Regulon and promoter analysis of the *E. coli* heat-shock factor, σ^{32}, reveals a multifaceted cellular response to heat stress

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The heat-shock response (HSR), a universal cellular response to heat, is crucial for cellular adaptation. In *Escherichia coli*, the HSR is mediated by the alternative σ factor, σ^{32}. To determine its role, we used genome-wide expression analysis and promoter validation to identify genes directly regulated by σ^{32} and screened ORF overexpression libraries to identify σ^{32} inducers. We triple the number of genes validated to be transcribed by σ^{32} and provide new insights into the cellular role of this response. Our work indicates that the response is propagated as the regulon encodes numerous global transcriptional regulators, reveals that σ^{70} holoenzyme initiates from 12% of σ^{32} promoters, which has important implications for global transcriptional wiring, and identifies a new role for the response in protein homeostasis, that of protecting complex proteins. Finally, this study suggests that the response protects the cell membrane and responds to its status: Fully 25% of σ^{32} regulon members reside in the membrane and alter its functionality; moreover, a disproportionate fraction of overexpressed proteins that induce the response are membrane localized. The intimate connection of the response to the membrane rationalizes why a major regulator of the response resides in that cellular compartment.

[*Keywords*: Heat-shock response; σ32; transcription; microarray]

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When cells are shifted from low to high temperature, synthesis of the heat-shock proteins (hsps) is rapidly and selectively induced. The heat-shock response (HSR), was first identified by Ritossa (1963), who showed that exposure to heat lead to transient changes in the puffing pattern of salivary chromosomes in *Drosophila*; Tissieres et al. (1974) demonstrated that these changes reflected the transient induction of several proteins. Initially, hsp function was unclear; however, experiments in several organisms revealed that many hsps were chaperones that promote protein folding (Pelham 1986; Beckmann et al. 1990; Gaitanaris et al. 1990; Skowyra et al. 1990). These studies not only suggested that a major function of the HSR is to maintain the protein folding state of the cell, but also indicated that some of these chaperones, such as Hsp70 and Hsp90, are present in all organisms (Bardwell and Craig 1984, 1987). Thus, both the HSR and some hsps are universally conserved among organisms.

In *Escherichia coli*, σ^{32}, an alternative σ factor, controls the HSR by directing RNA polymerase to transcribe hsps (Yamamori and Yura 1980; Grossman et al. 1984; Taylor et al. 1984; Cowing et al. 1985). Synthesis of hsps is induced upon temperature upshift and repressed upon temperature downshift (Lemaux et al. 1978; Yamamori et al. 1978; Straus et al. 1987, 1989; Taura et al. 1989), thereby allowing a rapid cellular response to changes in temperature. σ^{32} is controlled by negative feedback loops controlling its activity (Straus et al. 1989; Blaszczak et al. 1999) and stability (Straus et al. 1987) and a feed forward loop controlling its synthesis (Yuzawa et al. 1993; Morita et al. 1999a,b; El-Samad et al. 2005). σ^{32} regulon members mediate both feedback loops: The FtsH protease controls σ^{32} stability (Herman et al. 1995; Tatsuta et al. 1998), and the DnaKJ and GroEL/S chaperone machines control σ^{32} activity (Gamer et al. 1996; Tatsuta et al.

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1998; Guisbert et al. 2004), ensuring homeostatic control.

Given the importance of the heat-shock response for cellular adaptation, it is crucial to identify the genes that are induced so that these cellular processes can be identified and investigated. Several global approaches have been utilized in *E. coli* to identify genes under σ^{32} control. Both two-dimensional protein gels (Lemaux et al. 1978) and global transcriptional approaches have identified genes induced after transfer to high temperature (Chuang et al. 1993b; Richmond et al. 1999) or after overexpression of σ^{32} (Zhao et al. 2005); however, there has been no systematic determination of whether these induced genes are directly expressed from σ^{32}-dependent promoters. The few cases where the functions of σ^{32}-dependent genes were identified indicates the importance of this determination: Hsp33, a widely conserved hsp, is a redox activated chaperone (Jakob et al. 1999), thereby providing the first identification of a chaperone of this type, and FtsJ was shown to be a methyltransferase whose substrate is 23S RNA, revealing an unexpected link between the HSR and RNA (Bugl et al. 2000).

We report the identification of most σ^{32}-dependent genes obtained by combining whole-genome expression analysis of genes induced after σ^{32} overexpression with start site mapping of these genes and in vitro transcription studies, and also on the types of overproduced proteins that signal induction of σ^{32}. Our analysis has almost tripled the number of genes validated to be expressed from σ^{32}-dependent promoters, and revealed that the HSR response targets multiple processes, protects complex proteins, and targets the membrane as well as the cytoplasm. That the σ^{32}-mediated response is intimately related to membrane functionality rationalizes why the FtsH protease controlling σ^{32} stability resides in the cytoplasmic membrane.

Results

Identification of genes whose transcription increases following overexpression of σ^{32}

To identify genes regulated by σ^{32}, we compared expression from cells having a plasmid-borne IPTG-inducible *rpoH* gene with cells carrying the empty vector using whole-genome expression analysis. This approach is preferable to examining cells lacking σ^{32} because such cells are very sick and grow slowly. Because accumulation of DnaKJ and GroELS damps expression due to feedback inhibition (Gamer et al. 1996; Guisbert et al. 2004), we first determined the kinetics of hsp synthesis following σ^{32} overexpression. Hsp synthesis peaked at ~10–15 min following σ^{32} overexpression (Fig. 1A); thus, we analyzed an expression time course ranging from 0 to 20 min. Relative mRNA levels were determined by parallel two-color hybridization to glass-slide cDNA microarrays. SAM analysis (statistical analysis of microarrays) (Tusher et al. 2001) of gene expression from four independent cultures at 10 min after induction indicated that 105 genes were significantly induced. We used hierarchical clustering to examine the RNA expression pattern of these genes throughout the time course of induction (Fig. 1B) and then determined whether the induced genes consisted of subgroups with distinct induction characteristics using SOM (self-organized maps). We find that induced genes show both temporal distinction (rapid and slower responders) (Fig. 1C) and quantitative differences (strong and weak responders) (Fig. 1D).

Identification of induced genes having a σ^{32} promoter

First, we organized the significantly induced genes into transcription units (TUs) and, where appropriate, expanded the TUs to include other gene members that were less strongly induced based on the criterion that they are adjacent, in the same orientation, induced with similar kinetics, and have an expression pattern typical of an operon (the first gene has the highest induction, followed by decreasing induction of downstream genes). This gave 127 genes organized in 66 potential TUs. We then searched the 300-nucleotide (nt) region upstream of each TU for candidate σ^{32}-promoter sequences using MEME and BioProspector: This predicted 42 promoters upstream of 40/66 TUs (Table 1A,B), of which 14 were previously identified σ^{32} promoters and 28 were new predictions.

We tested whether the newly predicted promoters and TUs lacking known or predicted promoters had σ^{32}-dependent start sites by using 5′RACE (rapid amplification of cDNA ends) to compare the 5′ ends of mRNAs from cells overexpressing σ^{32} with those from cells lacking σ^{32} (Fig. 2). This confirmed 18 out of 28 promoter predictions, using as a criterion a σ^{32}-dependent start site located just downstream of a promoter prediction and identified four new σ^{32}-dependent start sites upstream of the 26 TUs with no predicted promoters (Table 1A). In 11 instances, either no σ^{32}-dependent start site was identified near a prediction or the start site location was inconsistent with that of the prediction. Given that the *rrnB*P1 has an overlapping σ^{32} and σ^{70}-dependent promoter (Newlands et al. 1993) and dual recognition promoters would score as σ^{32} independent in the 5′RACE assay, we retested these 11 promoters in vitro for transcription by both σ^{32} and σ^{70} holoenzyme. This identified five dual σ^{32}/σ^{70}-dependent promoters and two additional σ^{32}-dependent promoters (Fig. 3). In toto, we identified 29 new σ^{32} promoters (Table 1A). We also tested seven previously proposed σ^{32} promoters located upstream of genes that were not significantly induced on our arrays and detected no σ^{32}-dependent transcripts (Table 1C). Finally, neither 5′RACE nor in vitro transcription identified functional promoters for three of the σ^{32} promoters proposed by Zhao et al. (2005) (*ldhA, macB*, and *ybbN*; Table 1B) based on electrophoretic mobility gel shift by σ^{32}-holoenzyme.

Characteristics of σ^{32} promoters

The 29 newly identified σ^{32} promoters, 20 confirmed promoters, and two previously validated promoters up-

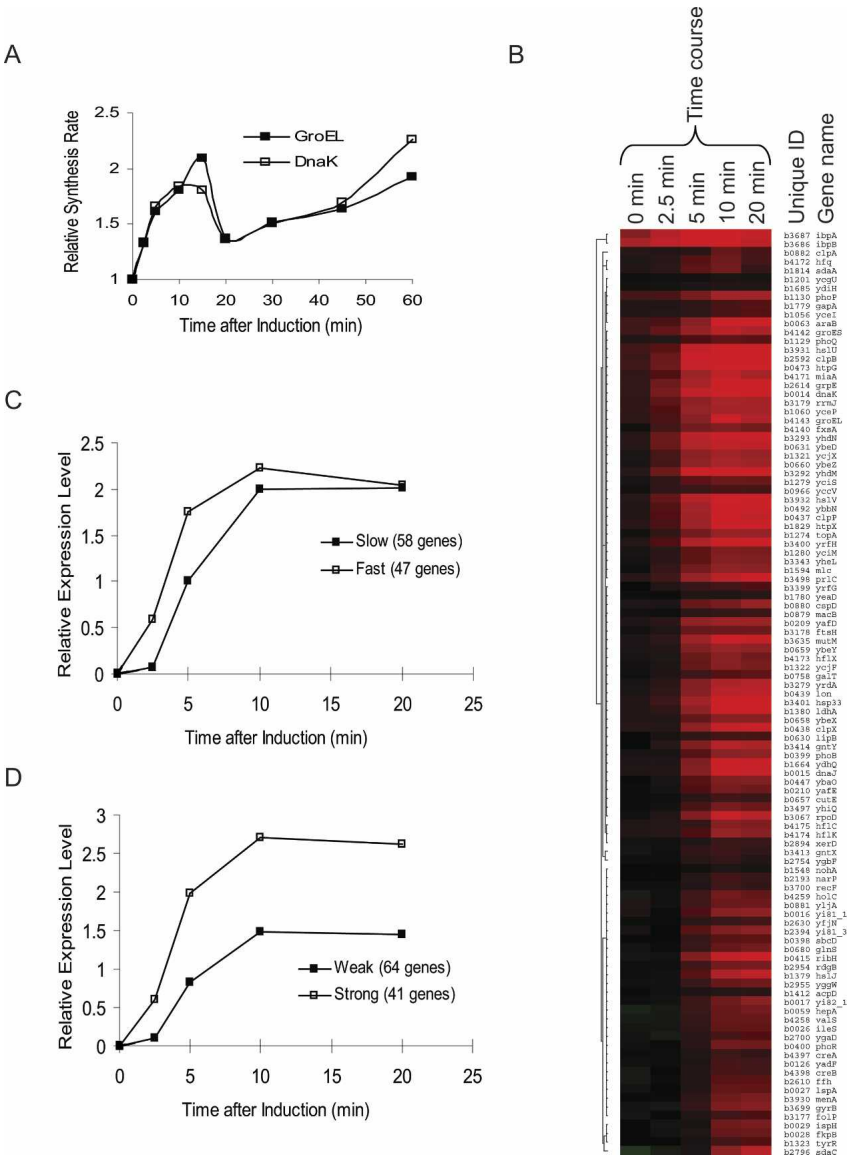

Figure 1. Expression profiles of σ^{32}-regulon members after overexpressing *rpoH*. (*A*) Activity of σ^{32} following overexpression of *rpoH*. An exponential phase culture of strain CAG50002 (which carries an IPTG-inducible copy of *rpoH*) growing at 30°C in M9 complete–methionine was induced with IPTG at OD_{450} = 0.3 (t = 0). At various times, pulse chase analysis was used to determine the rate of synthesis of two σ^{32}-dependent hsps, DnaK, and GroEL. Data is normalized to their synthesis rates at induction (0 min). (*B*) Hierarchical clustering of 105 genes whose expression is significantly altered following *rpoH* overexpression (CAG50002) vs. wild type (CAG50001). The color chart illustrates the average expression level at each time point for each gene from three time course experiments. Red denotes increased and green denotes decreased mRNA expression in CAG50002 vs. CAG50001: Maximum intensity represents greater than fourfold change. Time in minutes after induction of *rpoH* in the time-course experiments is indicated at the *top* of the figure; genes are identified by their unique ID and name. (*C*,*D*) SOM analysis of significantly induced genes following *rpoH* overexpression. The expression ratios for each gene across three time courses were averaged for each time point. Genes were partitioned based on their induction kinetics (fast/slow; *C*) or magnitude of induction (strong/weak; *D*). Each line represents an average trace of expression pattern for that group of genes. Relative Expression Level indicates mean and variance normalized (*C*) or raw (*D*) $\log_2$ (*rpoH* overexpressed/wild type) expression ratios.

stream of sequences not present on our arrays (Table 1C) comprise a total of 51 promoters that drive the expression of 49 TUs. The sequence logos of the conserved sequence motifs upstream of the 50 chromosomal σ^{32} promoters (note *repE* is on the F factor) together with their information content are displayed in Figure 4A. Virtually all of the total information content of these promoters (18.3 bits) is from the conserved –10 (CCCCATWT) and –35 (TTGAAA) core motifs, with very little contribution from flanking sequences. Figure 4, B and C display histograms of the distance distributions of the promoter elements from each other. Most promoters prefer a 7-nt spacing between –10 and +1 and a 13–14-nt spacing between –10 and –35. However, five promoters contained very short spacers (Fig. 4B). Four of these have very poor –35 sequences, raising the possibility that at these promoters, σ^{32} makes nonspecific interactions with sequences further upstream at the optimal spacing. Interestingly, four of these promoters contain A/T-rich sequences in the spacer region, suggesting that the intervening DNA may distort upon binding of RNAP. Finally, 66% of all σ^{32} promoters are located within 100 nt of the downstream gene translation start (Fig. 4D), as is the case for known σ^{70} promoters (Burden et al. 2005).

There are several A/T-rich motifs directly upstream of the –35 core motif reminiscent of the A/T-rich UP-elements that bind the α C-terminal domains (αCTDs) of RNAP (Ross et al. 1993; Estrem et al. 1998). We termed these motifs "Complete," "Proximal," and "Distal" by analogy to their UP element counterparts (Fig. 5). About 40% of all σ^{32} promoters contain such motifs, with about half having a distal motif only and the remainder having a complete motif or a combination of complete, distal, and proximal motifs (Fig. 6). We suggest that these A/T-rich σ^{32}-promoter motifs are likely to be αCTD-binding sequences; in fact, the upstream sequences of the *rrnB* P1 promoter, which contain all three motifs

Table 1. *σ^{32}-regulon members*

Transcription unit	Unique ID	Induction ratio	σ^{32} promoter		Evidence
			Distance	Sequence	
A. Significantly induced and identified promoter					
dnaK<P1> dnaJ yi81_1yi82_1	b0014-7	18.2 F, St	–150	CTTGATGACGTGGTTTACGACCCCATTTAGTAGTCA	K (Cowing et al. 1985)
dnaK<P2> dnaJ yi81_1 yi82_1	b0014-7	18.2 F, St	–75	GTTGAAACCAGACGTTTCGCCCCTATTACAGACTCA	P,K (Cowing et al. 1985)
ileS lspA fkpB ispH	b0026-9	3.3 S, W	–198	GTTGCAATGGACCTTTACGGTCGCCATATACAAGTAG	P,R
hepA	b0059	3.6 S, W	–360	CTTGCAATATCAGAATCGCGGCACCATTAAGTACGTA	R
yadF	b0126	2.4 S, W	–69	GTTTAAATCGTCCCGGAGTTGCCCTATATTAGCCAAA	P,T
yafD yafE	b0209-10	6.3 F, St	–86	GTTGAAAGAGTGAGCTAAAATCCCTATAA	P,R
ribE nusB thiL pgpA	b0415-8	9.9 S, St	–178	CTTGAGAAATTAGCCGACGCCCCCCAATTTAAATTC	P,R
clpP clpX	b0437-8	11.4 F, St	–108	CTTGAAATATGGTGATGCCGTACCCATAACACAGGGA	P,K (Li et al. 2000)
lon	b0439	9.3 S, St	–111	GTTGAATGTGGGGGAAACATCCCCATATACTGACGTA	K,T (Chin et al. 1988)
htpG<P1>	b0473	18.4 F, St	–80	CTTGAAATTATTCTCCCTTGTCCCCATCTCTCCCACAT	P,K (Cowing et al. 1985)
htpG<P2>	b0473	18.4 F, St	–71	ATTCTCCCTTGTCCCCATCTCTCCCACATCCTGTTTTT	P,K (Cowing et al. 1985)
ybeD lipB	b0631-0	9.5 F, St	–99	CTTGAAAGTGTAATTTCCGTCCCCATATACTAAGCA	P,T
ybeZ ybeY ybeX cutE	b0660-57	7.4 F, St	–126	CTTGTTTTTTCTCTCTTTATCCCCATCTTTATTGCA	P,R
glnS	b0680	3.4 S, W	–130	CTTTTTTTTTCATCAATCATCCCCATAATCCTTGTTA	P,T
yceJ yceI	b1057-6	1.5 S, W	–62	GTTGCAATTCATTCTCCGACACGCCATTTTCTTTCTCT	P,T
yceP	b1060	6.9 F, St	–111	CTTTAAAAATTCGGTGAATACCCTTACTTATTGGTATA	P,R
phoP phoQ	b1130-29	6.9 S, St	–76	GTTTATTTAATGTTTACCCCCATAACCACATAA	P,R
topA	b1274	6.0 F, St	–104	GTTGATATCCGCAGAGAGCGAGTCCATATCGGTAACT	P,K (Lesley et al. 1990)
yciS yciM pyrF yciH	b1279-82	3.7 F, W	–143	GTTGATTTTCCGAATTTAGCCCTTAAATCATCAACA	P,R
ycjX ycjF tyrR	b1321-3	6.9 F, St	–83	GTTGATAATCAATGGCCTGGCCCCCACATTCATATC	P,R
mlc ynfK	b1594-3	5.5 F, St	–62	ATTGAAGTGCTTCACCATAGCCTACAGATTATTTCG	P,K (Shin et al. 2001)
ydhQ	b1664	11.3 S, St	–110	GTTTAAACAGAGTTGTTTGCCACCATCTCAAATCTG	P,R
gapA yeaD	b1779-80	2.4 S, W	–190	CTTTAAAATTCGGGGCGCCGACCCCATGTGGTCTCAAG	P,K (Charpentier and Branlant 1994)
sdaA	b1814	3.9 F, W	–155	CTACTTGAGACAATCATCGCAATATTAGTTAAA	R
htpX	b1829	12.1 F, St	–77	CTTGAAAATAGTCGCGTAACCCATACGATGTGGGTA	P,K (Kornitzer et al. 1991)
narP	b2193	2.3 S, W	–115	GTTGTAGTTTTCACTACATGTCCATACATAAAATGGGG	P,R
clpB	b2592	21.5 F, St	–67	CTTGAATAATTGAGGGATGACCTCATTTAATCTCCA	P,K (Kitagawa et al. 1991)
grpE	b2614	14.2 F, St	–74	CTTGAAACCCTGAAACTGATCCCCATAATAAGCGAA	P,K (Lipinska et al. 1988)
yfjN	b2630	2.0 S, W	–45	GTATTATTGTAGAGTTTCCCCATATGTTTCTATG	P, T
ygaD recA	b2700-699	2.4 S, W	–81	GTAAAATTGCAACGCCAACACCATCTTCCTGACG	R
ygbF	b2754	1.9 F, W	–99	CTGGAGAAATACAACCGCCGGCCCCACCTGAAGATGCA	P,R
xerD dsbC recJ	b2894-2	2.1 S, W	–140	CTGGAACCGTAAAAAAGACCCATATTCATAACG	T
rdgB yggW	b2954-5	3.9 S, W	–97	ATTGAAAAAGGCGAACTTGGCCGCCACAA	P,R
rpoD	b3067	10.5 F, St	–395	CTTGAAAAACTGTCGATGTGGGACGATATAGCAGATA	K (Cowing et al. 1985)
rrmJ ftsH folP	b3179-7	7.4 F, St	–103	ATTGAAAACGGGTCATTCTACCGCCATCTCCCATATA	P,K (Herman et al. 1995)
yhdN zntR	b3293-2	11.9 F, St	–73	GTTTTTTTATACCCGTAGTATCCCCACTTATCTACAA	P,R
(yrfG) yrfH hsp33	b3399-401	17.0 F, St	–274	CTTGATTTATTACTTTCCACCCACACATTTGGTTATC	P,R
(gntX) gntY	b3413-4	8.8 F, St	–202	CTTGAATTGCCCCGTGCAAGGTCGCCATATGGTGATTG	P,R
prlC yhiQ	b3498-7	9.2 F, St	–92	ATTGAAATTCACTACACTTAACCCCCATGCTACACACA	P,T
mutM	b3635	12.4 F, St	–58	GTTTTTTGTTATCTGCTTGCCCCCATATTGACTGCA	P,R
ibpA ibpB	b3687-6	13.4 F, St	–131	CTTGAAAAGTTCATTTCCAGACCCATTTTTACATCG	P,K (Chuang et al. 1993a)
hslV hslU menA	b3932-0	15.5 F, St	–97	GTTGAAACCCTCAAAATCCCCCCCATCTATAATTG	P,K (Chuang et al. 1993a)

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Table 1. (*continued*)

Transcription unit	Unique ID	Induction ratio	σ^{32} promoter		Evidence
			Distance	Sequence	
A. Significantly induced and identified promoter					
fxsA	b4140	4.4 F, W	–96	CTTGAAATTTTGCTAATGACCACAATATAAGCTAAA	P,R
groES groEL	b4142-3	9.1 F, St	–109	CTTGAAGGGGCGAAGCCTCATCCCCATTTCTCTGGTCA	P,K (Cowing et al. 1985)
mutL miaA hfq hflX hflK hflC	b4170-5	N/A	–402	CTGGCGAACAGTCAGTCTGACCCCTATTTAAGCCAGG	K (Tsui et al. 1996)
miaA hfq hflX hflK hflC	b4171-5	9.9 F, St	–307	ATTGATTTCCAGTCAGATGCACAGCATGTGACCATCAG	K (Tsui et al. 1996)
hfq hflX hflK hflC	b4172-5	5.0 F, W	–926	GTTGATAAGCGTTGATTCTGCCCTTATTTACAAAGGGA	K (Tsui et al. 1996)
holC valS	b4259-8	3.9 F, W	–29	ATTTAAATCCACCACAAGAAGCCCCATTTATGAAAAA	P,R
creA creB creC	b4397-9	2.1 S, W	–315	CTTGGAATAACCTGCTTTCGCCGCTACATTGTCGAGCG	R
B. Significantly induced but no identified promoter					
araB	b0063	11.8 S, St	–136	ATTGATTATTTGCACGGCGTCACACTT	P, Tf
sbcD	b0398	2.9 S, W			
phoB phoR	b0399-0	5.1 S, W			
ybaO	b0447	4.5 S, W			
ybbN	b0492	11.6 F, St			Tf
galT	b0758	2.9 S, W			
macB	b0879	2.0 S, W			Tf
cspD	b0880	4.1 S, W	–133	TGTCAAATGCTTGACGGCTCGCCCTAATT	P, Tf
yljA clpA	b0881-2	3.5 S, W			
yccV	b0966	2.4 F, W			
ycgU	b1201	1.2 S, W			
ldhA hslJ	b1380-79	15.8 S, St	–104	GTTGATGAATTTTTCAATATCGCCATAG	P, Tf
acpD	b1412	1.5 S, W			
nohA	b1548	1.3 S, W			
ydiH	b1685	1.5 S, W			
yi81_3	b2394	4.7 S, W			
ffh	b2610	2.9 S, W			
sdaC sdaB	b2796-7	5.3 S, W	–272	GTTTAATGGGCAAATATTGCCCTTAAAT	P, Tf
yrdA	b3279	7.7 F, St			
yheL	b3343	4.7 F, W			
recF gyrB	b3700-699	4.4 S, W			
C. Not significantly induced but reported promoter					
repE	F factor	no data	–62	ATTGACTCTTTTTTATTTAGTGTGACAATCTAAAAACTT GTCACACTT	K (Wada et al. 1987)
*rrnB*P1	b3968	no data	–329	CTTGTCAGGCCGGAATAACTCCCTATAATGCGCCACCA	K (Newlands et al. 1993)
htpY	b0011	1.1	–120	TTTGAGGGGAAAATGAAAATTTTCCCCGGTTTCCGGT ATC	K,Tf (Missiakas et al. 1993)
ppiD	b0441	1.8	–105	CTTGTGGGCTGACAATTGCCCCCGTTTCT	K,Tf (Dartigalongue and Raina 1998)
pphA	b1838	1.0	–449	GGCGAAAAAATGAGTCTGGCACCGTTTATAGAAC	K,Tf (Missiakas and Raina 1997)
ptsH ptsI	b2415	1.2	N/A	N/A	K,Tf (Ryu 1998)
htrM	b3619	0.8	–152	CATGAAGGACTAGCTAAAACCCAAACTAGTT	K, Tf (Raina and Georgopoulos 1991)
htrC	b3989	1.1	–69	CTGAACTAATTGAGTCAAACTCGGCAAGGATTCGATA	K,Tf (Raina and Georgopoulos 1990)
metA	b4013	1.1	–76	ATTGGCAAATTTTCTGGTTATCTTCAGCTA	K,Tf (Biran et al. 1995)

(*A*) Genes induced upon overexpression of *rpoH* (as determined by transcription profiling) with validated upstream σ^{32} promoter. (*B*) Genes induced upon overexpression of *rpoH* (as determined by transcription profiling) but with no validated σ^{32} promoters. (*C*) Genes not induced after overexpression of σ^{32} (as determined by transcription profiling) but with identified confirmed σ^{32} promoters derived from the literature. (Transcription unit) TUs are listed in chromosomal order; genes within a unit are listed in order of transcription; genes in parenthesis are induced but are not predicted to be translated since the σ^{32} promoter is internal; in a few instances, a single TU has multiple σ^{32} promoters (both the *dnaK dnaJ yi81_1 yi82_1* and *htpG* operons have two; *mutL mia hfq hflX hflK hflC* operon has three); they are counted as one TU, thus total TUs activated by σ^{32} are 66 in number (listed in *A* and *B*). (Ratio) averaged expression ratio of (*rpoH*-induced)/*rpoH*^{wt} (time points, 10 min) of first gene in TU; induction kinetics from SOM (Fig. 1) are also included. (F) Fast; (S) slow; (St) strong; (W) weak. (σ^{32} Promoter) Identified or predicted σ^{32} promoter sequences. (Distance) Number of nucleotides of 5′ end of –35 motif upstream of translation start point of the first gene in the TU. (Sequence) σ^{32} promoter sequence with the conserved –35 elements, –10 elements, and the start of transcription underlined. (Evidence) Evidence for σ^{32} promoter; (K) previously known; (P) predicted by both MEME and BioProspector; (R) confirmed by 5′RACE PCR; (T) confirmed by in vitro transcriptions; (Tf) tested by in vitro transcriptions, but no σ^{32}-dependent transcripts were detected. Note that the expression ratio of *mutL* is not available because of the low signal intensity in microarrays; expression ratio of *repE* and *rrnB* are not available because of the missing spots on the microarrays; exact +1 sites of *yafD* and *yggV* are not available because of the inaccurate sequencing results in 5′RACE; +1 site of *holC* is 8 nt downstream of the proposed translation start site, suggesting a possible alternative translation start site.

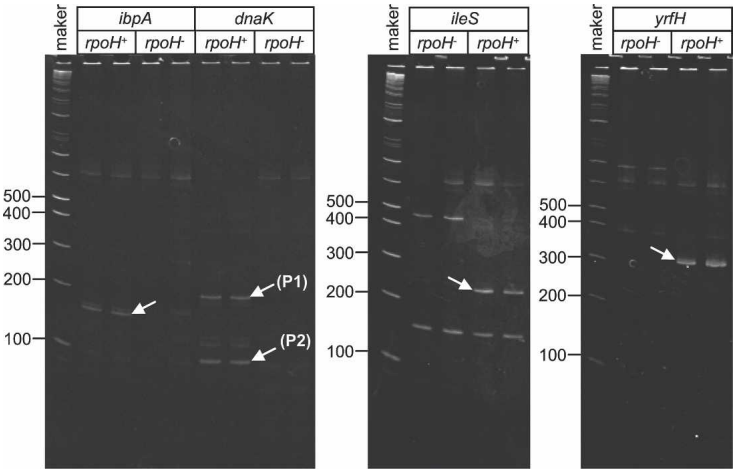

Figure 2. 5′RACE identified 22 σ^{32}-dependent transcription starts. To identify σ^{32}-specific transcripts, mRNA from *rpoH*$^+$ and *rpoH*$^-$ strains (CAG50002 and CAG50003, respectively) was 5′-labeled with an RNA oligo, reverse-transcribed, amplified by PCR, and then visualized by 7.5% PAGE (see Materials and Methods). Bands present in *rpoH*$^+$ but not in *rpoH*$^-$ reactions were regarded as σ^{32} specific. Two known σ^{32} promoters, *dnaK* and *ibpA*, were tested; 22 new σ^{32}-dependent promoters were identified from the 54 newly identified σ^{32}-induced TUs. Displayed are duplicate examples of two new (*ibpA, ileS*) and two known (*dnaK, ibpA*) σ^{32}-specific promoters.

(Fig. 6), has already been shown to stimulate σ^{32}-dependent transcription (Newlands et al. 1993). Interestingly, neither σ^{32} (Fig. 6) nor σ^S promoters (Typas and Hengge 2005) have solely proximal sites, although such sites are common in σ^{70} promoters. This is probably because the σ^{70} residues that contact αCTD and mediate enhanced transcription from the proximal site are not conserved in σ^S (Typas and Hengge 2005) and are poorly conserved in σ^{32} (Supplementary Fig. 1).

Functional analysis of the σ^{32}regulon members

We examined the location and functional classification of validated members of the σ^{32} regulon (Table 2A,B). A significant fraction (~25%) of known regulon members reside in the inner membrane, raising the possibility that one role of the response may be to monitor and preserve the membrane during stress. In addition to chaperones and proteases, the regulon is involved in maintaining the integrity of cellular DNA; the connection of the σ^{32} regulon to the RNA state is more extensive than previously realized; and the regulon is intimately connected to central metabolism and to transport of substrates. Finally, σ^{32} is a master regulator, altering expression of many transcription factors as well as activity of the transcriptional apparatus itself. Thus, the σ^{32}-mediated hsr has a general role in maintaining cellular homeostasis upon exposure to heat stress.

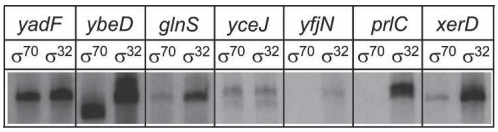

Figure 3. In vitro transcription assays identified seven new promoters transcribed by σ^{32}. Multiround in vitro transcription assays were performed to test 11 predicted and six previously documented promoters. Each promoter template was tested with RNAP containing either σ^{70} or σ^{32}: σ^{32}-dependent transcripts were obtained from seven promoters, five of which also generated σ^{70}-dependent transcripts (*yadF, ybeD, glnS, yceJ*, and *xerD*).

Identification of endogenous proteins that induce the σ^{32} HSR

A global analysis of proteins that induce a response when overexpressed is a powerful method for determining the signals normally sensed by the response. For example, overexpressed porins induce the σ^E envelope stress response (Mecsas et al. 1993); later work demonstrated that unassembled porins initiate the signal transduction pathway that induces σ^E (Walsh et al. 2003). We used the ASKA library, which contains every ORF of *E. coli* K-12 expressed from an IPTG-inducible promoter on separate plasmids (see Table 4, below) to search for inducers of the σ^{32} HSR. The plasmids were pooled, introduced into a reporter strain that expresses the *lacZ* operon from the σ^{32}-dependent *htpG* promoter, and transformants screened for higher than average σ^{32} activity by plating on triphenyltetrazolium lactose indicator plates and selecting white colonies (high-lactose fermenters). Candidates were confirmed by restreaking on MacConkey lactose plates (selecting red colonies) and then on Xgal and IPTG (selecting dark blue colonies). Thirty dark-blue colonies were selected at random, the ASKA plasmids extracted and sequenced to determine which ORF was responsible for inducing the HSR (Table 3), and the level of induction of the HSR measured by overexpressing each of the candidates in strains grown in liquid culture (Supplementary Fig. 2). Twenty-five candidates induced the HSR, and surprisingly, ~60% of these were membrane proteins; this is a significant overrepresentation, given that only 20% of all *E. coli* proteins are located in the membrane (Serres et al. 2004). Not all overexpressed membrane proteins induced the HSR (e.g., LacY) (Supplementary Fig. 2).

Discussion

The ability of cells to maintain homeostasis in response to temperature stress is essential for viability. In *E. coli,*

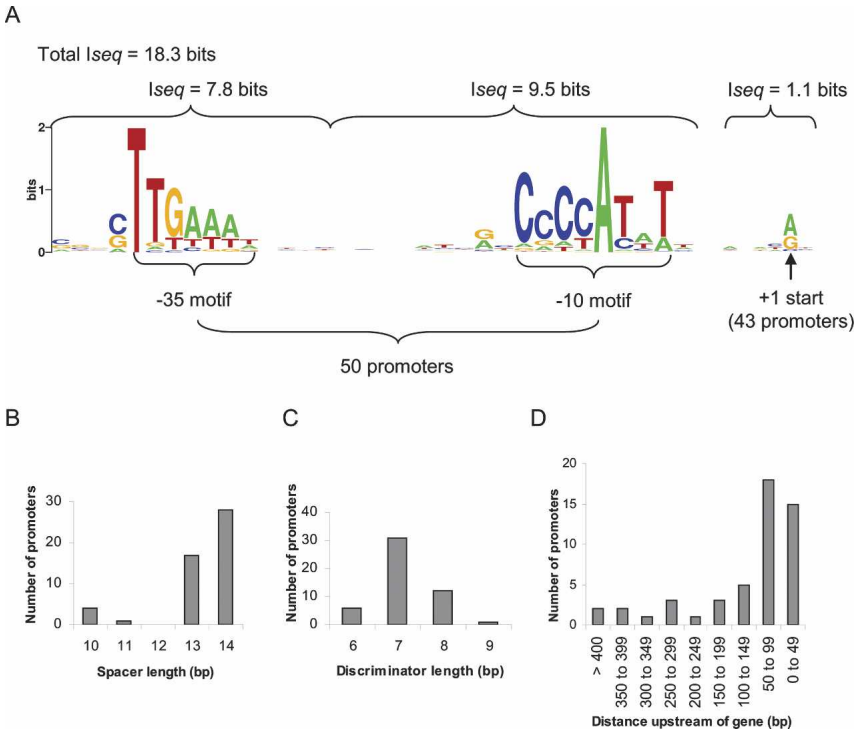

Figure 4. Core promoter motifs of 50 σ^{32} promoters. Fifty of the 51 validated σ^{32}-dependent promoters were used to derive sequence logos of the core motifs (see Table 1A,B) (the *repE* promoter was excluded due to the large number of multiple start points that made it difficult to confidently identify the upstream –10 and –35 motifs). The sequences were initially aligned by their start sites and WCONSENSUS was used to search small windows upstream for the conserved –10 and –35 motifs (see Materials and Methods). (*A*) Sequence logos (http://weblogo.berkeley.edu; Crooks et al. 2004) of the –35, –10, and +1 start site motifs. Note that only 43 promoters were used to derive the start site motif. Seven promoters were excluded that had either multiple starts with no clearly preferred position (*b0473* P1 and P2, *b1057*, *b3179*, and *b3400*) or no defined starts (*b0209* and *b2954*). The information content (I_{seq}) of each conserved sequence window is indicated. (*B*) Frequency distribution of distance between –35 (TTGAAA) and –10 (CCCATAT) motifs. (*C*) Frequency distribution of distance between –10 (CCCATAT) motif and +1 (A/G) start. (*D*) Histogram of the 50 σ^{32}-promoter start sites upstream of the gene translation start.

σ^{32}-controlled genes govern the immediate response to temperature stress. In this report we used global approaches to provide complete or nearly complete identification of the σ^{32} regulon and to identify endogenous signals of the response. Our analysis, together with data in the literature, indicates that the multifaceted σ^{32} stress response not only maintains protein homeostasis but protects DNA and RNA and is further propagated because σ^{32} controls the expression of other global regulators. Finally, our results reveal an unanticipated connection with the cellular membrane: σ^{32} senses imbalances in membrane proteins and σ^{32}-controlled genes contribute to membrane homeostasis.

The σ^{32} regulon

We identified 29 additional σ^{32} promoters and 57 additional members of the σ^{32} regulon. At present, the regulon is comprised of 49 TUs regulated by 51 σ^{32}-dependent promoters, which together encode on the chromosome 89 ORFs and one rRNA, and one ORF on the F factor (Table 1). We suggest that our analysis has identified most, if not all, σ^{32}-dependent TUs present on the arrays (~96% of the genome). This assertion is based on our success in validating previously proposed σ^{32}-dependent TUs. Of the 25 of the previously proposed TUs, 23 were present on our arrays; of these, 16 showed rapid induction after overexpression of σ^{32}. The seven uninduced TUs also failed to generate σ^{32}-dependent transcripts either in vitro or in vivo and contained poorly conserved σ^{32} promoters (Table 1C). We therefore suggest that these seven promoters either require additional regulators or are not recognized by σ^{32}. Thus, every bona fide previously proposed σ^{32} TUs present on our array was validated in this analysis.

The consensus promoter sequence derived from the 50 validated σ^{32} promoters indicates the following highly conserved bases: STTGAAA-N_{11-12}-GNCCCCATWT (Fig. 4; note the optimal spacer length [13–14 bp] is from the core-10 motif, CCCCATWT). The importance of these conserved bases is indicated by their close correspondence to the "functional" σ^{32}-promoter sequence, CTTGA-14 bp-GNCCCCATNT, derived by mutating each position of the core *groE* σ^{32}-promoter sequence to every other nucleotide (Wang and deHaseth 2003). There are subtle differences in the less-preferred bases between our aligned promoter profile and the derived "functional" σ^{32}-promoter sequence, suggesting that the importance of individual bases may vary according to the context of the local sequence.

We identified five overlapping σ^{32}/σ^{70} promoters in addition to the previously reported *rrnB*P1 σ^{32}/σ^{70} dual promoter (Newlands et al. 1993). Given the similarity between the σ^{32} and σ^{70} core promoter sequences (σ^{70} TTGACA-N_{17}-TATAAT; σ^{32} TTGAAA-N_{14}-CCCCATWT) it is likely that both holoenzymes are recognizing the same core promoter sequences. However, as 22 of our σ^{32} promoters generated only σ^{32}-specific transcripts as

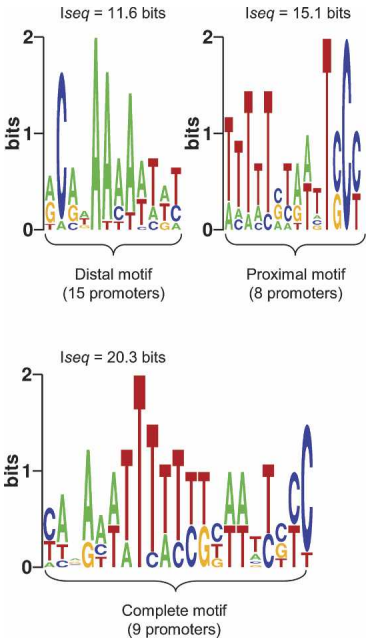

Figure 5. Sequence logos of A/T-rich motifs upstream of the σ^{32} promoters. WCONSENSUS was used to search sequences upstream of the –35 element of 50 σ^{32} promoters (excluding *repE*). Distal, Proximal, and Complete motifs were identified from the search windows –46 to –60, –36 to –51, and –36 to –60, respectively, by aligning the promoters with respect to their –35 elements and assigning the first "T" of the –35 motif as position –35.

detected by 5′RACE, it is unlikely that σ^{70} holoenzyme directly transcribes many more σ^{32} promoters, (Table 1A; Fig. 2). However, in *Pseudomonas putida*, both σ^{S} and σ^{32} holoenzymes recognize the Pm promoter (Dominguez-Cuevas et al. 2005), raising the possibility for promoter overlap between σ^{S} and σ^{32} in *E. coli*.

Many significantly induced genes did not have a detectable σ^{32} promoter. Interestingly, of the 47 rapidly induced genes (Fig. 1C), 43 were under σ^{32} control whether or not they were strongly induced, whereas most induced genes without an identified σ^{32} promoter (18/25) were only slowly and weakly induced (Table 1; Supplementary Fig. 3). Since σ^{32} controls a number of transcriptional regulators (see below), these transcription factors may be responsible for delayed expression of genes lacking a σ^{32} promoter. These data suggest that even in bacteria where events are very rapid, careful temporal analysis may be able to resolve cascades of induction.

σ^{32}-mediated temperature adaptation has many cellular targets

A primary role of the σ^{32} regulon in protein homeostasis was suggested by the fact that >50% of previously known regulon members were either chaperones or proteases (Table 1A). Our results suggest that the regulon also contributes to protein homeostasis by maintaining an adequate supply of complex proteins that contain cofactors, Fe^{++}-S centers, or lipoyl modification. Since the extent to which damaged complex proteins can be repaired is not well known, thermolabile proteins of this type may need to be synthesized at higher rates during temperature stress. The regulon encodes two Fe^{++}-S center proteins (SdaA, IspH), a predicted Fe^{++}-S center protein (YggW), and an IscA homolog (GntY) (Giel et al. 2006) likely to be involved in Fe^{++}-S cluster biogenesis. The regulon also encodes lipoyl protein ligase (LipB) and enzymes participating in the biosynthesis of the cofactors riboflavin (RibE), thiamin (ThiL), folate (FolP), and probably biotin (YafE). If cofactor supply is tightly coupled to demand, these pathways may need to be induced during temperature stress. The particular enzymes

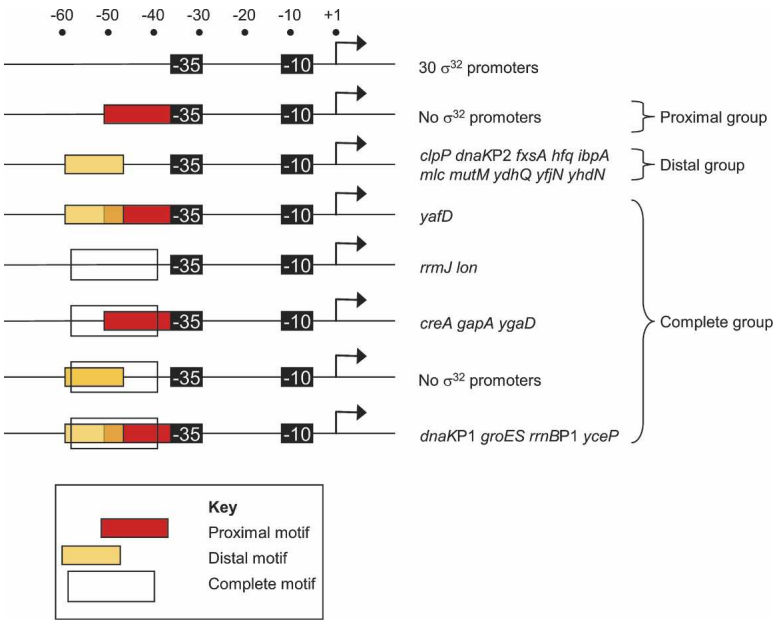

Figure 6. Location of A/T-rich motifs in σ^{32} promoters. The location and arrangement of the Distal, Proximal, and Complete A/T-rich motifs identified in Figure 5 are shown for the 50 σ^{32} promoters.

Table 2. *Location and functional classification of σ^{32}-regulon members*

A. Location	Regulon members
Periplasm (2)	DsbC YceI
Inner membrane (18)	CreC CutE FtsH FxsA GntY[a] HflX HflK HflC HtpX LipB[a] LspA MenA PgpA PhoQ YbeX[a] YbeZ[a] YceJ[a] YcjF[a]
Cytoplasm (48)	ClpB ClpP ClpX CreB DnaJ DnaK FkpB GapA GlnS GroELS GrpE HepA Hfq HolC Hsp33 HslU HslV HtpG IbpA IbpB IleS IspH Lon MiaA Mlc MutL MutM NarP NusB PhoP PrlC PyrF RecA RecJ RdgB RpoD *rrnB* RrmJ SdaA TopA TyrR ValS XerD YciH YfjN YrfH ZntR
Unknown (25)	CreA FolP RibE ThiL YadF YafD YafE YbeD YbeY YceP YciH YciM YciS YcjX YdhQ YeaD YfjN YgaD YgbF YggW YhdN YhiQ yi81_1 yi82_1 YnfK

B. Functional category	Regulon members
Metabolism	CutE FolP GapA IspH LipB LspA MenA PgpA PyrF RibE SdaA ThiL YadF YafE YceJ YggW
Chaperone/folding catalysts	ClpB DnaJ DnaK DsbC FkpB GroELS GrpE Hsp33 HtpG IbpA IbpB
Protein degradation	ClpP ClpX FtsH HflX HflK HflC HslU HslV HtpX Lon PrlC
DNA modification	HolC MutL MutM RecA RecJ RdgB TopA XerD
RNA state	GlnS Hfq IleS MiaA *rrnB* RrmJ ValS YciH YfjN YrfH
Transcription regulators	CreB CreC HepA Mlc NarP NusB PhoP PhoQ RpoD TyrR ZntR
Transporter	YbeX YbeZ
Miscellaneous	FxsA GntY YciH YfjN YhiQ yi81_1 yi82_1
Unknown function	CreA YafD YbeD YbeY YceI YceP YciM YciS YcjF YcjX YdhQ YeaD YgaD YgbF YhdN YnfK

Only regulon members encoded on the chromosome and with a confirmed σ^{32}-dependent promoter are considered. Proteins with no clear locational information are classified as unknown. Proteins with no significant sequence homology with any protein of known/predicted function are labeled unknown function.
[a]Some proteins are annotated as "membrane," and it is assumed they are located in the inner membrane.

in the regulon are likely to be rate-limiting and highly regulated, either because they are at the junction of two pathways (RibE and FolP) or because they utilize ATP (ThiL). Thus, altering the amount of these enzymes is likely to influence the amount of cofactor available to the cell.

σ^{32}-regulon members protect both DNA and RNA (Table 2B). Maintaining genomic integrity is essential for survival of the organism and high temperatures may increase both misincorporation and chromosome damage. The regulon encodes enzymes that mediate mismatch and excision repair (MutL, MutM), general recombination functions that resolve double-strand breaks (RecA, RecJ), and a site-specific recombination system to resolve chromosome dimers (XerD). This response also covalently modifies both classes of stable RNAs, possibly improving their thermal resistance. σ^{32}-regulon members have previously been shown to methylate 23S RNA (FtsJ) (Bugl et al. 2000) and to modify tRNA by transferring Δ^3-isopentyl-PP to A37 of tRNA (MiaA; just adjacent to and 3′ of the anticodon) (Tsui et al. 1996). A new regulon member, IspH, also produces Δ^3-isopentyl-PP; this tRNA modification stabilizes codon–anticodon pairing (Bjork and Hagervall 2005) and cells lacking this modification are temperature sensitive (Tsui et al. 1996). Finally, the regulon encodes the RNA chaperone Hfq (Table 2B) (Chuang and Blattner 1993; Tsui et al. 1996), which is required for the proper function of small RNAs.

Several regulon functions may promote transcription and translation at high temperatures. Two newly discovered regulon members, HepA and NusB, join TopA (Qi et al. 1996) as general effectors of transcription, and all three may respond to altered supercoiling at high temperature. Previous work indicated that thermal induction of TopA, an RNA polymerase-associated topoisomerase (Cheng et al. 2003) is necessary for normal transcription patterns at high temperature and for thermal resistance (Qi et al. 1996). HepA (RapA) is also an RNA polymerase-associated protein (Sukhodolets et al. 2001) specifically required for RNA polymerase recycling of tightly compacted negatively supercoiled DNA. Induction of NusB, essential for antitermination of rRNA transcription (Zellars and Squires 1999; Torres et al. 2004) may reflect altered requirements for rRNA antitermination when DNA supercoiling is altered, possibly to decrease R-loop formation (Drolet 2006). Finally, a general connection of the regulon to translation was previously established by Korber et al. (2000), who showed that regulon member Hsp15 (YfrH) binds free 50S ribosomal subunits with nascent chains and may promote ribosome recycling. This state is more prevalent at high temperatures (Korber et al. 2000), possibly because translation often terminates prematurely at lethal temperatures (VanBogelen and Neidhardt 1990).

Table 3. *Inducers of the heat-shock response*

Location	HSR inducers
Periplasm (3)	MalM RzpD YbjL
Inner membrane (14)	FecE FecR FliL GspO HybC LepA LpxK YdgK YhhQ YiiR YijD[a] YmgF YqjE YrbC
Cytoplasm (5)	Cca FabB YccR[a] YieM YqaE
Unknown (3)	CreA YagY YbbC

[a]These genes were obtained twice from the 27 sequenced candidates.

σ^{32} is also a master regulator controlling expression of seven transcriptional factors (including RpoD) (Table 2B) in addition to functions that alter RNA polymerase action (described above). The proportion of the σ^{32} regulon devoted to transcriptional factors (~8%) is similar to that for σ^S, previously thought to be the only alternative *E. coli* σ with a propagated response (Weber et al. 2005). The transcriptional regulators under σ^{32} control mostly sense extracellular conditions: Mlc senses glucose, Pho P/Q senses low Mg^{++} and is induced in acid stress, ZntR responds to excess Zn^{++}, and NarP induction requires nitrite/nitrate under anaerobic conditions. Intriguingly, many of the genes controlled by these regulators are membrane-localized transporters and other membrane proteins, pointing to the connection of this response to the status of the cytoplasmic membrane.

A comparison of genes directly regulated by HSF (heat-shock factor; controlling the eukaryotic counterpart response) with the σ^{32}-mediated heat-shock response indicates that only chaperones are encoded by both regulons (Hahn et al. 2004; Yamamoto et al. 2005). However, both regulons are of similar size and there is definite overlap in functional classes induced; for example, proteolysis, small molecule transport, additional transcription factors, proteins that modify RNA polymerase, and DNA related proteins. A broad response to heat stress appears to be universal among organisms.

The cytoplasmic membrane is a target of the σ^{32}-mediated hsr

The cytoplasmic membrane maintains cellular integrity and coordinates processes that traverse cellular compartments including secretion and processing of envelope proteins, energy generation, sensing the extracytoplasmic environment, and synthesis and/or transfer of the building blocks for phospholipids, LPS, and peptidoglycan. Previously identified σ^{32} regulon members FtsH (Herman et al. 1995), HflX, HflC (Chuang and Blattner 1993; Tsui et al. 1996), and HtpX (Kornitzer et al. 1991) play a vital role in membrane quality control; FtsH, together with its regulators HflX and HflC, degrades and dislocates unassembled membrane proteins, and the function of HtpX overlaps with that of FtsH. Our new data indicates that σ^{32}-regulon members have important additional roles in membrane homeostasis (Table 2):

(1) Increasing the potential to make fatty acids by up-regulating expression of carbonic anhydrase (YadF), which converts $C0_2$ to bicarbonate (Merlin et al. 2003). The first committed step of fatty acid biosynthesis is a major consumer of bicarbonate (Merlin et al. 2003).
(2) Altering transport properties of the membrane by up-regulating several transporters (Table 2B).
(3) Maintaining the potential for disulfide bond formation and isomerization by increasing menaquinone production (MenA), which is necessary to reoxidize DsbB under anaerobic conditions (Bader et al. 1999) and by up-regulating the disufide bond isomerase DsbC.
(4) Maintaining lipoprotein maturation by up-regulating both signal peptidase II (IspA), which cleaves the proprotein, and CutE, the enzyme that activates lipoproteins by adding palmitate to their N-terminal cysteine (Wu 1996). Because there are >100 lipoproteins, the precise physiological role of this regulation is unclear.
(5) Altering the composition of the lipid bilayer by up-regulating PgpA, an enzyme involved in synthesis of cardiolipin, a minority phospholipid in the inner membrane whose abundance increases during stationary phase (Hiraoka et al. 1993). As cells induce a variety of substances during stationary phase to prevent cell lysis, cardiolipin may impart additional stability to the lipid bilayer.
(6) Altering sensing capacity of the membrane by inducing the expression of two membrane-localized histidine kinases (CreC and PhoQ) and their cytoplasmic response regulators.

These results strongly implicate σ^{32}-regulon members in maintaining membrane functionality and homeostasis. This connection rationalizes why the σ^E-envelope stress response regulates σ^{32} in all organisms examined thus far, making this one of the most conserved features of the response (Rhodius et al. 2006). Because the inner membrane controls the flow of building blocks and proteins to the envelope, induction of σ^{32} is essential to maintaining envelope homeostasis.

It is provocative that genome-wide analysis indicated that when overproduced, very few cytoplasmic proteins and a disproportionate number of membrane proteins induce the σ^{32} response. Whether this simply reflects an increased propensity for accumulated membrane proteins to misfold or whether a novel mechanism is involved, the fact that σ^{32} is particularly sensitive to imbalances in membrane proteins provides a rationale for utilizing FtsH to degrade σ^{32}. Because FtsH carries out both membrane quality control and degradation of σ^{32}, these two processes can be coordinated, thereby providing the cell with a means to assess membrane homeostasis. Interestingly, the overexpressed membrane proteins that induce the σ^{32}-mediated response also induce the response mediated by the two-component CpxRA system (C. Herman, unpubl.). Moreover, at least two proteins, HtpX and DsbC, are jointly induced by both CpxRA and σ^{32}. Additional evidence that CpxRA senses membrane status has recently been presented by Ito (Shimohata et al. 2002). Thus, σ^{32} and CpxRA may jointly protect the cell membrane during times of stress.

Materials and methods

Medium, strains, and plasmids

M9 medium (Sambrook et al. 1989) was supplemented with 0.2% glucose, 1 mM $MgSO_4$, vitamins, all amino acids (40 μg/mL), ampicillin (100 μg/mL), and/or kanamycin (30 μg/mL) as required and is referred to as M9 complete. Bacterial strains and plasmids used in this study are listed in Table 4.

Table 4. *Bacterial strains and plasmids used in this study*

Strain/plasmid	Relevant genotype	Origin/construction
Bacterial strains		
MG1655	*E. coli* K-12 (MG1655) *rph*-1	Guyer et al. 1981; Jensen 1993; *E. coli* Genetic Stock Center
CAG39145	MG1655 Δ*rpoH*::*kan*, pRpoH-His	This work
CAG45146	MG1655 Δ*lacX74* [Φλ*htpG*P1::*lacZ*]	This work
CAG50001	CAG45146, pTrc99A	This work
CAG50002	CAG45146, pTG2	This work
CAG50003	CAG45146 [Δ*rpoH*::*kan*], pKV1561	CAG45146 + P1/CAG39154
Plasmids		
ASKA library	A complete set of *E. coli* K-12 ORF archive plasmid library: Contains every ORF of *E. coli* K-12 W3110 cloned downstream of the IPTG-inducible *T5/lac* promoter in the vector pCA24N, Cm^R	Kitagawa et al. 2005
pKV1561	pBR322 ori, Ap^R. *lacI*^{q} *lacUV5p-groESgroEL*	Kanemori et al. 1994
pQE-8	His-tagged expression vector, Col E1 ori, Ap^R	
pRpoH-His	*rpoH* cloned on a BamHI–HindIII fragment in pQE-8 downstream of the IPTG-inducible *T5/lac* promoter, Ap^R	This work
pTG2	*rpoH* cloned in p*Trc*99A downstream of the IPTG-inducible *trc* promoter, Ap^R	Liberek et al. 1992
p*Trc*99A	Vector, pBR322 ori, Ap^R. Expression vector containing an IPTG-inducible *trc* promoter	Amersham Pharmacia Biotech

Pulse chase

A 750-µL aliquot of cells was removed from a culture growing exponentially in M9 complete–methionine at 30°C, labeled for 1 min with 80 µC [^{35}S]methionine, chased for 1 min with excess unlabeled methionine, precipitated with 100 µL of ice-cold 50% TCA (15 min on ice), centrifuged, and resuspended in 50 µL of 2% SDS and 50 mM Tris (pH 7.5). Equal counts were loaded in each lane of a 7.5% polyacrylamide gel and bands corresponding to GroEL and DnaK proteins were quantified using the program ImageQuant version 1.2.

Time course microarray experiments

Cells were inoculated to OD_{450} = 0.02 from fresh overnight cultures of CAG50002 (Table 4; vector has IPTG-inducible *rpoH*) or CAG50001 (Table 4; control vector) into 100 mL of M9 complete in 500-mL conical flasks and grown aerobically at 30°C in a gyratory water bath (model G76 from New Brunswick Scientific) shaking at 240 rpm. At OD_{450} = 0.3, cultures were induced with IPTG (1 mM final concentration). Ten-milliliter samples for microarray analysis were removed immediately prior to and at 2.5, 5, 10, and 20 min after induction, added to ice-cold 5% water–saturated phenol in ethanol, and centrifuged at 6600*g*. Cell pellets were flash frozen in liquid N_2 and stored at –80°C. Preparation of labeled probes and microarray procedures were performed exactly as described in Rhodius et al. (2006) and Supplemental Material. Raw and normalized microarray expression data are available on the NCBI Gene Expression Onmibus (GEO) Web site (http://www.ncbi.nih.gov/geo) under the accession code GSE4321.

Additional Materials and Methods are provided in Supplemental Material.

Acknowledgments

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