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Global transcriptome response of recombinant *Escherichia coli* to heat-shock and dual heat-shock recombinant protein induction

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Abstract Recombinant *Escherichia coli* cultures are used to manufacture numerous therapeutic proteins and industrial enzymes, where many of these processes use elevated temperatures to induce recombinant protein production. The heat-shock response in wild-type *E. coli* has been well studied. In this study, the transcriptome profiles of recombinant *E. coli* subjected to a heat-shock and to a dual heat-shock recombinant protein induction were examined. Most classical heat-shock protein genes were identified as regulated in both conditions. The major transcriptome differences between the recombinant and reported wild-type cultures were heavily populated by hypothetical and putative genes, which indicates recombinant cultures utilize many unique genes to respond to a heat-shock. Comparison of the dual stressed culture data with literature recombinant protein induced culture data revealed numerous differences. The dual stressed response encompassed three major response patterns: induced-like, in-between, and greater than either individual stress response. Also, there were no genes that only responded to the dual stress. The most interesting difference between the dual stressed and induced cultures was the amino acid-tRNA gene levels. The amino acid-tRNA genes were elevated for the dual cultures compared to the induced cultures. Since, tRNAs facilitate protein synthesis via translation, this observed increase in amino acid-tRNA transcriptome levels, in concert with elevated heat-shock chaperones, might account for improved productivities often observed for thermo-inducible systems. Most importantly, the response of the recombinant cultures to a heat-shock was more profound than wild-type cultures, and further, the response to recombinant protein induction was not a simple additive response of the individual stresses.

Keywords Heat-shock response · Gene regulation · Chloramphenicol acetyltransferase · Transcriptome · DNA microarrays · tRNA · Chaperones

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Introduction

Living organisms respond to stressful environmental conditions by redirecting protein synthesis to alleviate cell damage. One of the most widely studied stressful environmental conditions is elevated temperature. The cellular response to elevated temperature is termed the heat-shock response. The heat-shock response in *Escherichia coli* was first described by the Neidhardt and Yura groups in 1978 [26, 46]. These research groups first observed that 20 proteins were very responsive to heat and later determined that the synthesis of these proteins was controlled at the transcription level [13, 23]. Later, these and other researchers identified numerous heat-shock proteins by examining protein levels on two-dimensional electrophoresis gels [20, 46] and RNA levels via hybridization with genomic libraries [4, 5, 29]. These methods provided the foundation for quantifying the heat-shock response and these identification methods have been extended by the complete sequencing of *E. coli* [2] and the advent of DNA microarrays [13, 31].

Heat-shock proteins are highly conserved across species. Heat-shock proteins monitor and respond to the level of protein folding in the cell [19]. Many heat-shock proteins are chaperones that promote protein folding, while other heat-shock proteins are proteases, which degrade unfolded or damaged proteins [1, 13]. Interestingly, many other stresses can also elicit the heat-shock response, such as ethanol, viral infections, and recombinant protein production [11, 13, 14, 17, 23, 24, 27, 34, 42, 43]. These and other stress response studies

indicate that these stresses do not elicit all the heat-shock response proteins or genes, which suggests overlapping, but distinct regulatory pathways [37]. Additionally, there are a number of heat inducible genes that are controlled by other factors, whose functions and regulations are less well understood [38]. Some studies indicate that the folded state of the recombinant protein elicits heat-shock-related protease activity [27], while other studies indicate that the recombinant protein synthesis rate may play an important role [6, 10, 18, 43]. Mutational studies of the heat-shock proteins indicate that many heat-shock proteins/genes (*dnaK, gapA, grpE, hscA, hslJ, mopAB*, and *rpoDH*) are essential for normal cellular functions at normal growth temperatures as well as numerous regulatory pathways [9, 13, 16].

DNA microarrays have been used to investigate transcriptome profiles of recombinant *E. coli* fermentations [3, 15, 27, 34]. These studies identified genes that were sensitive to recombinant protein production. Many of the identified genes overlapped with the classical heat-shock and stringent responses [27, 34]. It was also observed that recombinant protein production significantly decreased the transcriptome levels of transcription, translation, and energy synthesis related genes, which would impede recombinant protein production [15]. Additionally, recombinant protein production at elevated temperatures (42°C) has been observed to have higher productivities than normal (37°C) culture temperatures [18, 21, 30, 45]. Consequently, the objective of the present work is to gain a better understanding of the heat-shock response in recombinant cultures and how this response might impact recombinant protein production. To accomplish this objective, the transcriptome response of recombinant cultures subjected to a heat-shock and a dual heat-shock recombinant protein induction were analyzed. The transcriptome levels were determined using Affymetrix® *E. coli* Antisense DNA microarrays, such that the entire genome was evaluated. These two transcriptome responses were also compared to recombinant cultures at normal growth temperature that were not overexpressing the recombinant protein and a set of literature recombinant culture data that were chemically induced to overexpress the recombinant protein [15]. Additionally, the heat-shock response of the recombinant cultures was compared to the literature report of the heat-shock response in wild-type cultures [31]. The results of the global transcriptome analysis demonstrated that recombinant cultures respond differently to a heat-shock stress than wild-type cultures, where the transcriptome response of the recombinant cultures is further modified by production of a recombinant protein.

Materials and methods

Culture conditions

The *E. coli* MG1655 [pPROEx-CAT] cells were used for all studies. The pPROEx-CAT plasmid contains a pBR322 origin of replication, a *trc* promoter, a *lacI* gene, and the β-lactamase gene for ampicillin resistance (Invitrogen). Isopropyl-1-thio-β-D-galactopyranoside (IPTG) induces chloramphenicol acetyltransferase (CAT) expression via the *trc* promoter. One milliliter of frozen cells was used to inoculate 500 ml shake-flasks with 100 ml LB media containing 0.5% glucose (w/v) and 40 mg/l ampicillin. The LB media was prepared as described by Rodriguez and Tait [33]. The cultures were incubated overnight at 37°C at 200*g*. A 4-L fed-batch fermenter (Phillips Petroleum), with a 50% working volume, was inoculated with the overnight culture. Air and/or oxygen were sparged continuously into the fermenter at approximately 1,800 ml/min. Sterile antifoam (0.1% v/v) was added to the fermenter. The dissolved oxygen level (DO) was controlled to 60% of saturation by adjusting the agitation speed and air/oxygen flow rate. Sterile 5 M NaOH was used to control pH on-line to 7.0 ± 0.2. The batch media contained LB with 5 g/l glucose. Once the initial glucose was consumed, a feed media was used to maintain the glucose concentration at approximately 1 g/l. The feed media contained 500 g/l glucose and 70 g/l $MgSO_4 \cdot 7H_2O$. All fermentations were conducted in duplicate or triplicate. CAT expression was induced by 5 mM IPTG, which has been shown to maximize CAT expression [18]. The heat-shock and recombinant protein production phases were synchronized to the cell density of 11.5 OD, which is referred to as sample time 0. For the heat-shocked cultures, the temperature was increased from 37–50°C over 8 min beginning at sample time 0. The temperature and duration used in this study are the same conditions used to evaluate the heat-shock in wild-type cultures [5, 31]. The temperature was then decreased from 50–37°C over 4 min. Richmond et al. [31] observed that the transcriptome levels in heat-shocked wild-type samples taken 5, 10, 15, and 20 min after initiation of the heat-shock (50°C) were not different; therefore only one time point (15 min) was analyzed for the recombinant cultures. For the dual heat-shocked recombinant protein production cultures, 5 mM IPTG was added 8 min after sample time 0. In the Richmond et al. [31] study, IPTG was added to wild-type cells cultured in LB medium without glucose. They observed only nine significantly regulated genes, which included the *lac* operon genes. In contrast, IPTG-additions to wild-type cultures in the presence of glucose resulted no significant transcriptome changes in the *lac* operon, due to catabolite repression [15]. The unstressed recombinant cultures were conducted similarly, except without the heat-shock or IPTG-addition. The glucose concentration was measured using a glucose meter (One Touch Profile). The optical density was measured with a Beckman DU® 640 spectrophotometer at 595 nm.

Sample preparation

The specific CAT activity, cell harvest, total RNA isolation, cDNA synthesis, fragmentation, labeling, and

array hybridization methods were conducted as described previously [15]. The wash and stain procedures were carried out by the fluidics station using the ProkGE-fluidics script (Affymetrix, Inc.). Microarray suit 5.0 (Affymetrix, Inc.) was used to process the data. Signal data was prepared and normalized by Affymetrix Data Mining Tool (DMT 3.0), which evaluated the significance of the signal intensity. GeneSpring® was used to analyze the data. An ANOVA test using a Benjamini and Hochberg false discovery rate correction was used for the multiple comparisons ($P \leq 0.001$) with Tukey Post-Hoc tests.

Data analysis

Transcriptome levels were determined for (1) recombinant cultures not over-expressing the recombinant protein at 37°C (unstressed), (2) recombinant cultured only heat-shocked (heat-shocked), and (3) recombinant cultures both heat-shocked and chemically induced to overexpress the recombinant protein (dual stressed). The unstressed samples were obtained from the exponential phase of the fermentations at the synchronization cell density (~11.5 OD) and 1-hour after the synchronization cell density (~14 OD). These two time points had no significant transcriptome differences (*t*-test, $P \leq 0.001$), so these data were combined to delineate the unstressed transcriptome levels more precisely. The heat-shocked culture samples were obtained 15 min after the beginning of the heat-shock (15 min after the synchronization cell density with a sample OD ~ 10). The dual stressed culture samples were obtained 15 min after the beginning of the heat-shock (15 min after the synchronization cell density with a sample OD ~ 10). For clarity, the conditions in this study will be referred to as the unstressed, heat-shocked, and dual stressed cultures. For some comparisons, literature data for wild-type cultures and induced recombinant cultures were analyzed. The fermentation conditions for the wild type and induced recombinant cultures were similar, including growth rates, and cell densities at sampling times. The media used (LB with glucose) and fed-batch conditions were the same. When referenced in the text these cultures are referred to as the wild-type and induced cultures, respectively.

Each sample condition was obtained from at least two separate fermentations (two biological replicates). RNA from each biological replicate was purified and processed independently. Prior to hybridization, where only two biological replicates existed, one of the processed samples was divided (two technical replicates), resulting in three separate hybridized chips. The heat-shocked and dual stressed culture samples all consisted of three technical replicates from two biological duplicates. For the unstressed culture samples, triplicate samples were obtained for the 11.5 OD and duplicates for the 14 OD conditions. There were no statistical differences between the 11.5 and 14 OD unstressed samples ($P \leq 0.001$). Thus, the unstressed culture transcriptome profile consisted of six technical replicates from five biological replicates and four independent fermentations. The variances between biological replicates and between technical replicates were similar ($r^2 > 0.90$), thus transcriptome levels were weighted equally. For the wild-type culture literature data, duplicate fermentations with triplicate samples were used with an OD ~ 11.3. For the literature recombinant induced culture data, duplicate fermentations with triplicate samples were used with an OD ~ 13.5. All raw data intensities for the 12 DNA microarrays, individually, may be obtained from the University of Wisconsin *E. coli* Genome Project via the "A Systematic Annotation Package for Community Analysis of Genomes" as a guest (https://asap.ahabs.wisc.edu/annotation/php/logon.php) [12]. The total intensity on the DNA microarray was normalized and set to a constant value. Since, ribosome genes contribute greatly to the total intensity of the DNA microarray, several other factors were examined to assure acceptable normalization. For example, the average intensity signals for the intergenic (IG) regions were evaluated and found to be consistent across the conditions examined.

Results and discussion

The response of recombinant *E. coli* MG1655 [pPROEx-CAT] to elevated culture temperatures and recombinant protein production was investigated. Fed-batch cultures were synchronized with respect to cell densities at the initiation of the various stresses. The growth curves for the three culture conditions (unstressed, heat-shocked, and dual stressed) and the temperature profile for the heat-shocked cultures are shown in Fig. 1, where the unstressed cultures were maintained at 37°C. The glucose concentrations for these fermentations are also shown in Fig. 1. The final cell densities for the unstressed fermentations reached 16.4 OD (Fig. 1). In comparison, the heat-shocked and dual stressed fermentations had final cell densities of 12.2 and 11.2 OD (Fig. 1), respectively. The specific CAT activities for the dual stressed cultures reached 260 U CAT/mg total protein, as shown in Fig. 1, which is lower than similarly IPTG-only induced cultures, where the final specific CAT activity was 430 U CAT/mg total protein [15]. The specific CAT activity for the dual stressed cultures; however, was continuing to increase, but the extent was not determined, as the fermentation was stopped 4-hours postinduction. As expected, the cell densities for all the heat-shocked cultures decreased immediately following the heat-shock; however, eventually growth returned. Since, it was unknown at the time of these studies, to what extent the recombinant culture transcriptomes would be changed by a heat-shock, a rather severe heat-shock (50°C) was used. Also, the media selected was similar to the media used for the transcripome analysis of wild-type cultures, although the wild-type

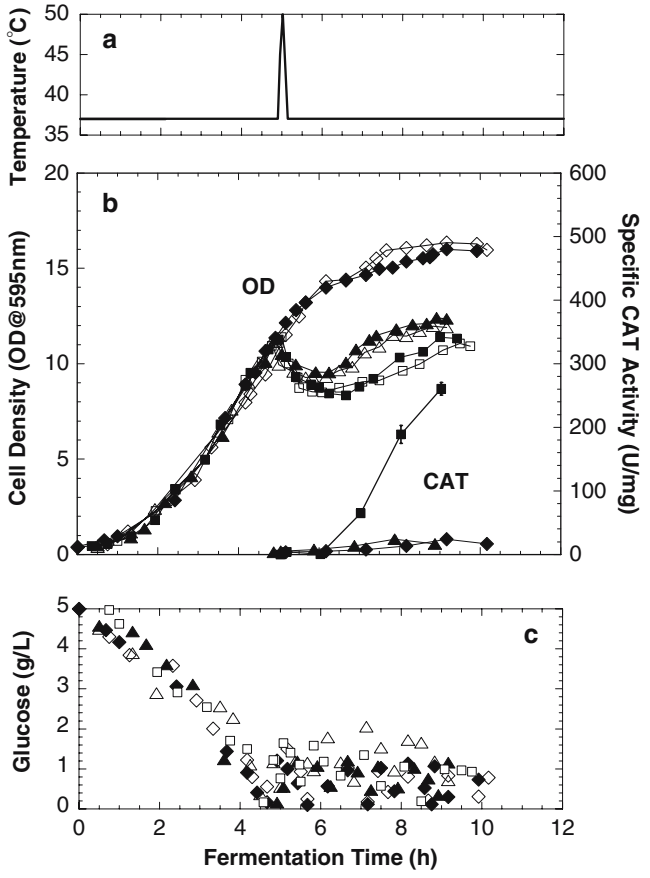

Fig. 1 Cell growth characteristics for duplicate *E. coli* MG1655 [pPROEx-CAT] cultures. **a** The temperature profile for the heat-shock. **b** Cell density and specific CAT activity for the unstressed (*filled diamond*, *open diamond*); heat-shocked (*filled triangle*, *open triangle*); and dual stressed (*filled square*, *open square*) cultures. Specific CAT activities are shown with standard error bars. **c** Glucose concentrations for the unstressed (*filled diamond*, *open diamond*); heat-shocked (*filled triangle*, *open triangle*); and dual stressed (*filled square*, *open square*) cultures

cultures were not fed glucose, as only low cell density shakes were used [31]. Additionally, the selected heat-shock was the same temperature and duration used for the published transcriptome analysis of wild-type cultures subjected to heat-shock [31]; however, more severe than typically used in industry for thermo-induction [22]. Therefore, it was not unexpected that the recombinant protein productivities of this study were lower for the dual stressed cultures compared to other induced cultures [15].

In order to provide a comprehensive examination of the affects of heat-shock and recombinant protein production burdens, literature transcriptome data for recombinant cultures induced with IPTG were included [15]. These literature recombinant cultures were grown in the same medium as the cultures in this study with identical growth rates until the point of the stress-additions. Also, the amount of IPTG used was the same, where recombinant protein induction was examined. The literature transcriptome data; however, were obtained 1-hour postinduction, instead of 7-minute postinduction. We examined the appropriateness of this comparison, which will be discussed in the next paragraph, after the statistical criteria have been established. The literature transcriptome data will be referred to as the induced cultures for clarity.

An ANOVA analysis with a Benjamini and Hochberg false discovery rate correction ($P \leq 0.001$) was used for the multiple comparisons. Since, one objective of this work was to determine how the combined stress of a heat-shock and recombinant protein induction interacted, it was critical to compare the dual stressed culture responses to both individual stresses. To streamline the presentation of significantly different genes, the literature induced culture data are included in the ANOVA-analysis. The number and identity of the genes declared to have significance differences between the unstressed, heat-shocked, and dual stressed cultures did not change significantly if only the three culture conditions were examined by the ANOVA analysis. Therefore, the number and identity of the genes significantly different between conditions will be presented and described for the four cultures: unstressed, heat-shocked, dual stressed, and induced. Tukey Post-Hoc tests were used to identify genes that were statistically different between pairs of conditions. It was determined that 2,700 of the 4,404 annotated genes on the Affymetrix DNA microarray were significantly different across the four culture conditions. Table 1 lists the number of genes that have different expression levels between the condition pairs, as well as the number of genes with similar expression levels between the pairs. Induced recombinant protein production resulted in the greatest number of differences (2,011 genes) relative to the unstressed cultures; however, both the heat-shocked and dual stressed cultures also had over one thousand genes identified as different.

Since, the sample time postinduction for the dual stressed cultures and literature induced cultures were different, we investigated the transcriptome response of these two conditions to determine if it was reasonable and/or meaningful to compare the transcriptome levels, as a means to determine the affect of heat-shock on recombinant protein expression. In other words, were the dual stressed cultures responding to the IPTG and recombinant protein expression sufficiently to make any conclusion regarding the effects of recombinant protein induction? To address this issue the *lac* operon response was examined. The *lac* operon in recombinant cultures, possessing a *lacI*-containing plasmid, is very sensitive to IPTG, due to the high level of expression of the *lacI* gene encoded by the plasmid. Thus, the transcriptome response for the *lacAYZ* and *lacI* genes were examined across the four cultures, specifically focusing on the dual stressed and induced cultures. The transcriptome levels of the *lac* operon genes for the dual stressed and induced cultures were determined to not be different. In contrast, the unstressed cultures had significantly different expression levels for these four genes. A summary of the pairwise comparison for the *lac* operon genes is shown in

Table 1 The number of genes with different expression levels (in bold) between the culture pairs, as well as the number of genes with similar expression levels (in italics) between the pairs

Cultures	**Unstressed**	**Heat-Shocked**	**Dual Heat-Shocked/Induced**	**Induced**
Unstressed	X	**1335**	**1881**	**2011**
Heat-Shocked	*1365*	X	**457**	**890**
Dual Heat-Shocked/Induced	*819*	*2243*	X	**211**
Induced	*689*	*1810*	*2489*	X

The ANOVA analysis identified a total of 2,700 genes with significant regulation ($P \leq 0.001$) across the four cultures. "X" indicates self-comparison

Table 2 The number of *lac* operon genes (*lacAYZ* and *lacI*) with different expression levels (in bold) between the culture pairs, as well as the number of genes with similar expression levels (in italics) between the pairs ($P \leq 0.001$)

Cultures	**Unstressed**	**Heat-Shocked**	**Dual Heat-Shocked/Induced**	**Induced**
Unstressed	X	**3**	**4**	**4**
Heat-Shocked	*1 (lacZ)*	X	**2**	**4**
Dual Heat-Shocked/Induced	*0*	*2 (lacZ, lacI)*	X	**0**
Induced	*0*	*0*	*4*	X

"X" indicates self-comparison

Table 2. Although, not a perfect comparison, the dual stressed cultures at the transcriptome level were responding to the presence of the IPTG at levels comparable to the induced cultures 1-hour postinduction. Thus, it is likely that some of the transcriptome differences between the heat-shocked and dual stressed cultures are due to the effects of recombinant protein induction above the heat-shock stress, and likewise differences in transcriptome between the dual stressed and induced cultures is due to the heat-shock. However, due to the time differences between the samples, only conservative inferences will be presented.

Classical heat-shock response

The heat-shock response in wild-type *E. coli* has been extensively studied and is characterized by the up-regulation of 35 proteins and/or genes due to elevated culture temperatures [13, 31]. For this study these genes will be referred to as the classical heat-shock response genes. Since, no previous reports have examined the heat-shock response in recombinant *E. coli* using DNA microarrays, it was unclear to what extent recombinant cells would elicit a heat-shock response due to a heat-shock as assessed by DNA microarrays. Thus, the first analysis of the regulated genes in this study was to determine to what extent the classical heat-shock response genes had been regulated by the three stresses in the recombinant cultures. It was determined that 34 of the 35 classical heat-shock response genes were regulated by one of the three stresses. Thirty-one genes were regulated in the heat-shocked cultures. Also, 31 genes were regulated in the dual stressed cultures; however, not the same 31 genes. The elevated culture temperature was definitely a common factor that regulated these genes in recombinant cultures; however, some of these genes were not regulated in the expected direction (up-regulated). To better highlight the behavior of these atypical genes, the normalized intensity for all 35 classical heat-shock response genes is shown in Fig. 2 for the four cultures [unstressed, heat-shocked (HS), dual stressed (Dual), and induced] and for a fifth culture, a wild-type fermentation (wild) under similar conditions as the unstressed cultures [15]. The line color of a gene's profile remains the same across the five conditions (for example, *gapA* is red) and indicates the relative expression level of this gene in the unstressed culture. Red indicates high expression in the unstressed,

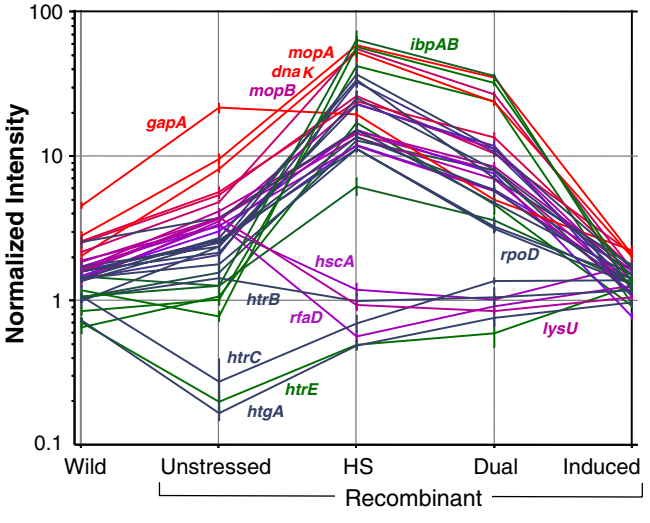

Fig. 2 Transcriptome profiles for the classical heat-shock response genes for unstressed wild-type *E. coli* cultures and recombinant *E. coli* cultures. The recombinant cultures shown are uninduced (*unstressed*), heat-shocked (*HS*), dual stressed (*Dual*), and induced cultures, in addition to the wild-type (*Wild*) cultures. Standard error bars are shown

blue indicates medium expression, and green indicates low expression. Due to the high number of heat-shock response genes with similar profiles, only the outlier genes or genes with atypical profiles are labeled individually in Fig. 2. As can be readily observed, most of the classical heat-shock response genes were significantly up-regulated in both heat-shocked culture conditions. Table 3 lists all of the classical heat-shock response genes with fold change due to the three stresses relative to the unstressed culture. Interestingly, four of the classical heat-shock response genes (*gapA, htgA*, and *htrBC*) were not significantly regulated by the heat-shock, and a few of the classical heat-shock response genes (*hscA, lysU* and *rfaD*) were down-regulated due to the heat-shock for the recombinant cultures. The *gapA* gene was most likely not significantly regulated due to the elevated temperature, since the expression level of *gapA* in the recombinant cultures was significantly higher than that observed for the wild-type cultures shown on the right-side of Fig. 2. The genes *gapA, hscA*, and *rfaD* also encode essential genes [9], which may play a part in the atypical behavior observed in the already stressed recombinant cultures due to the metabolic burden of plasmid maintenance. The additive response of the heat-shock response genes for the dual stressed cultures relative to the heat-shocked and induced cultures may play an important role in the improved productivities observed in thermo-inducible systems. Since, many of the heat-shock response genes are chaperones, the higher chaperone transcriptome levels for the dual stressed cultures likely improves protein folding. For example, the classical heat-shock genes *dnaJK*, *htpG*, and *mopAB* (GroEL/GroES) all encode chaperones. These chaperone genes were either unaffected or down-regulated due to the chemically induction of the soluble recombinant protein CAT; however, for the dual stressed cultures, these same chaperone genes were all up-regulated. Unlike CAT, it has been reported that expression of insoluble proteins at normal culture temperatures elicits increased heat-shock response transcriptome levels, and increased chaperone levels are known to improve recombinant protein productivities [25, 40, 44]. Taken together, the results of this study support the correlation between improved recombinant productivities at elevated temperature due to higher chaperone levels.

Table 3 The classical heat-shock response gene expression levels for the heat-shocked (*HS*), dual stressed and induced cultures relative to the unstressed cultures

Gene	bname	HS	Dual	Induced	Description
clpA	b0882	3.9		−2.2	ATP-binding component of serine protease
clpB	b2592	20.5	6.7		Heat-shock protein
clpP	b0437	5.4	2.7	−3.6	ATP-dependent proteolytic subunit of clpA-clpP serine protease, heat-shock protein F21.5
clpX	b0438	3.8	2.0	−3.1	ATP-dependent specificity component of clpP serine protease, chaperone
dnaJ	b0015	7.6	3.7		Chaperone with DnaK; heat-shock protein
dnaK	b0014	8.4	3.8	−2.2	Chaperone Hsp70; DNA biosynthesis; autoregulated heat-shock proteins
ftsJ	b3179	4.0	2.2	−3.6	Cell division protein
gapA	b1779		−3.4	−7.6	Glyceraldehyde-3-phosphate dehydrogenase A
grpE	b2614	17.2	5.3		Phage lambda replication; host DNA synthesis; heat-shock protein; protein repair
hflB	b3178	5.2	2.0	−2.4	Degrades σ^{32}, integral membrane peptidase, cell division protein
hscA	b2526	−2.9	−3.4	1.9	Heat-shock protein, chaperone, member of Hsp70 protein family
hslJ	b1379	5.1	3.0		Heat-shock protein hslJ
hslU	b3931	5.7	2.8		Heat-shock protein hslVU, ATPase subunit, homologous to chaperones
hslV	b3932	4.0	2.2	−2.9	Heat-shock protein hslVU, proteasome-related peptidase subunit
htgA	b0012		3.1	6.6	Positive regulator for σ^{32} heat-shock promoters
htpG	b0473	19.0	5.2		Chaperone Hsp90, heat-shock protein C62.5
htpX	b1829	39.4	22.4		Heat-shock protein, integral membrane protein
htrA	b0161	8.6	5.3		Periplasmic serine protease Do; heat-shock protein HtrA
htrB	b1054				Heat-shock protein
htrC	b3989		4.2	4.2	Heat-shock protein HtrC
htrE	b0139	2.6	4.1	5.2	Probable outer membrane porin protein involved in fimbrial assembly
ibpA	b3687	38.1	21.2		Heat-shock protein
ibpB	b3686	54.6	30.6		Heat-shock protein
ldhA	b1380	16.5	3.4		Fermentative D-lactate dehydrogenase, NAD-dependent
lon	b0439	11.4	5.9		DNA-binding, ATP-dependent protease La; heat-shock K-protein
lysU	b4129	−3.3	−3.7	−3.0	Lysine tRNA synthetase, inducible; heat-shock protein
mopA	b4143	14.3	7.0	−2.3	GroEL, chaperone Hsp60, peptide-dependent ATPase, heat-shock protein
mopB	b4142	7.8	4.6	−3.6	GroES, 10 kDa chaperone binds to Hsp60 in pres. Mg-ATP, suppressing its ATPase activity
pspA	b1304	3.5	2.0	−2.6	Phage shock protein, inner membrane protein
rfaD	b3619	−5.5	−3.4	−2.5	ADP-L-glycero-D-mannoheptose-6-epimerase
rpoD	b3067	4.4		−2.0	RNA polymerase, σ^{70} factor; regulation of proteins induced at high temperatures
rpoE	b2573	3.8		−2.7	RNA polymerase, σ^{E} factor; heat-shock and oxidative stress
rpoH	b3461	5.0	2.2	−2.1	RNA polymerase, σ^{32} factor; regulation of proteins induced at high temperatures
yrfH	b3400	11.9	3.8		orf, hypothetical protein (*hslR*)
yrfI	b3401	7.2	2.1		orf, hypothetical protein (*hslO*)

Blanks indicate the fold change was not significant ($P \geq 0.001$)

Heat-sensitive genes observed in wild-type cultures

In the heat-shock response work described by Richmond et al. [31] for wild-type cultures, only 20 of the 35 classical heat-shock response genes were reported to have been up-regulated by the heat-shock. Richmond et al. [31] applied a 5-fold criterion to identify regulated genes in additional to statistical significance. If a 5-fold criterion were applied to the recombinant culture data of the current study, 19 of the heat-shock response genes would have been classified as up-regulated by the heat-shock. Thus, the recombinant and wild-type cultures responded to a heat-shock in a very consistent manner with respect to the classical heat-shock response genes. Richmond et al. [31] also identified 97 additional genes (5-fold regulated) that were heat-sensitive in the wild-type cultures. A comparison of these Richmond identified genes with the significantly regulated genes (including less than 5-fold regulated genes) observed for the high-cell density recombinant *E. coli*, an overlap of 73 genes was observed, of which 65 genes were also regulated similarly in one of the heat-shocked cultures of this study. Thus, it appears that the recombinant cells elicit a heat-shock response that is similar to the wild-type cultures, although not identical. All of the Richmond identified heat-sensitive genes are listed in Table 4 with fold-changes for the heat-shocked, dual stressed, and induced cultures relative to the unstressed cultures.

Heat-sensitive genes in recombinant cultures

Further analysis of the heat-sensitive genes for the heat-shocked and dual stressed cultures identified numerous genes that were highly responsive to the elevated temperature in the recombinant cultures that were not observed in the wild-type cultures. Specifically, of the 1,335 genes observed to be significantly regulated in the heat-shocked cultures (Table 1), 1,093 genes had at least a 2-fold transcriptome level change, 260 genes had at least a 5-fold change, and 75 genes had at least a 10-fold change. Fifty-six of the 10-fold genes are not classified as classical heat-shock genes or identified by Richmond et al. [31]. Not unexpectedly, seven of these 56 genes were amino acid-tRNA genes, which are known to be heat-shock sensitive. Unexpectedly, 32 of the 56 10-fold genes were hypothetical or putative genes. This very high fraction of hypothetical/putative genes for these highly regulated genes indicates that recombinant cultures utilize many genes not normally utilized to combat a heat-shock in wild-type cultures.

Of the 1,881 genes observed to be significantly regulated in the dual stressed cultures (Table 1), 1,633 genes had at least a 2-fold transcriptome level change, 521 genes had at least a 5-fold change, and 170 genes had at least a 10-fold change. One hundred and sixty of the 10-fold genes have not been classified as classical heat-shock genes or identified by Richmond et al. [31]. Unexpectedly, 113 of the 160 10-fold genes were hypothetical or putative genes. Since, it is generally accepted that 87% of the *E. coli* genome has been assigned function to some degree of confidence [31, 32], the high percentage of hypothetical/putative genes observed to be regulated in the recombinant cultures under the dual stressed conditions would seem to indicate that many more genes are needed to cope with the dual stress than either stress alone. This observation is also supported by the greater growth inhibition observed for the dual stressed cultures compared to the single stress cultures.

Metabolism genes

Based on the dramatic change in cell growth rate due to heat-shock (Fig. 1), it was expected that numerous metabolic pathways would be affected at the transcriptome level. Roughly, 25% of the genes in any one pathway were affected by the elevated temperature, indicating a global response [5, 8, 39]. The metabolic genes most uniformly affected by the heat-shock were energy and protein synthesis related. Oxidative phosphorylation genes responsible to energy synthesis were significantly down-regulated in the heat-shocked cultures. The oxidative phosphorylation genes include ATP synthase, cytochrome, fumarate reductase, NADH dehydrogenase, and polyphosphate kinase genes (40 genes total). The ATP synthase genes (*atpABDEFGH*) were significantly down-regulated approximately 3-fold. The ATP synthase genes were also down-regulated in the dual stressed and induced cultures at approximately the same level. The cytochrome genes (*appBC, cydA*, and *cyoABCDE*) were also down-regulated in the heat-shocked cultures; however, the down-regulation observed for the dual stressed and induced cultures for these genes was slightly lower than the heat-shocked cultures. The NADH dehydrogenase genes (*nuoBCEFGHIJKLM*) were all significantly down-regulated in the heat-shocked cultures and in nearly all of the *nuo* genes were down-regulated in the dual stressed cultures, whereas for the induced cultures, only half of these genes were significantly regulated. Interestingly, overall, the induced cultures had only 18 of the 40 oxidative phosphorylation genes down-regulated compared to 28 and 24 genes down-regulated for the heat-shocked dual stressed cultures, respectively. The response of the oxidative phosphorylation genes indicates an impaired energy synthesis system under heat-shock, which could in part account for the observed reduced growth rates.

Protein synthesis relies on aminoacylated-tRNAs and ribosomes for translation. The aminoacyl-tRNA biosynthesis (synthetases) genes were significantly down-regulated on average 2.6-fold in the heat-shocked cultures (13 of the 25 genes), whereas 17 of the 25 genes were significantly down-regulated (~1.9-fold) and five genes were up-regulated (~1.8-fold) in the dual stressed cultures. The fold changes for the individual tRNA synthatase genes are listed in Table 5. For the induced cultures, 17 aminoacyl-tRNA synthetase genes were

Table 4 The heat-sensitive genes identified by Richmond et al. [31] including the classical heat-shock genes

Gene	bname	HSP	Down in Richmond	HS	Dual	Induced	Description
adiA	b4117		*		4.1	4.2	Biodegradative arginine decarboxylase
b1593	b1593						Orf, hypothetical protein
b1903	b1903						Orf, hypothetical protein
b3022	b3022						Orf, hypothetical protein
b4140	b4140			40.1	24.4	2.7	Orf, hypothetical protein
cadA	b4131			5.9	11.7	17.2	Lysine decarboxylase 1
cadB	b4132				3.7	4.1	Transport of lysine
carA	b0032						Carbamoyl-phosphate synthetase, glutamine (small) subunit
carB	b0033						Carbamoyl-phosphate synthase large subunit
cheA	b1888		*		4.4	4.1	Sensory transducer kinase between chemo- signal receptors and CheB and CheY
cheW	b1887		*		2.2		Positive regulator of CheA protein activity
clpA	b0882	*		3.9	1.9		ATP-binding component of serine protease
clpB	b2592	*		20.5	6.7		Heat-shock protein
clpP	b0437	*		5.4	2.7	−3.6	ATP-dependent proteolytic subunit of clpA-clpP serine protease, heat-shock protein F21.5
clpX	b0438	*		3.8	2.0	−3.1	ATP-dependent specificity component of clpP serine protease, chaperone
codA	b0337		*				Cytosine deaminase
codB	b0336		*				Cytosine permease
corA	b3816			5.0			Mg2+ transport, system I
creB	b4398			4.4	3.4		Catabolic regulation response regulator
cspD	b0880						Cold shock protein
cutC	b1874			4.4			Copper homeostasis protein
cvpA	b2313					1.9	Membrane protein required for colicin V production
cycA	b4208						Transport of D-alanine, D-serine, and glycine
dnaJ	b0015	*		7.6	3.7	−2.2	Chaperone with DnaK; heat-shock protein
dnaK	b0014	*		8.4	3.8	−3.1	Chaperone Hsp70; DNA biosynthesis; autoregulated heat-shock proteins
endA	b2945		*		2.5	3.7	DNA-specific endonuclease I
eno	b2779		*	−3.1	−6.6	−4.0	Enolase
fba	b2925		*		−3.5	−2.7	Fructose-bisphosphate aldolase, class II
flgE	b1076		*		3.5	4.0	Flagellar biosynthesis, hook protein
fliD	b1924		*		2.5	3.6	Flagellar biosynthesis; filament capping protein; enables filament assembly
ftsJ	b3179	*		4.0	2.2	−3.6	Cell division protein
glyA	b2551		*				Serine hydroxymethyltransferase
grpE	b2614	*		17.2	5.3		Phage lambda replication; host DNA synthesis; heat-shock protein; protein repair
guaA	b2507						GMP synthetase (glutamine-hydrolyzing)
guaB	b2508						IMP dehydrogenase
hflX	b4173			2.3	2.3	−3.6	GTP–binding subunit of protease specific for phage lambda cII repressor
hfq	b4172			3.1	1.5	−4.4	Host factor I for bacteriophage Q beta replication, a growth-related protein
hslU	b3931	*		5.7	2.8		Heat-shock protein hslVU, ATPase subunit, homologous to chaperones
hslV	b3932	*		4.0	2.2	−2.9	Heat-shock protein hslVU, proteasome-related peptidase subunit
htpG	b0473	*		19.0	5.2		Chaperone Hsp90, heat-shock protein C 62.5
htpX	b1829	*		39.4	22.4		Heat-shock protein, integral membrane protein
hyaB	b0973		*			2.1	Hydrogenase-1 large subunit
ibpA	b3687	*		38.1	21.2		Heat-shock protein
ibpB	b3686	*		54.6	30.6		Heat-shock protein
intF	b0281						Putative phage integrase
lon	b0439	*		11.4	5.9	−1.9	DNA-binding, ATP-dependent protease La; heat-shock K-protein
lpdA	b0116		*	−2.9	−3.8	−2.9	Lipoamide dehydrogenase (NADH); component of 2-oxodehydrogenase and pyruvate complexes; L-protein of glycine cleavage complex
marA	b1531			3.2			Multiple antibiotic resistance; transcriptional activator of defense systems
marR	b1530			2.8			Multiple antibiotic resistance protein; repressor of mar operon
miaA	b4171			2.1		−4.7	Delta (2)-isopentenylpyrophosphate tRNA-adenosine transferase
mopA	b4143	*		14.3	7.0	−2.3	GroEL, chaperone Hsp60, peptide-dependent ATPase, heat-shock protein
mopB	b4142	*		7.8	4.6	−3.6	GroES, 10 kDa chaperone binds to Hsp60 in pres. Mg-ATP, suppressing its ATPase activity
mutM	b3635			4.1	2.9	2.2	Formamidopyrimidine DNA glycosylase
narP	b2193			8.6	6.9		Nitrate
nuoC	b2286		*	−5.1	−3.3	−2.2	NADH dehydrogenase I chain C, D

Table 4 The heat-sensitive genes identified by Richmond et al. [31] including the classical heat-shock genes

pflB	b0903		*		−10.4	Formate acetyltransferase 1
phoB	b0399			13.2	6.4	Positive response regulator for pho regulon, sensor is PhoR (or CreC)
phoR	b0400			33.4	13.0	Positive and negative sensor protein for pho regulon
prlC	b3498			3.6		Oligopeptidase A
pta	b2297		*			Phosphotransacetylase
purB	b1131					Adenylosuccinate lyase
purC	b2476					Phosphoribosylaminoimidazole-succinocarboxamide synthetase = SAICAR synthetase
purE	b0523				3.1	Phosphoribosylaminoimidazole carboxylase = AIR carboxylase, catalytic subunit
purK	b0522					Phosphoribosylaminoimidazole carboxylase = AIR carboxylase, CO(2)-fixing subunit
purN	b2500					Phosphoribosylglycinamide formyltransferase 1
pykF	b1676		*		−3.4	Pyruvate kinase I (formerly F), fructose stimulated
pyrB	b4245		*		2.6	2.5	Aspartate carbamoyltransferase, catalytic subunit
pyrC	b1062		*		1.7	1.8	Dihydro-orotase
pyrD	b0945		*				Dihydro-orotate dehydrogenase
pyrI	b4244		*		2.8	3.3	Aspartate carbamoyltransferase, regulatory subunit
pyrL	b4246		*	15.0	11.0	5.0	pyrBI operon leader peptide
rpoD	b3067	*		4.4		−2.0	RNA polymerase, sigma (70) factor; regulation of proteins induced at high temperatures
rpoE	b2573	*		3.8		−2.7	RNA polymerase, sigma-E factor; heat-shock and oxidative stress
rseA	b2572			2.9		−4.2	Sigma-E factor, negative regulatory protein
sdaC	b2796			11.3	4.8	2.9	Probable serine transporter
sgcR	b4300				5.5	6.4	Putative DEOR-type transcriptional regulator
tdcE	b3114		*		1.6	1.8	Probable formate acetyltransferase 3
topA	b1274			3.8			DNA topoisomerase type I, omega protein
treC	b4239				8.3	11.5	Trehalase 6-P hydrolase
tsx	b0411					2.3	Nucleoside channel; receptor of phage T6 and colicin K
uhpA	b3669			−3.4			Response regulator, positive activator of uhpT transcription (sensor, uhpB)
upp	b2498						Uracil phosphoribosyltransferase
uraA	b2497		*		11.3	11.7	Uracil transport
yafD	b0209			5.9			Orf, hypothetical protein
yafE	b0210						Putative biotin synthesis protein
yahA	b0315			4.0	4.6		Orf, hypothetical protein
yahB	b0316					2.6	Putative transcriptional regulator LYSR-type
yaiU	b0374					8.2	Putative flagellin structural protein
ybbM	b0491						Putative metal resistance protein
ybbN	b0492			4.1			Putative thioredoxin-like protein
ybeY	b0659			2.1		−3.3	orf, hypothetical protein
ybeZ	b0660			2.3			Putative ATP-binding protein in pho regulon
ybjZ	b0879			23.5	8.3		Putative ATP-binding component of a transport system
yccV	b0966			9.0	4.4	−3.1	Orf, hypothetical protein
yceP	b1060			52.6	26.8		Orf, hypothetical protein
ycfC	b1132		*				Orf, hypothetical protein
ycfR	b1112			3.5			Orf, hypothetical protein
ycjF	b1322			8.0	3.3		Orf, hypothetical protein
yeaF	b1782		*	−2.2	−2.2	−1.4	Orf, hypothetical protein
yedU	b1967				−2.1	−8.7	Orf, hypothetical protein
yfjD	b2613			3.5	1.9		Putative transport protein
yhdN	b3293			6.0	1.6	−1.4	Orf, hypothetical protein
yheL	b3343			5.2	2.4		Orf, hypothetical protein
yhgE	b3402			2.5	3.7	3.3	Putative transport
yhgH	b3413						Orf, hypothetical protein
yhiE	b3512		*		−3.9	−4.9	Orf, hypothetical protein
yi81	1			13.7	8.2	1.9	IS186 hypothetical protein
yi82	1			4.0	2.4	2.9	IS186 and IS421 hypothetical protein
yidE	b3685			11.0	6.6	4.6	Putative transport protein
yjdE	b4115		*		3.3	3.6	Putative amino acid
yjeH	b4141			4.1		1.8	Putative transport
ylcB	b0572		*	2.7	4.1	4.6	Putative resistance protein
yljA	b0881			2.5		−4.0	Orf, hypothetical protein
yrdB	b3280			−1.6			Orf, hypothetical protein
yrfG	b3399			1.9			Putative phosphatase
yrfI	b3401	*		7.2	2.1		Orf, hypothetical protein
ytfE	b4209			1.9	2.4	2.8	Orf, hypothetical protein

The gene expression levels relative to the unstressed cultures are shown for the heat-shocked (*HS*), dual stressed, and induced cultures. *Asterisks* in blanks indicate the fold change was not significant ($P \geq 0.001$)

significantly down-regulated (~1.8-fold) and four genes were up-regulated (~2.1-fold). In contrast, most of the amino acid-tRNA genes (51 of 78 genes) were significantly up-regulated in the heat-shocked cultures with only seven genes significantly up-regulated in the dual stressed cultures. Whereas, 36 of the amino acid-tRNA genes were significantly down-regulated in the induced cultures. Over half of the ribosome genes (39 of 73) were also significantly down-regulated on average over 3-fold in the heat-shocked and dual stressed cultures. This mixed protein translation transcriptome response indicates that heat-shock and recombinant protein induction have many common negative effects on the translation apparatus, except for the amino acid-tRNA genes.

In addition to the oxidative phosphorylation genes used to synthesis cell energy, central carbon metabolism plays an important role, namely glycolysis and the tricarboxylic acid (TCA) cycle. Only 13 of the 42 genes in glycolysis were significantly regulated in the heat-shocked cultures. The *aceEF, eno, galM, lpdA, pfkA, pgi, pgk*, and *yibO* genes were down-regulated between 1.8- and 7.4-fold. The *aldH, bglB*, and *glvCG* genes in glycolysis were up-regulated between 2.1- and 3.8-fold in the heat-shocked and dual stressed cultures. These up-regulated genes catalyze reactions not part of the main glucose to acetyl-CoA pathway, but side reactions, for example acetate synthesis (*aldH*). For the TCA cycle, only eight (*icdA, fumB, gltA, lpdA, pckA*, and *sucAB*) of the 27 genes were significantly regulated; however, all of these affected genes were down-regulated between 2- and 5-fold. In comparison, a cold temperature shift for wild-type *E. coli* cultured in shake flasks was observed to limitedly affect metabolic genes, where TCA cycle genes were reported to be up-regulated [7]. Since, only wild-type cultures were examined in the cold temperature shift study, it is difficult to extrapolate the reported behaviors to recombinant cultures. The response of the central carbon metabolism genes indicates impaired carbon utilization, which could in part account for the observed reduced growth rates during and after a heat-shock.

Dual stress sensitive genes

Many gene families were regulated by the heat-shock and recombinant protein induction with varying responses in the dual stressed cultures. Basically, the overall responses of the genes to the dual stress divided in to four categories: (1) heat-shocked-like response, (2) induced-like response, (3) in between the heat-shocked and induced responses, and (4) a greater than either heat-shocked or induced response. In order to evaluate the dual stress transcriptome response, the initial screening was limited to the genes that were greater than 5-fold regulated, and excluded the classical heat-shock genes and Richmond identified heat-sensitive genes. This included 260 genes for the heat-shocked and 521 genes for the dual stressed cultures. The union of these two pools was 563 genes. Pair wise comparisons between the unstressed and the three stressed cultures for the 563 genes identified 433, 457, and 426 genes that were significantly regulated ($P \leq 0.001$) for the heat-shocked, dual stressed, and induced cultures, respectively. This procedure identified genes with less than 5-fold regulation for the other stresses and also more stringently selected genes with statistically significant changes. Thus, for the dual stressed cultures, the original 521-gene list was reduced to 456 genes.

Table 5 The tRNA synthetase gene expression levels for the heat-shocked (*HS*), dual stressed and induced cultures relative to the control cultures

Gene	bname	HS	Dual	Induced	Description
alaS	b2697	−1.9	−2.6	−2.8	Alanyl-tRNA synthetase
argS	b1876		3.0	3.3	Arginine tRNA synthetase
asnS	b0930				Asparagine Trna synthetase
aspS	b1866	−2.3	−2.9	−2.5	Aspartate tRNA synthetase
cysS	b0526		1.9	1.9	Cysteine tRNA synthetase
fmt	b3288	−3.8	−3.8	−2.8	10-Formyltetrahydrofolate: L-methionyl-tRNA(fMet) N-formyltransferase
glnS	b0680		−1.4		Glutamine tRNA synthetase
gltX	b2400		−1.3	−1.5	Glutamate tRNA synthetase, catalytic subunit
glyQ	b3560	−3.1	−2.6	−2.0	Glycine tRNA synthetase, alpha subunit
glyS	b3559	−2.7	−2.2	−2.0	Glycine tRNA synthetase, beta subunit
hisS	b2514	−2.6	−3.5	−2.7	Histidine tRNA synthetase
ileS	b0026		−1.9	−4.3	Isoleucine tRNA synthetase
leuS	b0642	−1.9	−2.4	−2.3	Leucine tRNA synthetase
lysS	b2890		−1.5	−1.3	Lysine tRNA synthetase, constitutive; suppressor of ColE1 mutation in primer RNA
lysU	b4129	−3.3	−3.7	−3.0	Lysine tRNA synthetase, inducible; heat-shock protein
metG	b2114			−1.1	Methionine tRNA synthetase
pheS	b1714	−3.3	−3.5	−1.8	Phenylalanine tRNA synthetase, alpha-subunit
pheT	b1713	−1.7	−3.7	−3.8	Phenylalanine tRNA synthetase, beta-subunit
proS	b0194		1.2		Proline tRNA synthetase
serS	b0893	−2.3	−2.9	−2.2	Serine tRNA synthetase; also charges selenocystein tRNA with serine
thrS	b1719	−3.8	−5.3	−4.1	Threonine tRNA synthetase

Blanks indicate the fold change was not significant ($P \geq 0.001$)

The objective of the analysis of the dual stressed transcriptome was to determine if a global response existed that was different for the dual stressed cultures that was not solely due to the individual stresses, thus a holistic approach was used. The classification of the genes into the four categories was not absolute. For example, the *ybfH* gene was up-regulated 36.6-fold, up-regulated 31.6-fold, and not significantly regulated for the heat-shocked, dual stressed, and induced cultures, respectively, so this gene was classified as heat-shocked-like, where it might also be considered an "in between" response. The *cfa* gene is an example of the greater than either individual stress response, as it was down-regulated 5.6-fold, down-regulated 13.6-fold, and down-regulated 5.4-fold for the heat-shocked, dual stressed, and induced cultures, respectively. The heat-shocked-like and induced-like responses were the most arbitrary classifications, and also yielded little new information. The higher than either stress provided the most useful information to better understand the dual stressed cultures. All 563 genes with fold changes grouped by category can be found in the electronic supplemental materials (Table S1) via a link at http://www.ces.clemson.edu/bio/people/harcum.htm.

The heat-shocked-like category for the dual stressed cultures was the smallest category with only 14 genes. Only three of these genes had known functions (*cbl, gapC*, and *uidC*), where *gapC* and *cbl* were down-regulated and *uidC* was up-regulated for the dual stressed and heat-shocked cultures. The *gapC* gene is a non-functional glyceraldehyde 3-phosphate dehydrogenase gene and *cbl* is a transcriptional regulator for *cys* regulon (cysteine biosynthesis regulator). Interestingly, none of the classical heat-shock gene or the Richmond identified genes had this behavior in the dual stressed cultures.

The induced-like category for the dual stressed cultures contained 55 genes. The most interesting genes in the induced-like group were the ribosome genes *rplEFJX, rpmDIJ*, and *rpsH*, which were all down-regulated at least 5-fold for the dual stressed and induced cultures, but only down-regulated approximately 3-fold for the heat-shocked cultures. Thirty-five of these induced-like genes are putative or hypothetical genes. The remaining 12 known-function genes had no commonality. Additionally, one of the classical heat-shock genes (*htrC*) and eleven of the Richmond identified genes (*aidA, cheA flgE, pyrBCI, sgcR, uraA, yidE, yjdE*, and *ytfE*) had characteristics of the induced-like response in the dual stressed cultures.

Most of the genes that were significantly regulated in the dual stressed cultures fell into the "in between" the heat-shocked and induced response category. This group included 225 genes out of 456 significantly regulated genes. This also described the behavior of most of the classical heat-shock genes (29 of the 31 significantly regulated genes), as well as the majority of the heat-sensitive identified Richmond genes (53 of the 67 significantly regulated genes). Three cytochrome genes (*cyoBCD*) were down-regulated, and six flagella/fimbriae genes (*flgBCI*, *fihA*, and *fliLQ*) were up-regulated. Additionally, 134 genes that had "in between" behavior were putative or hypothetical genes. Unfortunately, this high number of uncharacterized genes makes if difficult to develop a theoretical understanding of the in-between dual stress response.

The category of genes that responded more profoundly in the dual stressed cultures than either stress alone contains 163 genes. Additionally, two of the classical heat-shock genes (*hscA* and *lysU*) and three Richmond identified genes (*hflX, yahA*, and *yeaF*) had this behavior. Unlike the other three categories, this category contains numerous gene families indicating a coordinated response. The *aceABEF* genes were all significantly down-regulated ~9-fold, as was the *poxB* gene, indicating pyruvate metabolism was affected. Two *bgl* genes (*bglBG*) were both up-regulated by the dual stress and *bglF* was up-regulated ~4-fold in the heat-shocked cultures, but did not meet the significance criteria for the dual stressed culture. The *bgl* genes are crytic genes associated with glucosidase metabolism. The *cadC* gene, which is a transcriptional activator, was up-regulated, where Richmond et al. [31] observed the *cadA* gene up-regulated due to heat-shock in wild-type cultures. This study also observed *cadA* up-regulation due to heat-shock, and more so in the dual stressed cultures. Two cold shock genes (*cspAC*) were down-regulated (~8-fold) more by the dual stress; however, *cspE* was also down-regulated by the dual stress, but at lower levels. Many glutamine biosynthesis genes were down-regulated (~9-fold), including *glnAHKPQ*. Two of the molybdopterin genes (*moaCD*) were classified into this category, while two other molybdopterin biosynthesis genes (*moaAB*) were classified in to the in-between category. Despite falling into different categories, these four molybdopterin genes were observed to be down-regulated (> 5-fold) under all three stressed culture conditions. Two outer membrane protein genes (*ompCX*) were also down-regulated (> 7-fold) by the dual stress more profoundly than either stress alone. Rhs element protein genes, *rhsAB*, were both up-regulated (11-fold) by the dual stress. This is interesting in that RhsA protein overexpression has been observed to reduce cell survival in the stationary phase [41], and the heat-shocked and dual stressed cultures stopped growing immediately following the heat-shock stress. Many of the ribosome genes (*rplKMNRY* and *rpsDKMNPR*) were profoundly down-regulated (> 5-fold) by the dual stress, which may provide insight into the long observed phenomena of decreased ribosome levels in heat-shocked cells [19]. Also, eight ribosome genes were classified and induced-like and were down-regulated ~6-fold by the dual stress. The RNA polymerase genes (*rpoAS*) were down-regulated by the dual stress, in contrast to the known heat-shock RNA polymerase genes, *rpoDEH*. Only *rpoH* was significantly up-regulated in the dual stressed cultures, where *rpoDE* were not significantly regulated. Additionally, the *relA* gene was more profound

down-regulated 7-fold in the dual stressed cultures, where this gene regulates the stringent response. Also, the *slp* gene, which is normally induced after a carbon starvation, was down-regulated 10-fold, indicating glucose was sufficient. The tRNA synthetase genes were nearly uniformly down-regulated (~2-fold) Overall, there were no genes that had unique responses to the dual stressed cultures; however, for many of the ribosome and metabolic pathway genes the dual stressed cultures were more profoundly affected that the single stressed cultures. More importantly, the dual stressed cultures transcriptome profiles were most often "in between" the transcriptional responses of heat-shocked and induced cultures, which mitigated the negative effects of these stresses in many cases.

Recombinant protein production implications

In order to reduce the need for large quantities of IPTG for industrial-scale processes, thermo-inducible system are often used to elicit recombinant protein production [18, 21, 28, 30, 35]. Recombinant protein production has been observed to result in significant transcriptome changes that would seem to be targeted to impede recombinant protein production [15]. It has also been observed that recombinant protein production at elevated culture temperatures can actually improve recombinant protein productivity [18, 21, 30, 36]; however, a viable mechanism that explains these observations has not been developed. Although the heat-shock (50°C) used in this study is greater than would be used in industry for thermo-induction (42°C), examining the differences between the induced and dual stressed cultures could provide insight into the mechanism by which thermo-induction improve recombinant protein production over chemical induction at normal growth temperatures. Thus, an analysis of the transcriptome level differences between the induced and dual stressed cultures was under taken to provide a basis for a preliminary mechanism. Based on the pair wise Tukey Post-Hoc tests, only 211 genes had different expression levels between the induced and dual stressed cultures. A majority of these genes (148 genes) also had different expression levels relative to the unstressed, whereas 63 of the 211 genes were not differentially expressed between the unstressed and induced cultures.

The most prevalent gene family identified by comparing transcriptome levels under the induced and dual stressed cultures were the amino acid-tRNA genes. Thirty-seven of the 211 genes were amino acid-tRNA genes and all had higher expression in the dual stressed cultures compared to the induced cultures. Since, amino acid-tRNA genes play a critical role in protein translation, up-regulation of these genes could provide an explanation for improve recombinant protein productivities, as the necessary tools to counteract the other negative effects that recombinant protein production has on the translation reactions. In *E. coli*, there are 78 identified amino acid-tRNA genes, of which 58 of these genes were observed to be significantly regulated across the four cultures examined. The heat-shocked cultures had the highest amino acid-tRNA transcriptome levels. Both of the heat-shocked and induced cultures significantly regulated the tRNA transcriptome levels; however, in opposite directions, thus when the amino acid-tRNA transcriptome levels for the dual stressed cultures are comparable to the unstressed cultures, and very few amino acid-tRNA genes appear to be regulated, if one only compares the dual stressed to the unstressed cultures. In the electronic supplemental materials, Table S2 (http://www.ces.clemson.edu/bio/people/harcum.htm), all 211 genes with fold change are listed. Also in the electronic supplemental materials, in Table S3 (http://www.ces.clemson.edu/bio/people/harcum.htm), all 78 amino acid-tRNA genes are listed with fold changes relative to the unstressed cultures.

Interestingly, the SYNPBR322 probe, which is a control probe on the Affymetrix DNA microarrays designed to hybridize with the β-lactamase (ampicillin resistance) portion of the pBR322 plasmid, was identified as over 2-fold higher for the dual stressed cultures compared the induced cultures. The transcriptome level for the SYNBR322 probe between the unstressed, heat-shocked and dual stressed cultures were not significantly different. The pPROEx-CAT plasmid is a pBR322 derivative, so transcriptome signal attributed to the plasmid was not unexpected. In previous wild-type versus recombinant culture comparisons, the signal from plasmid-related synthetic probes, where a chromosomal gene existed, correlated well with the known copy number of the pPROEx-CAT plasmid, for example the *lacI* signal. Therefore, recombinant protein productivities may be higher for the dual stressed cultures relative to the chemically induced cultures due, in part, to higher plasmid copy number.

In addition to the amino acid-tRNA genes, numerous other transcription and translation genes were also up-regulated by the elevated temperature. These other up-regulated genes included *ffh, ffs, holC, miaA, nusB, rffH, rmf, rnc*, *rnpB, ssrAS*, and *stpA*. These genes generally catalyze transcription or translation reactions. Specifically, *miaA* and *rnpB* are involved in tRNA processing, while *rseAB* encode the σ^E factor and its regulator, respectively. While *ffs* and *rmf* encode factors that modulate the ribosome, up-regulation of these genes would result in improved protein synthesis, and therefore high recombinant protein synthesis.

Summary

The recombinant cultures responded to a heat-shock in a similar manner as the reported wild-type cultures; however, many more genes, in particular putative and hypothetical genes, were significantly regulated. The dual stressed cultures also had transcriptome responses to the heat-shock similar to the wild-type cultures for the

classical heat-shock genes, and many more genes were highly affected. For the recombinant cultures, both the heat-shock and dual stressed cultures had significant down-regulation of energy and protein translation genes. Numerous other metabolic and nonmetabolic gene families were regulated including the fimbriae, flagellar, cold-shock, molybdopterin, amino acid-tRNA, and tRNA synthetase, and ribosome gene families. This study demonstrated that recombinant cultures have an active and responsive heat-shock apparatus for which some transcriptome responses are mitigated by recombinant protein induction and others are exacerbated. The highly sensitive heat-sensitive genes observed for the heat-shocked and dual stressed recombinant cultures may provide insight into the differences observed in the growth characteristics of recombinant and wild-type cultures, once characterized.

The dual stressed cultures were observed to have numerous differences from the induced cultures, specifically higher amino acid-tRNA and chaperone gene transcriptome levels. Also, the plasmid copy number may have been higher. Although ribosome levels were still down-regulated for the dual stressed cultures compared to the unstressed cultures, the up-regulation of the amino acid-tRNA genes relative to the induced cultures, in concert with the higher chaperone gene levels may mitigate some of the metabolic burden associated with recombinant protein production. To confirm that these results are applicable to industrial thermo-induction, 42°C heat-shocked and dual stressed culture transcriptome profiles need to be assessed.

Supporting material

All raw data intensities for the 12 DNA microarrays, individually, may be obtained from the University of Wisconsin *E. coli* Genome Project via the ''A Systematic Annotation Package for Community Analysis of Genomes'' as a guest https://asap.ahabs.wisc.edu/annotation/php/logon.php) [12].

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