production (IPTG) cultures relative to control cultures at 1-h poststress for the classical stringent response genes and/or protein [1].

Gene	bname	SH Δfold	IPTG Δfold	Description
Normally upregulated				
argB	b3959	3.4	−2.2	Acetylglutamate kinase
argC	b3958	5.6	−2.2	*N*-Acetyl-gamma-glutamylphosphate reductase
argE	b3957	2.7	−2.4	Acetylornithine deacetylase
argF	b0273	50	6.1	Ornithine carbamoyltransferase 2, chain F
argH	b3960	2.3		Argininosuccinate lyase
argI	b4254	2.9	2.7	Ornithine carbamoyltransferase 1
asd	b3433		−7.7	Aspartate–semialdehyde dehydrogenase
clpB	b2592	1.5	−1.9	Heat shock protein
dapB	b0031		−4.8	Dihydrodipicolinate reductase
deoA	b4382		−3.0	Thymidine phosphorylase
dnaK	b0014		−2.6	Chaperone Hsp70; DNA biosynthesis; autoregulated heat shock proteins
gdhA	b1761		−2.0	NADP-specific glutamate dehydrogenase
glnA	b3870	1.5	−12	Glutamine synthetase
gltB	b3212		−1.3	Glutamate synthase, large subunit
hflB	b3178		−4.5	Degrades σ^{32}, integral membrane peptidase, cell division protein
himA	b1712		−7.0	Integration host factor (IHF), alpha subunit; site-specific recombination
hipA	b1507		2.6	Persistence to inhibition of murein or DNA biosynthesis, DNA-binding regulator
hipB	b1508		1.9	Persistence to inhibition of murein or DNA biosynthesis; regulatory protein
hisB	b2022	2.7	−2.1	Imidazoleglycerol phosphate dehydratase and histidinol-phosphate phosphatase
hisD	b2020		−1.7	L-Histidinal/NAD^+oxidoreductase; L-histidinol/NAD^+oxidoreductase
hisG	b2019	1.9	−2.2	ATP phosphoribosyltransferase
htpG	b0473			Chaperone Hsp90, heat shock protein C 62.5
ileS	b0026		−4.0	Isoleucine tRNA synthetase
ilvA	b3772		1.6	Threonine deaminase (dehydratase)
ilvB	b3671	2.1		Acetolactate synthase I, valine-sensitive, large subunit
lacZ	b0344		3.4	β-D-Galactosidase
lysA	b2838		8.3	Diaminopimelate decarboxylase
lysC	b4024		3.1	Aspartokinase III, lysine sensitive
lysU	b4129		−3.6	Lysine tRNA synthetase, inducible; heat shock protein
metC	b3008	−1.7	−1.7	Cystathionine beta-lyase (β-cystathionase)
metF	b3941	−7.4	−5.2	5,10-Methylenetetrahydrofolate reductase
metK	b2942	−2.3	−6.8	Methionine adenosyltransferase 1 (AdoMet synthetase); methyl and propylamine donor, corepressor of met genes
mopA	b4143	1.5	−3.7	GroEL, chaperone Hsp60, peptide-dependent ATPase, heat shock protein
pheS	b1714	1.2	−3.0	Phenylalanine tRNA synthetase, α-subunit
pheT	b1713		−7.2	Phenylalanine tRNA synthetase, β-subunit
relA	b2784		−6.0	(p)ppGpp synthetase I (GTP pyrophosphokinase); regulation of RNA synthesis; stringent factor
rpoD	b3067		−3.8	RNA polymerase, σ^{70} factor; regulation of proteins induced at high temperatures
rpoH	b3461		−4.4	RNA polymerase, σ^{32} factor; regulation of proteins induced at high temperatures
rpoS	b2741		−5.9	RNA polymerase, σ^{S} factor; synthesis of many growth phase-related proteins
speC	b2965		2.4	Ornithine decarboxylase isozyme

Table 1 (continued)

Gene	bname	SH Δfold	IPTG Δfold	Description
spoT	b3650		−3.2	(p)ppGpp synthetase II; also guanosine-3′,5′-*bis*-pyrophosphate 3′-pyrophosphohydrolase
thrA	b0002	−1.5	−3.0	Aspartokinase I, homoserine dehydrogenase I
thrB	b0003			Homoserine kinase
thrC	b0004			Threonine synthase
thrS	b1719	1.3	−7.5	Threonine tRNA synthetase
udp	b3831		−2.4	Uridine phosphorylase
Normally downregulated				
apt	b0469		−1.9	Adenine phosphoribosyltransferase
argS	b1876			Arginine tRNA synthetase
cfa	b1661		−7.1	Cyclopropane fatty acyl phospholipid synthase
cls	b1249		−2.2	Cardiolipin synthase, a major membrane phospholipid; novobiocin sensitivity
cspA	b3556		−30	Cold shock protein 7.4, transcriptional activator of hns
fabA	b0954		−4.6	β-Hydroxydecanoyl thioester dehydrase, *trans*-2-decenoyl-ACP isomerase
fusA	b3340		−13	GTP-binding protein chain elongation factor EF-G
gppA	b3779		−1.5	Guanosine pentaphosphatase; exopolyphosphatase
gpt	b0238		−1.3	Guanine–hypoxanthine phosphoribosyltransferase
guaB	b2508		−2.1	IMP dehydrogenase
infC	b1718		−8.5	Protein chain initiation factor IF-3
lgt	b2828		−1.5	Phosphatidylglycerol-prolipoprotein diacylglyceryl transferase; a major membrane phospholipids
metZ	b2814		−6.8	Initiator methionine tRNAf1; triplicate gene
pgi	b4025		−3.7	Glucosephosphate isomerase
plsB	b4041		−4.4	Glycerol-3-phosphate acyltransferase
ppc	b3956		−2.4	Phosphoenolpyruvate carboxylase
psd	b4160		−2.0	Phosphatidylserine decarboxylase; phospholipid synthesis
pssA	b2585		−2.6	Phosphatidylserine synthase; phospholipid synthesis
purA	b4177		−3.9	Adenylosuccinate synthetase
pyrB	b4245		2.5	Aspartate carbamoyltransferase, catalytic subunit
pyrI	b4244		1.7	Aspartate carbamoyltransferase, regulatory subunit
rpoA	b3295		−12	RNA polymerase, α subunit
rpoB	b3987		−7.3	RNA polymerase, β subunit
rpoC	b3988		−3.5	RNA polymerase, β' subunit
speA	b2938		−1.6	Biosynthetic arginine decarboxylase
trmA	b3965		−1.8	tRNA (uracil-5-)-methyltransferase
tsf	b0170		−3.8	Protein chain elongation factor EF-Ts
tufA	b3339		−11	Protein chain elongation factor EF-Tu (duplicate of tufB)
tufB	b3980		−10	Protein chain elongation factor EF-Tu (duplicate of tufA)
upp	b2498		−2.2	Uracil phosphoribosyltransferase
valS	b4258	1.6	−2.9	Valine tRNA synthetase

Genes that are normally upregulated during the stringent response are shown in A, and the normally downregulated genes are shown in B.

dropped to 0.09 h^{-1} 1 h after the serine hydroxamate addition, compared to a growth rate of 0.2 h^{-1} for the control and IPTG-induced cultures. Again, the failure to observe a changed in the transcriptome related to the classical stationary phase could be due to a lack of the protein RpoS, to transmit the message.

The heat shock response, stringent response, and protease regulation have many overlapping mechanisms [6]; thus, the gene expression profiles of the heat shock response and identified protease genes were examined. It was observed that only 9 of the 35 heat shock genes (*clpAB*, *gapA*, *hscA*, *htpX*, *ibpA*, *ldhA*, *lon*, and *mopA*) were significantly regulated by the serine hydroxamate addition. All of these genes were upregulated, except *gapA*. The protease genes *clpAB* and *ompT* were the only protease genes observed to be regulated due to the serine hydroxamate addition, and these were only upregulated approximately 1.5- to 2-fold.

The serine tRNA synthetase gene (*serS*) was significantly downregulated by the serine hydroxamate addition approximately 1.3-fold. In all, five aminoacyl-tRNA synthetases were affected (*cysS*, *pheS*, *serS*, *thrS*, and *valS*), where the other four genes were slightly upregulated. Additionally, none of the *rpo* or rRNA genes were affected by the serine hydroxamate addition. Only 10 of the 55 ribosomal protein genes (*rplDMPSVW*, *rpmCFI*, and *rpsS*) were affected by the serine hydroxamate addition. Furthermore, these ribosomal protein genes were only downregulated approximately 1.3-fold, except *rpmFI*, which were upregulated about 1.5-fold. By comparison, recombinant protein production resulted in the downregulation of 51 of the 55 ribosomal protein genes and was downregulated between 5- and 13-fold.

Both the serine hydroxamate addition and IPTG-induced recombinant protein expression significantly regulated a high number of unknown genes. Specifically, the serine hydroxamate addition had more than 300 hypothetical/putative/probable genes regulated out of the 631 identified genes, including the downregulation of *yhiDEFMNV* (putative transport genes) between two- and sevenfold.

Although the classical stringent response and stationary phase genes were only mildly affected by the serine hydroxamate addition, many metabolic pathway genes were regulated. Using Pathway Tools to visual the regulated genes [21], 286 of the 631 regulated genes mapped onto the *E. coli* metabolic pathways. Pathway Tools visually overlays the gene expression changes on to the metabolic pathways of a specific organism. The most striking aspect of the serine hydroxamate addition on metabolism was the high number of regulated genes related to amino acid biosynthesis, utilization, and transport. Additionally, many genes associated with fatty acid and lipid synthesis were regulated. Figure 2 shows the gene expression changes for most of the amino acid biosynthesis, fatty acid and lipid biosynthesis, and two amino acid degradation pathways using the Pathway Tool projections [22]. The common metabolites are shown with dashed line connections, while metabolite conversion between the pathways is shown with arrows. Many of the amino acid biosynthesis pathways were uniformly upregulated or downregulated. For example, in the histidine biosynthesis pathway, all but two genes were upregulated. For arginine biosynthesis, the entire pathway was upregulated, and the degradation pathway was entirely downregulated. Genes in the methionine pathway were either not regulated or downregulated.

Histidine biosynthesis is a very energy-intensive process and thus is highly controlled in *E. coli* [23]. Additionally, the histidine biosynthesis pathway provides a critical link between amino acid synthesis and purine biosynthesis [24]. It is most interesting to note that guanosine 3′,5′-*bis*(diphosphate) [ppGpp] is an inhibitor of the HisG enzyme, the enzyme that catalyzes the first reaction in the histidine pathway, in the presence of histidine

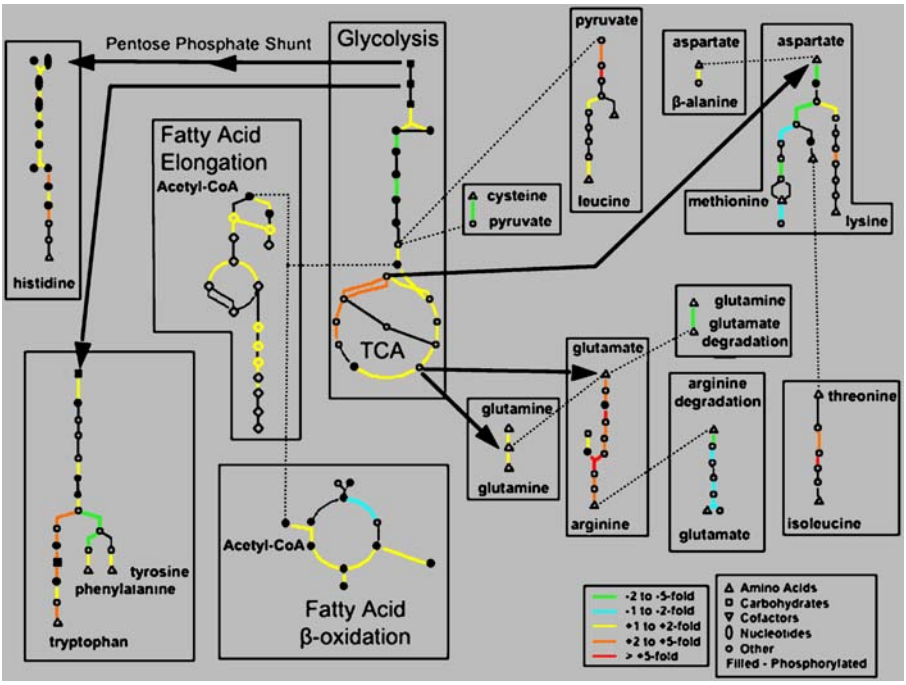

Fig. 2 The major metabolic pathways in *E. coli* that were significantly regulated due to the serine hydroxamate addition. The projections were generated using Pathway Tools [21, 22]. Glycolysis and the TCA cycle provide the precursors to the amino acid biosynthesis pathways, as well as the fatty acid and lipid biosynthesis pathways. Pathways are labeled by the terminal amino acid. The amino acid degradation pathways are shown for arginine and glutamate. Downregulated genes are shown in *blue* (-1 to -2-fold) and *green* (-2 to -5-fold), and upregulated genes are shown in *yellow* (+1 to +2-fold), *orange* (+2 to +5-fold), and *red* (greater than +5-fold)

and is a positive effector of the *his* operon [1]. The intracellular concentration of ppGpp increases during amino acid starvation or a chemically imposed stringent response by serine hydroxamate [23]. The uniform upregulation of the *his* operon observed in this study indicates that ppGpp levels increased as expected for a stringent response due to the serine hydroxamate addition. It also appears that the tryptophan biosynthesis pathway, which shares the phosphoribosylpyrophosphate substrate with the histidine pathway, is also sensitive to ppGpp levels.

In Pizer and Merlie [10], the serine concentration inside the cells was observed to be normal during a serine hydroxamate addition-stimulated stringent response. The transcriptome data are consistent with their observation, in that *serB* was only slightly upregulated (1.5-fold) and *serS* was slightly downregulated (1.3-fold), while none of the serine biosynthesis genes were significantly regulated. The transcription data indicate that serine-related enzyme regulation was not altered by the serine hydroxamate addition.

While the transcriptome analysis suggested the serine hydroxamate-treated cells were in the process of remodeling the lipids present in the cell membranes, the examination of the total fatty acid content and polar lipid content indicated that no change was detectable. This lack of change most likely indicates that either the cells were not incubated long enough after treatment for changes in membrane lipids to be detected or that the enzymes encoded by the transcriptome changes were not synthesized sufficiently.

It is often observed during recombinant protein overexpression that the cell growth rate drops [25, 26]; however, in this study and others [27–29], IPTG-induced cultures continued to grow at preinduction rates. The stringent response may be a critical factor in these divergent observations. In this study, glucose was supplied on demand, and for the IPTG-induced and serine hydroxamate addition cultures, the glucose demand was higher for these cultures to maintain nonzero glucose levels. Many recombinant proteins are overexpressed in batch systems (shake flasks), where the nutrient levels decrease with time and are not replenished. It has also been documented that recombinant protein overexpression can deplete intracellular amino acid pools in a nonlinear manner with respect to the amino acid composition of the recombinant protein [12, 30–32]. These serine hydroxamate addition transcription data under mild stringent response conditions exhibited significant growth rate inhibition, which indicates that when recombinant proteins are overexpressed and growth rates decrease, the decrease in growth rate is likely caused in part by a transient stringent response. The plasmid burden may have also contributed to the decreased response due to the serine hydroxamate addition, as many of the classical stringent response-associated genes had significantly difference levels in the plasmid-bearing cells relative to the nonplasmid-bearing cells characterized in Haddadin and Harcum [14].

Summary

The serine hydroxamate addition resulted in a significant decrease in culture growth rate and cell yields from glucose for high-cell-density recombinant cultures. The transcriptome data support the hypothesis of a stringent response, possibly attenuated due to inhibited protein synthesis. These data also support the hypothesis that under nutrient-limited conditions, recombinant protein overexpression is more likely to result in a stringent response and to be observed as decreased cell growth rates and cell yields.

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Appendix

Table 2 Gene significantly regulated twofold by serine hydroxamate.

Gene	bname	Fold SH	Change Induced	Description
aidB	b4187	−4.1	−10.0	Putative acyl coenzyme A dehydrogenase
appA	b0980	−2.1	−1.6	Phosphoanhydride phosphorylase; pH 2.5 acid phosphatase; periplasmic
appB	b0979	−2.6	−2.8	Probable third cytochrome oxidase, subunit II
argA	b2818	3.6		*N*-Acetylglutamate synthase; amino acid acetyltransferase
argB	b3959	3.4	−2.2	Acetylglutamate kinase
argC	b3958	5.6	−2.2	*N*-Acetyl-gamma-glutamylphosphate reductase
argD	b3359	4.5	−1.4	Acetylornithine delta-aminotransferase
argE	b3957	2.7	−2.4	Acetylornithine deacetylase
argF	b0273	49.6	6.1	Ornithine carbamoyltransferase 2, chain F
argG	b3172	3.3	−1.9	Argininosuccinate synthetase
argH	b3960	2.3		Argininosuccinate lyase

Table 2 (continued)

Gene	bname	Fold SH	Change Induced	Description
argI	b4254	2.9		Ornithine carbamoyltransferase 1
argR	b3237	2.6		Repressor of arg regulon; cer-mediated site-specific recombination
argT	b2310	2.1	−3.1	Lysine-, arginine-, ornithine-binding periplasmic protein
arp	b4017	−2.1		Regulator of acetyl CoA synthetase
artP	b0864	2.1	−5.1	ATP-binding component of third arginine transport system
asnA	b3744	2.2	−2.3	Asparagine synthetase A
b0836	b0836	−3.3	−10.0	Putative receptor
b1297	b1297	2.2		Putative glutamine synthetase (EC 6.3.1.2)
b1410	b1410	2.4	4.3	orf, hypothetical protein
b1488	b1488	3.8	−2.0	orf, hypothetical protein
b1541	b1541	−2.5	−2.3	orf, hypothetical protein
b1583	b1583	2.3	−2.3	orf, hypothetical protein
b1593	b1593	−13.4	−5.5	orf, hypothetical protein
b1605	b1605	2.1		Putative arginine
b1625	b1625	−3.8	−6.0	orf, hypothetical protein
b1680	b1680	−2.5	−13.1	orf, hypothetical protein
b1745	b1745	−2.2	−7.2	orf, hypothetical protein
b1746	b1746	−2.3	−10.1	Putative aldehyde dehydrogenase
b1777	b1777	2.5		orf, hypothetical protein
b1832	b1832	2.5	−2.9	orf, hypothetical protein
b1963	b1963	2.1	−2.7	orf, hypothetical protein
b1970	b1970	2.0		orf, hypothetical protein
b1995	b1995	−4.7	−3.8	orf, hypothetical protein
b2080	b2080	2.4	−3.2	orf, hypothetical protein
b2343	b2343	−3.1	−5.5	orf, hypothetical protein
b2387	b2387	−4.1	1.6	Putative PTS system enzyme IIB component
b2529	b2529	2.2	−4.7	orf, hypothetical protein
b2531	b2531	2.1	−3.5	orf, hypothetical protein
b2636	b2636	−4.2	6.6	orf, hypothetical protein
b2659	b2659	2.2	−4.7	orf, hypothetical protein
b2862	b2862	2.0	1.8	orf, hypothetical protein
b3004	b3004	−5.6		orf, hypothetical protein
b3007	b3007	−2.6		orf, hypothetical protein
btuE	b1710	2.1	−2.3	Vitamin B12 transport
cbpA	b1000	−2.6	−11.3	Curved DNA-binding protein; functions closely related to DnaJ
crcA	b0622	−10.0	−2.1	orf, hypothetical protein
creA	b4397	2.1		orf, hypothetical protein
crl	b0240	2.1	−2.4	Transcriptional regulator of cryptic csgA gene for curli surface fibers
cybB	b1418	2.2		Cytochrome *b* (561)
dacC	b0839	2.1		D-Alanyl-d-alanine carboxypeptidase; penicillin-binding protein 6
dadA	b1189	2.1	−2.3	D-Amino acid dehydrogenase subunit
ddg	b2378	−2.3	−3.6	Putative heat shock protein
entC	b0593	−2.3	−3.4	Isochorismate hydroxymutase 2, enterochelin biosynthesis
entE	b0594	−2.5	−6.8	2,3-Dihydroxybenzoate-AMP ligase
feoA	b3408	−8.2	−27.9	Ferrous iron transport protein A
feoB	b3409	−4.9	−8.3	Ferrous iron transport protein B
ffs	b0455	−3.1	−11.9	4.5S rRNA; mammalian counterpart, SRP, includes 4.5S RNA; cotranslational integration of proteins into membrane
fimA	b4314	2.2	−1.6	Major type 1 subunit fimbrin (pilin)

Table 2 (continued)

Gene	bname	Fold SH	Change Induced	Description
flgD	b1075	−2.0		Flagellar biosynthesis, initiation of hook assembly
focA	b0904	−3.9	−18.1	Probable formate transporter (formate channel 1)
folX	b2303	2.0	−2.0	D-Erythro-7,8-dihydroneopterin tri P epimerase
frdA	b4154	−2.0	−3.8	Fumarate reductase, anaerobic, flavoprotein subunit
fruB	b2169	−2.0	−2.3	PTS system, fructose-specific IIA
fumA	b1612	2.6		Fumarase A=fumarate hydratase class I; aerobic isozyme
gadB	b1493	−3.2	−14.4	Glutamate decarboxylase isozyme
gapC	2	−2.0	−11.9	Glyceraldehyde-3-phosphate dehydrogenase (second fragment)
gatA	b2094	−2.1	−3.5	Galactitol-specific enzyme IIA of phosphotransferase system
gatY	b2096	−2.1	−10.9	Tagatose-bisphosphate aldolase 1
gatZ	b2095	−2.4	−6.9	Putative tagatose 6-phosphate kinase 1
glnK	b0450	2.3	−4.7	Nitrogen regulatory protein P-II 2
gloB	b0212	2.2		Probable hydroxyacylglutathione hydrolase
glpA	b2241	−2.1	−1.6	*sn*-Glycerol-3-phosphate dehydrogenase (anaerobic), large subunit
glpE	b3425	2.2		Protein of glp regulon
glpG	b3424	2.0	−1.5	Protein of glp regulon
gltP	b4077	2.3		Glutamate–aspartate symport protein
hdhA	b1619	2.8	−3.8	NAD-dependent 7alpha-hydroxysteroid dehydrogenase, dehydroxylation of bile acids
hisB	b2022	2.7	−2.1	Imidazoleglycerolphosphate dehydratase and histidinol-phosphate phosphatase
hisC	b2021	2.0	−2.4	Histidinol-phosphate aminotransferase
hisJ	b2309	2.2	−3.1	Histidine-binding periplasmic protein of high-affinity histidine transport system
hisQ	b2308	2.4	−2.4	Histidine transport system permease protein
hyaE	b0976	−2.3		Processing of HyaA and HyaB proteins
ibpB	b3686	2.1	−2.1	Heat shock protein
ilvB	b3671	2.1	−2.2	Acetolactate synthase I, valine-sensitive, large subunit
ilvC	b3774	7.1		Ketol-acid reductoisomerase
intF	b0281	2.7		Putative phage integrase
kdpA	b0698	−2.0	−2.1	ATPase of high-affinity potassium transport system, A chain
kgtP	b2587	2.9	−2.3	alpha-Ketoglutarate permease
lrp	b0889	2.3	−3.4	Regulator for leucine (or lrp) regulon and high-affinity branched-chain amino acid transport system
marA	b1531	2.5	−1.7	Multiple antibiotic resistance; transcriptional activator of defense systems
mdaA	b0851	2.0	−2.3	Modulator of drug activity A
mdh	b3236	2.1	−4.1	Malate dehydrogenase
metA	b4013	−2.3	−3.0	Homoserine transsuccinylase
metF	b3941	−7.4	−5.2	5,10-Methylenetetrahydrofolate reductase
metK	b2942	−2.3	−6.8	Methionine adenosyltransferase 1 (AdoMet synthetase); methyl and propylamine donor, corepressor of met genes
mscL	b3291	2.9		Mechanosensitive channel
mtr	b3161	2.0		Tryptophan-specific transport protein
nadA	b0750	2.4		Quinolinate synthetase, A protein
narX	b1222	2.1	−1.4	Nitrate
nhaR	b0020	−2.1	−2.9	Transcriptional activator of nhaA
nirB	b3365	−2.9	−2.3	Nitrite reductase (NAD(P)H) subunit
nrdA	b2234	2.2	−1.9	Ribonucleoside diphosphate reductase 1, alpha subunit, B1
nuoA	b2288	2.2	−3.2	NADH dehydrogenase I chain A

Table 2 (continued)

Gene	bname	Fold SH	Change Induced	Description
nuoB	b2287	2.2	−2.7	NADH dehydrogenase I chain B
ompT	b0565	2.1		Outer membrane protein 3b (a), protease VII
pncB	b0931	2.1	−1.9	Nicotinate phosphoribosyltransferase
potF	b0854	3.5		Periplasmic putrescine-binding protein; permease protein
potG	b0855	5.4		ATP-binding component of putrescine transport system
potH	b0856	2.1		Putrescine transport protein; permease
pssR	b3763	2.0		Regulator of pssA
purL	b2557	−2.1		Phosphoribosylformyl-glycineamide synthetase=FGAM synthetase
purU	b1232	2.8		Formyltetrahydrofolate deformylase; for purT-dependent FGAR synthesis
sdhA	b0723	2.2		Succinate dehydrogenase, flavoprotein subunit
sdhC	b0721	2.6		Succinate dehydrogenase, cytochrome b556
sdhD	b0722	2.8		Succinate dehydrogenase, hydrophobic subunit
soxS	b4062	5.8		Regulation of superoxide response regulon
ssrS	b2911	−2.3	−14.4	6S RNA
thiC	b3994	2.7		Thiamin biosynthesis, pyrimidine moiety
thiF	b3992	12.5	6.3	Thiamin biosynthesis, thiazole moiety
torD	b0998	−3.7	−1.7	Part of trimethylamine-*N*-oxide oxidoreductase
treA	b1197	2.0		Trehalase, periplasmic
trpB	b1261	2.3	−2.0	Tryptophan synthase, beta protein
trpC	b1262	3.4		*N*-(5-phosphoribosyl)anthranilate isomerase and indole-3-glycerolphosphate synthetase
trpD	b1263	3.8	−1.3	Anthranilate synthase component II, glutamine amidotransferase, and phosphoribosylanthranilate transferase
trpL	b1265	2.0	1.8	trp operon leader peptide
udhA	b3962	2.1		Putative oxidoreductase
xasA	b1492	−4.6	−11.4	Acid sensitivity protein, putative transporter
yaeC	b0197	−2.4	−7.5	Putative lipoprotein
yaeE	b0198	−2.8	−8.2	Putative transport system permease protein
yagB	b0266	2.0	2.2	orf, hypothetical protein
yagN	b0280	3.0		orf, hypothetical protein
yagS	b0285	−2.7		orf, hypothetical protein
ybaT	b0486	−2.0	−10.2	Putative amino acid
ybbD	b0500	2.9		orf, hypothetical protein
ybbI	b0487	−2.0	−11.9	Putative transcriptional regulator
ybbJ	b0488	−2.5	−3.8	orf, hypothetical protein
ybfL	b0705	−2.0	2.0	Putative receptor protein
ybhC	b0772	2.0		Putative pectinesterase
ybjM	b0848	−3.1	−3.3	orf, hypothetical protein
yccD	b0999	−3.0	−6.3	orf, hypothetical protein
ycfL	b1104	2.6	−2.7	orf, hypothetical protein
ycfR	b1112	2.9	−3.7	orf, hypothetical protein
ycgL	b1179	2.3		orf, hypothetical protein
ycjZ	b1328	2.3	2.6	Putative transcriptional regulator LYSR-type
ydaA	b1333	−2.0	−6.4	orf, hypothetical protein
ydhD	b1654	2.7	−2.3	orf, hypothetical protein
ydjS	b1744	−2.9	−5.6	orf, hypothetical protein
yeaA	b1778	4.1		orf, hypothetical protein
yebJ	b1831	2.0	−3.4	orf, hypothetical protein
yedV	b1968	2.1	3.4	Putative 2-component sensor protein

Table 2 (continued)

Gene	bname	Fold SH	Change Induced	Description
yfhE	b2527	2.7	−3.2	orf, hypothetical protein
yfhF	b2528	2.4	−4.2	Putative regulator
yfiD	b2579	−4.9	−9.1	Putative formate acetyltransferase
yfjR	b2634	−4.3		orf, hypothetical protein
yggB	b2924	2.4	−3.4	Putative transport Protein
yhbQ	b3155	−2.2		orf, hypothetical protein
yhdW	b3268	2.1		Putative periplasmic-binding transport protein
yhfY	b3382	−2.1	1.7	orf, hypothetical protein
yhgG	b3410	−2.1	−5.2	orf, hypothetical protein
yhiD	b3508	−3.4	−7.2	Putative transport ATPase
yhiE	b3512	−2.5	−7.9	orf, hypothetical protein
yhiF	b3507	−3.3	−4.6	orf, hypothetical protein
yhiM	b3491	−7.0	−5.0	orf, hypothetical protein
yhiN	b3492	−4.3		orf, hypothetical protein
yhiV	b3514	−5.7	−5.2	Putative transport system permease protein
yigI	b3820	2.3		orf, hypothetical protein
yjaE	b3995	2.5		Putative transcriptional regulator
yjfN	b4188	−2.3	−3.0	orf, hypothetical protein
yjgH	b4248	2.0		orf, hypothetical protein
ykfB	b0250	2.6		orf, hypothetical protein
ylaC	b0458	2.0	−1.6	orf, hypothetical protein
ymfH	b1142	−2.2	−4.2	orf, hypothetical protein
ymfR	b1150	−2.0		orf, hypothetical protein
ynaF	b1376	−2.1	−2.2	Putative filament protein
ynaJ	b1332	2.6		orf, hypothetical protein
ynhA	b1679	−3.0	−11.8	orf, hypothetical protein
ynhC	b1681	−2.0	−11.7	orf, hypothetical protein
yohM	b2106	−5.5	2.9	orf, hypothetical protein
yojH	b2210	2.3		orf, hypothetical protein
yojI	b2211	2.1	1.5	Putative ATP-binding component of a transport system
yrfE	b3397	2.7		orf, hypothetical protein

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