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Functional Characterization of the csp Homologs of E. coli K-12

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Functional Characterization of the *csp* Homologs of *E. coli* K-12

A Thesis Presented to the Bayer School of Natural and Environmental Sciences
Department of Biological Sciences
Duquesne University

In partial fulfillment of the requirements for the degree of
Masters of Science

By

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ABSTRACT

Functional Characterization of the *csp* Paralogs of *E. coli* K-12 by Christina M. Ventrice

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E. coli K-12 contains nine *csp* paralogs, *cspA* –*cspI*. Although the *csp* paralogs are conserved sequences that produce highly similar proteins, expression of each of these genes requires a different means of induction. While *cspA*, *cspB*, *cspG*, and *cspI* are induced by cold shock, *cspC* and *cspE* are constitutively expressed. *cspD* is induced by stationary phase. The type of induction, if any, needed for *cspF* and *cspH* is not known. Studies of CspE have demonstrated that the overexpression of this protein can confer a 10-fold resistance to the DNA decondensing agent, camphor, and upregulates the *E. coli* *rcaA* gene by 1.7-fold. Phenotypic assays to determine Csp function show that stabilized Csp proteins that are normally degraded by Lon protease (CspC, CspE, CspG, CspI) display resistance to camphor and upregulate the *rcaA* gene when they are expressed from an arabinose inducible vector harbored in a *lon*⁻ strain. Csp proteins that are not stabilized (CspA, Csp B, CspF, and CspH), even in the *lon*⁻ mutant strain, did not display these phenotypes. Csp protein function was further assessed by studying the pattern of Csp protein accumulation across various phases of cell growth. When expression is controlled from an inducible promoter, CspA, CspG, and CspI stability in a *lon*⁻ strain tends to peak during late log and stationary phase. CspC and CspE stability tends to remain the same throughout each of the growth phases observed, while CspB, CspF, and CspH are not present at all. Overall, my work suggests that CspC, CspE, CspG, and CspI possess similar functions, which they each perform under different environmental conditions and at different phases of cell growth.

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INTRODUCTION

The cold shock response

Evolution has provided organisms with the traits necessary to cope with various types of environmental stress. Exposure to heat, cold, change in osmolarity or pH, and nutrient deprivation are several forms of stress. Much of what is known about stress responses has focused on the effects of heat and the production of heat shock proteins. These heat-induced proteins are typically conserved from bacteria to humans, and function as molecular chaperones and proteases that control heat induced damage to proteins (Hendrick and Hartl, 1993; Gottesman, S., *et al.*, 1997). In contrast, less is known about the cold shock response, and the exact functions of cold-induced proteins.

Cold shock has been defined as an abrupt downshift in temperature that results in the induction of cold shock proteins and the repression of heat shock proteins (Jones, P.G., *et al.*, 1987). Many organisms produce a physiological response upon exposure to cold shock. Induction of protein synthesis in response to cold shock has been observed in psychrotrophic yeast like *Trichosporan pullans* (Julseth, C.R. and Imiss, W.E., 1990) and the slime mold, *Dictyostelium discoideum* (Maniak, M. and Nellen, W., 1998). Production of cold shock proteins has also been observed in higher organisms like the plant, *Chorella vulgaris* (Salerno, G.L. and Pontis, H.G., 1988) and even some species of fish (Tiku, P.E., *et al.*, 1996).

Microorganisms respond to a downshift in temperature and for *E.coli* the temperature drop is from 37⁰ C to 10⁰ C. In contrast to heat shock, *E. coli* proteins, membranes, and DNA exposed to cold temperatures become more stable. Consequently,

bacterial cells experience a decrease in membrane fluidity. As a result, protein secretion and active transport are diminished (Nedwell and Rutter, 1994; Wada, H., *et al.*, 1990). DNA and RNA secondary structures become stabilized at low temperatures, and the synthesis of DNA along with most RNA and protein is brought to a halt during the first four hours of exposure to cold temperatures (Ng, H., *et al.*, 1962). These cells resume growth with an extremely slow doubling time of 24 hours (Ng, H., *et al.*, 1962).

Proteins induced by cold shock

In response to a temperature downshift, the synthesis of some proteins is significantly increased. Cold Acclimatization Proteins (CAPs) were first described in *Pseudomonas fragi* (Hebraud, M *et al.*, 1995). CapA and CapB are nucleic acid binding proteins of *P. fragi*, which are induced by an abrupt downshift in temperature and continue to be expressed several hours after exposure to cold temperatures. Both proteins possess approximately 60% sequence identity to the major cold induced protein of *E. coli*, CspA (Hebraud, M. *et al.*, 1997). Cold-induced proteins, which are found in organisms like *E. coli*, *B. subtilis*, and *P. fragi* undergo increased synthesis following an abrupt drop in temperature, but are only expressed transiently (Graumann, P. and Marahiel, M.A., 1996; Hebraud, M. *et al.*, 1997).

E. coli produces a number of cold induced proteins including RecA, H-NS, NusA, initiation factors 2β and 2α , dihydrolipoamide, pyruvate dehydrogenase, CsdA, and RbfA. RecA is induced upon cold shock, and has multiple functions that include: DNA binding, recombination, and DNA repair via the SOS pathway (Walker, G.C.,

1984). H-NS is involved in DNA binding, nucleoid assembly, and regulation of DNA transcription and recombination by altering the chromosomal architecture (Schmid, M.B, 1990). Other cold shock induced proteins include NusA (regulation of transcriptional termination) (Friedman, D.J., *et al.*, 1984), initiation factors 2 β and 2 α (control translation) (Plumbridge, J.A., *et al.*, 1985), DNA gyrase (regulates DNA supercoiling) (Menzel, R. and Gellert, M., 1983), and energy metabolism enzymes like dihydrolipoamide acetyltransferase and pyruvate dehydrogenase (acetyl coA production) (Jones, P.G., *et al.*, 1992). CsdA is a ribosome associated cold shock induced protein involved in RNA unwinding (Jones, P.G., *et al.*, 1987). RbfA is another example of a cold induced ribosomal binding protein (Jones, P.G., *et al.*, 1996).

Table 1. Cold Induced Proteins and their Functions

Cold Induced Protein	Function
CspA	Gene regulation, RNA stability
RecA	DNA recombination and repair
H-NS	Nucleoid assembly and regulation of transcription and translation
NusA	Regulation of transcriptional termination
Initiation factors 2 α and 2 β	Transcriptional control
DNA gyrase	DNA supercoiling
Dihydrolipoamide	Acetyl coA production
Pyruvate dehydrogenase	Acetyl coA production
CsdA	RNA unwinding
RbfA	Modification of 30s ribosome

CspA, the major cold shock protein of *E. coli*, makes up 13.1% of total protein synthesized following a shift from 37⁰ C to 15⁰ C. Increased synthesis occurs within 30 to 60 minutes of the temperature downshift, and *cspA* is only expressed for a short period

(approximately 2 hours) following this decrease in temperature (Goldstein, J., *et al.*, 1990). CspA makes up 8.5% of total cellular protein after a temperature shift from 37⁰ C to 10⁰ C (Goldstein, J., *et al.*, 1990).

CspA, the small 7.4 kDa protein, (Goldstein, J., *et al.*, 1990) has more than fifty orthologs among other prokaryotes (Yamanaka, K., *et al.*, 1998). CspB of *Bacillus subtilis* is a 67 amino acid protein that shares 61% sequence identity with CspA of *E. coli* (Willimsky, G., *et al.*, 1992). Sac7d of *Sulfolobus acidocaldarius* is a small heat stable DNA binding protein, which increases the melting temperature of DNA by approximately 40⁰ C (McAfee, J.G., *et al.*, 1995; Choli, T., *et al.*, 1988; McAfee, J.G., *et al.*, 1996). Sac7d has a tertiary structure very much like that of CspA. It is a β -barrel made up of five β -strands (Edmondson, S.P., *et al.*, 1995) (Figure 1), three of which are important for binding the minor groove of double stranded DNA (Baumann, H., *et al.*, 1994; Robinson, H., *et al.*, 1998). Sac7d creates a sharp kink in the DNA, which results from the intercalation of DNA bases by hydrophobic residues found in β -strands 3, 4 and 5 (McAfee, J.G., *et al.*, 1996).

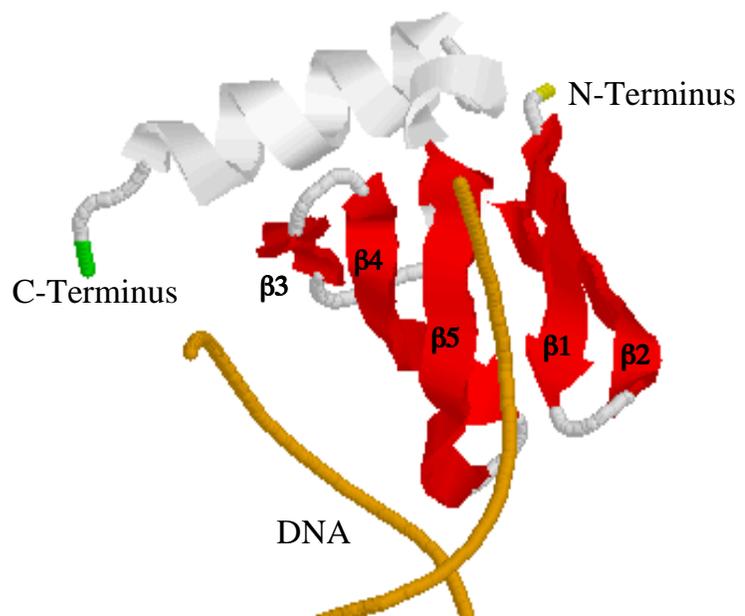


Figure 1. Three dimensional structure of Sac7d of *S. acidocaldarius* bound to DNA (yellow strands). β 1-5 represents the 5 β -strands that form a β -barrel.

Sequence similarities strongly suggests that Csp proteins have been conserved from *E. coli* to man. The cold shock domain of eukaryotic Y-box proteins has 43% sequence identity to CspA (Wolffe, A.P., 1994). As shown in Figure 2, the Y-box proteins contain cold shock (Csp) domains embedded in a larger protein (for review see Wolffe, A.P., 1992). The Y-box cold shock domain is located at the N-terminus of the protein and is required for DNA binding (Tafari, S.R. and Wolffe, A.P., 1992). Y-box proteins have been shown to bind single stranded DNA (ssDNA) and RNA (Koike, K., *et al.*, 1997).

The first Y-box protein to be discovered was human YB-1 (Didier, D.K., *et al.*, 1988), which functions as a transcription factor for MDR-1 (multidrug resistance gene 1), MMP-2 (matrix metalloproteinase 2), PTP-1B (protein tyrosine phosphatase 1B, and MHC class II genes (Ohga, T., *et al.*, 1998; Mertens, P.R., *et al.*, 1997; Fukada, T. & Tonks, N.K., 2003; Didier, D., *et al.*, 1988). The entire YB-1 protein complete with an alanine-proline rich variable domain (N-terminal), cold shock domain, and a C-terminal domain with alternating acidic and basic residues (Wolffe, A.P., 1994; Evdokimova, V.M. and Ovchinnikov, L.P., 1999) binds to the Y-box sequence ($5'$ CCAAT $3'$) within the promoters and enhancers of these genes (Ohga, T., *et al.*, 1998; Mertens, P.R., *et al.*, 1997; Fukada, T. & Tonks, N.K., 2003; Didier, D., *et al.*, 1988). The cold shock domain, alone, does not bind preferentially to the Y-box sequence, but does have an affinity for single-stranded pyrimidine-rich DNA sequences (Klocks, C., *et al.*, 2001). YB-1 has been shown to inhibit the cellular transformation induced by the PI3k (phosphatidylinositol 3-kinase) oncoprotein by interfering with translational events within this pathway (Bader,

A., *et al.*, 2003). AKT, (a Ser/Thr protein kinase) activates the translational regulator, TOR (Ser/Thr kinase), which has effects on cell growth, survival and differentiation (Alessi, D.R. and Downes, C.P., 1998; Wymann, M.P. & Pirola, L., 1998; Scheid, M.P. and Woodgette, J.R., 2001; Brazil, D.P., *et al.*, 2002). YB-1 interferes with the P3K pathway downstream of AKT and may help to prevent human cancers by disrupting the TOR protein (Bader, A. *et al.*, 2003).



Figure 2. Human YB-1 protein with CSD (Cold Shock Domain) located near the amino terminus (N); V represents the variable N-terminal domain. The carboxyl domain (C) is comprised of blocks of acidic and basic residues.

The Csp proteins of *E. coli* K-12

The *cspA* gene is one of nine *csp* paralogs present on the *E. coli* chromosome. The members of this family of small nucleic acid binding proteins are referred to as CspA through CspI (Yamanka, K., *et al.*, 1998). Nine highly conserved sequences are rare in *E. coli*, which contains very few repeated sequences in its genome. Ribosomal genes, of which there are seven copies, are the other major conserved repeat sequences in *E. coli*. *cspA* has been mapped to 80.1 minutes on the *E. coli* chromosome (Figure 3), and is the only one of the nine homologs found in close proximity to the DNA replication origin (84 min). The other eight *csp* homologs are located at various places around the chromosome. *cspE*, *D*, *H*, *G*, *I*, *B*, *F*, and *C*, map to 14.14, 19.90, 22.6, 22.64, 35.35,

35.35, 35.35, and 41.06 minutes respectively (Figure 3). *cspE* is the only *csp* gene that is part of an operon. All of the other *csp*'s have their own predicted promoters (Rudd, K.E., 1998; Yamanaka, K., *et al.*, 1998).

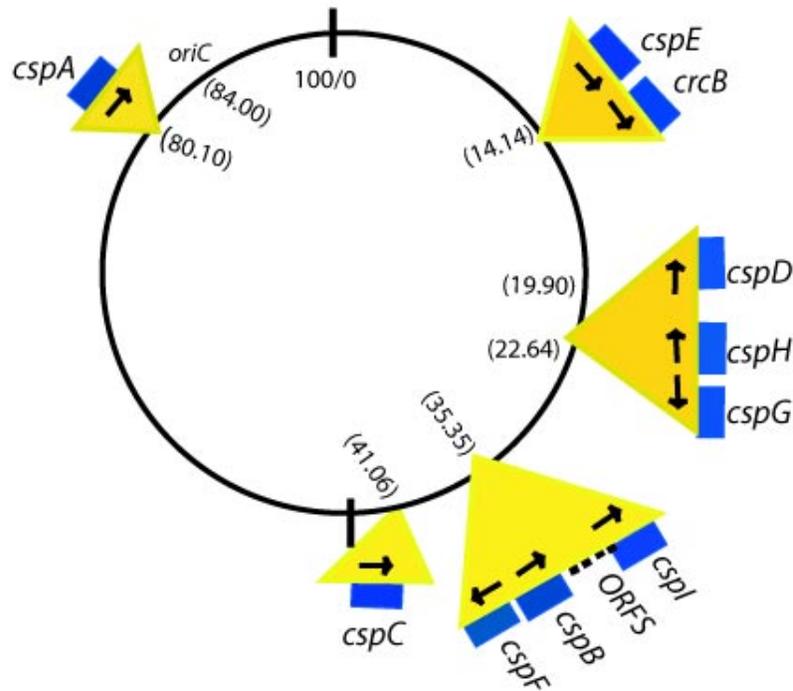


Figure 3. Position of *csp* genes on the *E. coli* chromosome. Numbers inside the circle refer to map position in minutes. Arrows represent the direction of transcription. *oriC* is the origin of replication.

The Csp protein sequences range in size from 69 (CspC and CspE) to 74 (CspD) amino acids and possess identities that range from 83% (CspE and CspC) to 29% (CspD and CspF) (Figure 4 A and B). CspA and B are the second most identical (80%) pair of Csp proteins followed by CspG and I (79%) and finally CspF and H (77%) (Trun, N. and Johnston, D., 2003). Yamanaka *et al.* (1999) have proposed that multiple copies of the *csp* sequence may have resulted from gene duplication.

A

A	MSGKMTGI	VKWFNAD	KGFGFI	TPDDGSKDV	FVHFSAIQ	NDGYKSL	D	EGGKVSFTIE	S	GAK	GPAAGNV	TSL	70	
B	MSNKMTGL	VKWFNAD	KGFGFI	SPVDGSKDV	FVHFSAIQ	NDNYRTL	F	EGGKVTFSE	S	GAK	GPAAAANW	ITD	71	
G	MSNKMTGL	VKWFNAD	KGFGFI	TPDDGSKDV	FVHF	TAIQ	SNEFRTL	N	ENQKVEFSE	Q	QGR	GPAAAANW	TL	70
I	MSNKMTGL	VKWFNPE	KGFGFI	TPKDGSKDV	FVHFSAIQ	SNDFKTL	T	ENGEVEFGE	N	GPK	GPAAVHW	AL	70	
C	MAKIK	QQKWFNES	KGFGFI	TPADGSKDV	FVHFSAIQ	GNGFKTL	A	EGQNVFEI	Q	QCK	GPAAVNV	TAI	69	
E	MSKIT	GNKWFNES	KGFGFI	TPEDGSKDV	FVHFSAIQ	TNGFKTL	A	EGQRVFEI	T	NAK	GPSAANV	AIL	69	
D	MEKGT	VKWFNNA	KGFGFI	CPEGGEDI	F	AHYSTIQ	MDGYRTL	KAGQSVQ	DVH	QPK	GNHASVIVPVEVEA	AV	74	
F	MSRKMTGI	VKTFDGG	SGKGLI	TPSDGRI	DV	QLHVSALNLRDAEEI	TT	GLRVFCRI	N	GLR	GPSAANW	YLS	70	
H	MSRKMTGI	VKTFDRK	SGKGF	TPSDGRKEV	QVH	SATPRDAEV	I	PLRVFCRVN	N	GLR	GPTAANW	YLS	70	

B

	CspA	CspB	CspC	CspD	CspE	CspF	CspG	CspH	CspI	
CspA		80%	69%	49%	70%	44%	73%	47%	70%	% Identity
CspB	84%		64%	50%	69%	43%	77%	47%	70%	
CspC	79%	74%		49%	83%	40%	67%	43%	71%	
CspD	64%	63%	70%		51%	29%	44%	30%	47%	
CspE	81%	77%	91%	74%		46%	67%	49%	73%	
CspF	61%	62%	59%	46%	59%		44%	77%	46%	
CspG	84%	84%	80%	66%	79%	61%		47%	79%	
CspH	57%	63%	62%	46%	60%	87%	59%		47%	
CspI	79%	81%	83%	66%	79%	60%	90%	59%		

% Similarity

Figure 4(A) Csp protein sequence alignment. Red, blue, and black letters represent identical, similar, and different amino acids, respectively (B) Percent identity and similarity of Csp proteins. Circled values represent those Csp proteins that are identical to one another.

CspA

The tertiary structure of CspA has been determined by both X-ray crystallography (Schindelin, H., *et al.*, 1994) and NMR (Newkirk, K., *et al.*, 1994) (Figure 5). The CspA protein is comprised of five antiparallel β -strands linked by turns and loops. Like Sac7d, β -strands 1, 2 and, 3 form one half of the β -barrel, while 4 and 5 form the other side. The first three β -strands contain seven out of eight total aromatic residues of the protein and two RNA binding motifs. These RNA binding domains contain the basic and aromatic amino acids that are characteristic to RNA-binding proteins (Golden, B.L., *et al.*, 1994; Golden, B.L., *et al.*, 1993; Hoffman, D.W., *et al.*, 1994). RNP1 (KGFGF) is an RNA binding domain that is located on β -strand 2, while RNP2 (VFVHF) is on β -strand 3. Aromatic residues (in bold) and basic residues (underlined) are found within RNP1 and RNP2. The aromatic residues are thought to contribute to nucleic acid binding by intercalating between the bases of ssDNA and RNA (Jiang, W., *et al.*, 1997). The CspB protein of *Bacillus subtilis* possesses a crystal structure similar to *E. coli* CspA. Differences between CspB and CspA occur mainly in the loop regions connecting the β -strands, in particular, loops 2 and 3 (Schindelin, H., *et al.*, 1994). Studies have shown that replacing the aromatic residue, phenylalanine (F), with a non-aromatic residue (Alanine, A) within the RNP1 and RNP2 domains of *B. subtilis* CspB results in the loss of nucleic acid binding activity (Schroder, K., *et al.*, 1995).

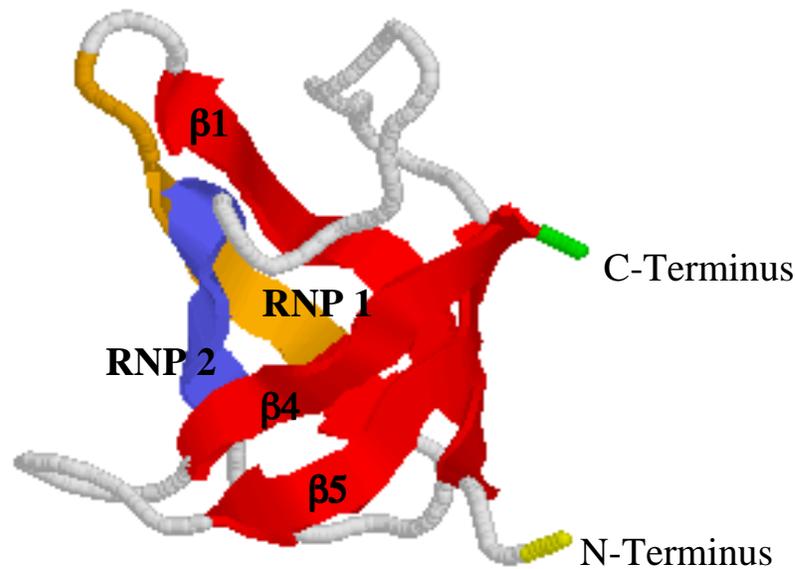


Figure 5. Three dimensional structure of CspA. β 1-5 represents the 5 antiparallel β -strands that form the β -barrel. RNP1 (RNA binding domain) is illustrated in yellow on β -strand 2 and RNP2 is pictured in blue on β -strand 3.

According to the Chou-Fasman method for secondary structure analysis (Chou, PY and Fasman, GD, 1974) all of the Csp proteins should form a β -barrel (Yamanaka, K., 1999). Despite the fact that all of the Csp proteins should possess similar folding patterns, sequence analysis has revealed that CspF and CspH lack the two RNP domains found in the other Csp proteins (Yamanaka, K., *et al.*, 1998). CspF and H also do not possess five (four are located in the RNP domains) of the seven conserved aromatic residues found in the other Csp proteins. This suggests that these Csp proteins are incapable of binding nucleic acids in the same manner as the others (Yamanaka, K., *et al.*, 1998). Moreover, *cspF* and *cspH* genes do not possess ATG start codons and so may not be translated into proteins.

Cold shock induction of *cspA* is controlled at the post-transcriptional level. The *cspA* promoter region contains several unique motifs (Figure 6). The UP (upstream)

element is an AT rich region found just upstream of the -35 box (Goldenberg, D., *et al.*, 1997). This UP element serves as a recognition site for the α -subunit of RNA polymerase (Ross, W., *et al.*, 1993). The *cspA* promoter is active at both 37°C and 15°C , however, Csp protein is not present at 37°C (Mitta, M *et al.*, 1997). Studies have also shown that although transcription of *cspA* is induced 3- to 5- fold after cold shock, there is a 30- to 50- fold increase in *cspA* mRNA with a temperature drop from 37°C to 10°C within the first hour of cold shock (Goldenberg, D., *et al.*, 1996). Thus, this increase in *csp* mRNA could not be attributed to an increase in transcription.

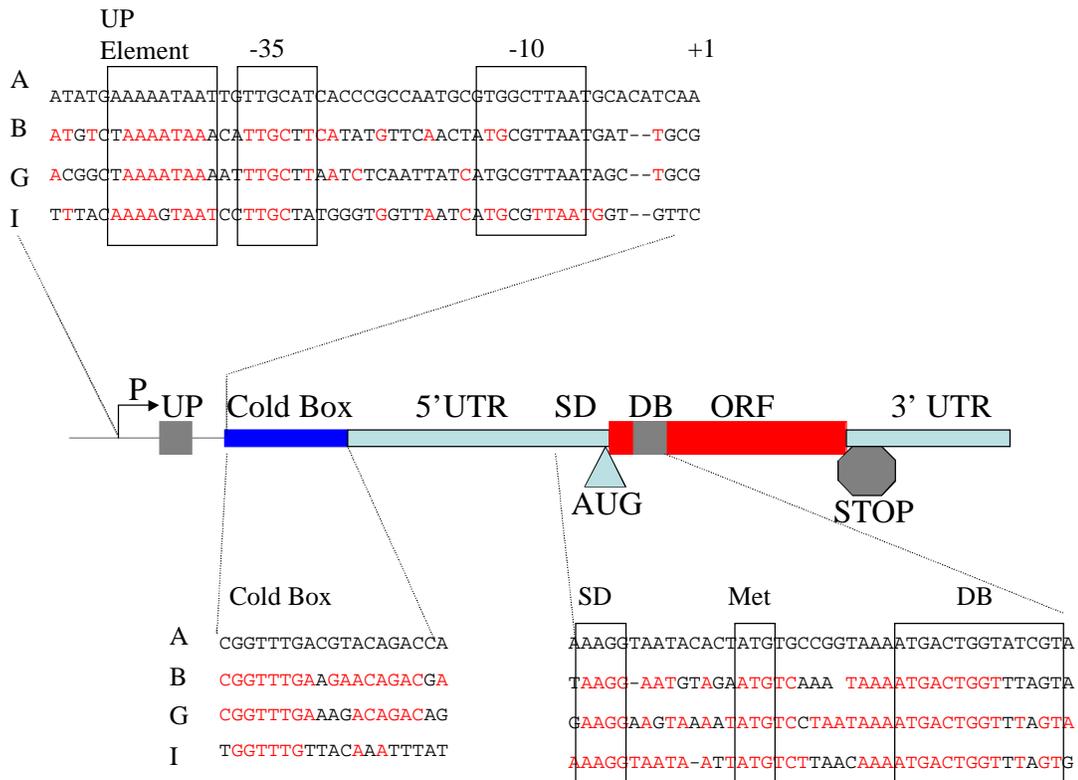


Figure 6. *cspA* DNA with AT rich UP (upstream) element of the promoter region. The -35 and -10 boxes are downstream of the UP element. The cold box is located in the unusually long 5' UTR (untranslated region), which is found upstream of the *cspA* ORF (open reading frame). SD represents the Shine-Delgarno or ribosome binding sequence. The DB (downstream box) is located 12 bases downstream from the ATG site (Met) found at the start of the *cspA* ORF. A 3' UTR is found downstream from the stop codon. Bases identical to the *cspA* sequence are red and different bases are black.

Post-transcriptional stability accounts for most of the increased presence of *cspA* mRNA upon cold shock. *cspA* mRNA is very unstable at 37⁰ C with a half life of 12 seconds. Structural and functional stability of *cspA* mRNA increases at 15⁰ C, when the half life is increased to 20 minutes and translation results in increased presence of the protein (Brandi, A., *et al.*, 1996; Goldenberg, D., *et al.*, 1996; Fang, L., *et al.*, 1997). Increased stability of *cspA* mRNA has been attributed to several factors. Evidence suggests that an unusually long 5' UTR is responsible for *cspA* mRNA instability at 37⁰ C (Brandi, A., *et al.*, 1996; Goldenberg, D., *et al.*, 1996; Fang, L., *et al.*, 1997). The 5'UTR contains an RNase cleavage site just upstream of the Shine-Delgarno sequence, that contributes to the instability of *cspA* mRNA at 37⁰ C. Three base substitution mutations in the putative RNase E binding site of the 5' UTR located immediately upstream of the SD sequence resulted in a 150-fold increase in the stabilization of *cspA* mRNA at 37⁰ C (Fang, L., *et al.*, 1997). Studies have shown that the removal of a region of the 5' UTR causes increased β -galactosidase activity from a *cspA-lacZ* fusion at 37⁰ C (Mitta., *et al.*, 1997).

cspA mRNA stability is not the only factor that contributes to the increased presence of CspA upon temperature downshift. A cold box sequence found in the 5' UTR region of *cspA* mRNA (Fig. 6) regulates its own expression through translation initiation (Jiang, W. *et al.*, 1996a). A downstream box (DB) (Fig. 6) located twelve bases from the translation start site has been found to increase translation efficiency. β -galactosidase activity was six fold greater for strains carrying the DB fused to the *lacZ*

reporter gene as compared to fusions that did not contain the DB sequence (Mitta, M., *et al.*, 1997).

The increased presence of cold shock proteins such as CspA at low temperatures is thought to promote the expression of other genes. For instance, RbfA, a known cold induced protein, modifies the 30S ribosome and stimulates translation of non-cold shock RNAs (Dammel, C.S. and Noller, H.F., 1995). The Cold Shock Ribosome Adaptation Model suggests that as a result of this increase in gene expression, cells adapt to the decline in temperature and the cold shock response is suppressed (Brandi, A., *et al.*, 1996). Studies have shown that temperature sensitive ribosomes control the expression of heat-induced and cold-induced proteins at the translational level (Van Bogelen, R.A and Neidhardt, F.C., 1990). While the decrease in temperature blocks translation initiation of non-cold-induced proteins as evidenced by an increase in 70S monosomes and a decrease in polysomes, ribosomes are still capable of translating the mRNA of cold-induced and ribosomal proteins (Jones, P.G. and Inouye, M., 1996). Presence of the DB sequence in cold-induced genes (*cspA*, *cspB*, *cspG*, *cspI*, *csdA*, *rbfA*, *nusA*, and *pnp*) allows 16S rRNA to bind more readily to mRNA and enhance the translation of cold induced proteins (Mitta, M., *et al.*, 1997). As a result, RbfA binds to ribosomal subunits and modifies them for the translation of non-cold-induced mRNAs, thereby, leading to an adaptation to the cold shock response (Jones, P.G and Inouye, M., 1996).

CspA regulates the expression of both itself and other genes. CspA enhances transcription of at least two known cold shock genes, *hns* (La Teana, A., *et al.*, 1991) and *gyrA* (Jones, P.G., *et al.*, 1992). Both the H-NS and GyrA proteins are associated with DNA binding and DNA structure (Dersch, P., *et al.*, 1994; Sugino, A., *et al.*, 1977). RNA

bound by CspA is ten times more sensitive to RNase activity than free RNA, suggesting that CspA eliminates secondary structure and leaves the RNA vulnerable to enzyme activity (Jiang, W., *et al.*, 1997). CspA has also been shown to negatively regulate its own gene expression by interacting with the 5' UTR cold box region of its mRNA thereby shutting off its cold induced response. Cells containing a *cspA* deletion experienced a 3-fold increase in transcription of the promoter region, which was measured by the amount of 5' UTR mRNA present 30 minutes after cold shock. Moreover, reintroduction of *cspA* into the strain that was mutant for *cspA* resulted in a 50% decline in transcription of the 5' UTR of *cspA* mRNA (Bae, W., *et al.*, 1997).

***csp* paralogs**

CspB and CspG are 71 and 70 amino acid proteins, respectively, and are 77% identical to one another. CspB is 80% identical to CspA, while the CspG protein possesses 73% identity to CspA (Trun, N. and Johnston, D., 2003). Like their closely related paralog, CspA, CspB and G are induced by cold shock, although not to the same extent (Etchegaray, J.P., *et al.*, 1996, Nakashima, K., *et al.*, 1996). Cells containing a *cspG-lacZ* fusion experienced a 2.5 fold increase in β -galactosidase activity upon a shift from 37⁰ C to 15⁰ C during the logarithmic phase (Nakashima, K., *et al.*, 1996). While *cspA* is optimally induced by a shift from 30 to 10⁰ C, *cspB* and *cspG* are produced most efficiently within a more narrow temperature range between 20 and 10⁰ C.(Etchegaray, J.P., *et al.*, 1996, Nakashima, K., *et al.*, 1996). *cspB* and *G* mRNAs also possesses some of the same unique motifs that are characteristic to *cspA* mRNA (Table 1). *cspB* and

cspG mRNAs have a 5' UP (upstream) element along with a cold box containing 5' UTR (Etchegaray, J.P., 1996, Nakashima, K., *et al.*, 1996; Jiang, W., *et al.*, 1996a) (Table 1, Fig. 6), and a DB (downstream box) sequence that improves translation efficiency during cold shock (Mitta, M. *et al.*, 1997 and Etchegaray, J.P. and Inouye, M *et al.*, 1999b) (Table 1, Fig. 6). Like CspA, CspB and CspG may regulate gene expression by serving as cold temperature RNA chaperones (Etchegaray, J.P. and Inouye, M., 1996)

CspI, a 70 amino acid protein, is also a cold shock induced protein (Wang, N., *et al.*, 1999), and is 70 and 79% identical to CspA and G, respectively (Trun, N. and Johnston, D., 2003). Unlike its cold induced homologs (CspA, B and G), CspI is optimally induced between a very narrow temperature range of 15 and 10⁰ C (Wang, N, *et al.*, 1999). *cspI* lacks the same 5' UTR that is present in all of the other *csp* mRNAs; however, it does possess an UP element, cold box, and downstream box like that of its homologs (Yamanaka, K., 1999) (Table 1, Fig. 6). The function of CspI has not yet been determined.

Table 2. Known Features of All *csp* Paralogs

<i>csp</i>	Induction	Regulation	Special Characteristics	Protein Functions
A	Cold shock	Post transcriptional	UP element, Long 5' UTR, DB	Gene regulation
B	Cold shock	Post transcriptional	UP element, Long 5' UTR, DB	RNA chaperone
C	Constitutive	?	?	Chromosome condensation
D	Stationary phase	?	?	?
E	Constitutive	?	?	Chromosome condensation, Gene regulation
F	?	?	?	?
G	Cold shock	Post transcriptional	UP element, Long 5'UTR, DB	RNA chaperone
H	?	?	?	?
I	Cold shock	?	UP, DB	?

CspC and CspE, both 69 amino acids in length, are constitutively expressed at all temperatures (Bae, W., *et al.*, 1999). These Csp proteins are 83% identical to one another. CspC is 69% identical to CspA, while CspE is 70% identical to CspA (Trun, N. and Johnston, D., 2003). Both CspC and CspE are multicopy suppressors of the *mukB106* mutation (Yamanaka, K., *et al.*, 1994), and CspE is a multicopy suppressor of the *mukB* deletion (Hu, K., *et al.*, 1996). MukB is a 177 kDa (Niki, H., *et al.*, 1991) DNA binding protein involved in chromosome partitioning (Niki, H., *et al.*, 1991). Cells with the *mukB106* temperature sensitive mutation, which affects the ATP binding site needed for partitioning of chromosomes, experience a 100-fold increase in the frequency of DNA-less cells. Cells possessing the *mukB106* mutation also experience temperature sensitive colony formation (Niki, H., *et al.*, 1991). The addition of multiple copies of *cspC* or *cspE*, *crcB*, and *crcA* results in a decline in the number of anucleate cells present in a *mukB106* strain (Yamanaka, K., *et al.*, 1994; Hu, K., *et al.*, 1996). *crcA* and *crcB* are open reading frames that flank the *cspE* gene. *cspE* in combination with *crcA* and *crcB* partially suppresses a *mukB103* (mutation in the globular DNA binding domain) as well as a complete *mukB* deletion. Thus overexpression of *cspE* in combination with *crcA* and *crcB* partially compensates for the loss of *mukB* (Hu, K., *et al.*, 1996).

There are several ways that overproduction of CspC and CspE could suppress the nucleoid unfolding that results from a *mukB106* mutation. Evidence suggests that CspE alleviates these effects by causing DNA condensation. Overproduction of *cspE*, *crcA*, and *crcB*, prevents the DNA decondensing effects of camphor (a known DNA decondensing agent) (Hu, K.H., *et al.*, 1996). CspE's ability to confer camphor

resistance, along with its ability to bind nucleic acids (ssDNA, dsDNA, RNA) (Hanna, M and Liu, K, 1998;) suggests that it counteracts the *mukB106* mutation by interacting with the DNA or some other closely associated component (RNA or protein) and causing the *E. coli* chromosome to become more tightly compacted. CspE has also been shown to increase supercoiling of plasmid DNA (Sand, O., *et al.*, 2003).

CspE not only functions to maintain chromosome structure, but it is also involved in regulating the expression of several genes. CspE inhibits Q-mediated transcription antitermination of λ through binding to the P_R ' promoter and blocking access of Q to this region. However, CspE does not increase termination efficiency (Hanna, M. and Liu, K, 1998). CspE reduces CspA expression by 50% by affecting either transcription elongation or termination (Bae, W., *et al.*, 1999). β -galactosidase experiments have shown that over-expression of CspE upregulates the *rcsA* gene by 1.7 fold (Sand, O., *et al.*, 2003).

CspD is a 74 amino acid protein that most closely resembles CspE in sequence (51% identity), but is the least homologous to any of the Csp proteins (Trun, N., and Johnston, D., 2003). *cspD-lacZ* translational fusions have shown that β -galactosidase activity is highest upon onset of stationary phase, suggesting that *cspD* is induced by factors necessary for stationary phase (Yamanaka, K. and Inouye, M., 1997). Although most genes induced by stationary phase require the stationary phase sigma factor (σ^S), *cspD* expression does not (Yamanaka, K. and Inouye, M., 1997). CspD function has not been determined.

CspF and CspH, both 70 amino acid proteins, are 77% identical (Trun, N. and Johnston, D., 2003). Neither the regulation nor the physiological importance of CspF and CspH have been determined (Yamanaka, K., *et al.*, 1998).

Overall goal and rationale

Four of the nine *csp* homologs of *E. coli* are induced by cold shock (CspA, B, G, and I) and one is induced by stationary phase (CspD). CspE and CspC are expressed constitutively. The means of induction for CspF and CspH have not been determined. Each of the Csp proteins are believed to have nucleic acid binding properties. The question of functional similarity between these homologs has not yet been addressed. The overall goal of this project is to determine if Csp proteins have the same, different, or overlapping functions. *csp* genes were cloned and expressed from the inducible *araBAD* promoter in the pBAD24 vector to ensure that each of the Csp proteins would be expressed independently of their natural promoters without the need for stress induction (pers. comm., C. Hall).

Specific aims

I. I will examine the production of mRNA from each of the clones.

Northern blots will be used to study *csp* mRNA expression from each of the *csp* clones. *araC* mRNA will serve as a positive control. Levels of *csp* mRNA will be compared with levels of *araC* mRNA in order to quantitate *csp* mRNA expression from the plasmids.

II. I will examine the production of Csp proteins from each of the clones.

Following analysis of *csp* mRNA, Csp protein expression from the arabinose inducible clones will also be studied. Preliminary studies have shown that Csp protein is not present in wild type cells harboring the *csp* clones (B. Kramer, pers. comm.). It is hypothesized that Csp protein accumulation may be limited due to cell proteases. *E. coli* has four known proteases responsible for the degradation of abnormal cytoplasmic proteins: Lon, ClpAP, ClpQ, and FtsH (Gottesman, S., 1996). Protein expression will be studied in various protease mutants including: *lon*, *clpP*, *clpQ*, and *ftsH*. Csp protein expression will also be monitored from a *lon clpP* double mutant.

III. I will perform phenotypic assays using known phenotypes of CspE in order to functionally characterize the Csp proteins

I will determine if cspE paralogs confer camphor resistance

Phenotypic assays will be performed to elicit the functional role of each of the Csp proteins. The role of Csp proteins in nucleoid structure will be assessed by exposing cells to the DNA decondensing agent, camphor and measuring cell viability.

Overexpression of CspE confers camphor resistance upon cells by preventing DNA decondensation (Hu, K., *et al.*, 1996). The ultimate goal is to determine if the other Csp paralogues also function to compact the DNA. It is hypothesized that any of the Csp proteins with DNA binding capabilities could potentially perform a function similar to

CspE. CspC is 83% identical to CspE, and like CspE, it is also constitutively expressed (Bae, W., *et al.*, 1999). Both CspC and E also suppress *mukB106* temperature sensitive mutants (Yamanaka, K., *et al.*, 1994; Hu, K., *et al.*,). At the very least, it is hypothesized that CspC will play a functional role that will overlap CspE's role in DNA condensation.

I will determine if cspE paralogs regulate the rcaA gene

The effect of Csp proteins on the regulation of the *rcaA* gene, which is important for the regulation of capsule synthesis, will be observed. β -galactosidase assays will be performed. These assays will be carried out on strains harboring *rcaA-lacZ* fusions in combination with Csp expression vectors. Previous studies have shown that CspE upregulates *rcaA* 1.7-fold (Sand, O., *et al.*, 2003), and it is hypothesized that homologs most similar to CspE, like CspC, will have the ability to do the same.

I will determine if cspE paralogs increase supercoiling of plasmid DNA

The DNA supercoiling of each of the Csp proteins will also be assessed by performing chloroquine gels to determine the amount of supercoils present in plasmid DNA. Multiple copies of CspE produce an increase in the number of supercoils in plasmid DNA (Sand, O., *et al.*, 2003). It is hypothesized that all Csps with DNA binding characteristics will supercoil plasmid DNA.

IV. I will monitor protein expression at various growth stages from a *lon* mutant harboring *csp* clones and determine if the pattern of Csp protein expression changes over time

Results obtained from physiological experiments suggest that Csp protein expression is significantly increased during the later stages of cell growth (i.e. late log and stationary phase). Protein expression from each of the *csp* clones will be assessed over different phases of cell growth ranging from early log to late stationary phase. It is hypothesized that the amount of Csp protein in L-arabinose induced samples will be greater during later phases of cell growth as compared to induced samples from earlier stages.

MATERIALS AND METHODS

Bacterial strains and growth conditions

A list of all bacterial strains is provided in Table 2. Overnight cultures were prepared by inoculating 5 ml of LB and incubating at 37°C with aeration. Plasmid containing strains were grown in LB with the appropriate antibiotic. Antibiotics used included: ampicillin (50 µg/ml), chloramphenicol (25 µg/ml), kanamycin (25 µg/ml), and tetracycline (25 µg/ml).

Table 3. Strains

Strain Name ^a	Genotype	Source or Reference
MC4100	F ⁻ <i>araD139</i> Δ(<i>argF-lac</i>) <i>U160 rpsL150 relA1 flB5301 deoC1 ptsF25 rbsR</i>	Silhavy, T.J. <i>et al.</i> , 1984
NT610	MC4100 Δ <i>ara174 leu::Tn10</i>	N. Trun
NT610 (pBAD24)	Vector plasmid	“
NT610 (pCH20)	Vector containing <i>cspA</i>	“
NT610 (pCH21)	Vector containing <i>cspB</i>	“
NT610 (pCH22)	Vector containing <i>cspC</i>	“
NT610 (pCH24)	Vector containing <i>cspE</i>	“
NT610 (pCH25)	Vector containing <i>cspG</i>	“
NT610 (pCH26)	Vector containing <i>cspI</i>	“
NT610 (pCH27)	Vector containing <i>cspH</i>	“
NT610 (pCH28)	Vector containing <i>cspF</i>	“
BK1	NT610 <i>ftsHts</i>	B. Kramer
BK1 (pBAD24)	Vector plasmid	“
BK1 (pCH20)	Vector containing <i>cspA</i>	“
BK1 (pCH21)	Vector containing <i>cspB</i>	“
BK1 (pCH22)	Vector containing <i>cspC</i>	“
BK1 (pCH24)	Vector containing <i>cspE</i>	“

BK1 (pCH25)	Vector containing <i>cspG</i>	“
BK1 (pCH26)	Vector containing <i>cspI</i>	“
BK1 (pCH27)	Vector containing <i>cspH</i>	“
BK1 (pCH28)	Vector containing <i>cspF</i>	“
NT785	MC4100 Δ <i>ara174</i> <i>rscC::kan lon146::mtet</i>	N. Trun
NT785 (pBAD24)	Vector plasmid	“
NT785 (pCH20)	Vector containing <i>cspA</i>	“
NT785 (pCH21)	Vector containing <i>cspB</i>	“
NT785 (pCH22)	Vector containing <i>cspC</i>	“
NT785 (pCH24)	Vector containing <i>cspE</i>	“
NT785 (pCH25)	Vector containing <i>cspG</i>	“
NT785 (pCH26)	Vector containing <i>cspI</i>	“
NT785 (pCH27)	Vector containing <i>cspH</i>	“
NT785 (pCH28)	Vector containing <i>cspF</i>	“
CV1	NT785 (pBR322)	This study
CV2	NT785 (pNT2)	This study
SG20780	MC4100 <i>cspB10::lac (iλ)</i> <i>lon</i> Δ 510pst	S. Gottesman
NT584	<i>leu::Tn10</i> Δ <i>ara174</i>	N. Trun
NT580	Δ <i>ara174</i>	N. Trun
CV3	MC4100 <i>lon</i> Δ 510pst	This study
CV4	MC4100 <i>lon</i> Δ 510pst <i>leu::Tn10</i> Δ <i>ara174</i>	This study
CV5	MC4100 <i>lon</i> Δ 510pst Δ <i>ara174</i>	This study
CV6	MC4100 <i>lon</i> Δ 510pst Δ <i>ara174 rscC::kan</i>	This study
GP150	AB1157 <i>sbC201 recB21</i> <i>recC22 sbcB15</i> (<i>F</i> <i>thr-1 ara14 leuB6</i> Δ (<i>gpt-proA</i>)62 <i>tsx-33</i> <i>supE44 galK2 nisG4(oc)</i> <i>rfbD1 mgl51 rpsL31</i> <i>kdgK51 xyl-5 mtl-1 argE3</i> <i>thi-1 λ rac</i>) <i>recA::kan</i>	G. Phillips
CV7	MC4100 <i>lon</i> Δ 510pst Δ <i>ara174 rscC::kan</i> <i>mukB106 Tn10</i>	This study
CV8	GP150 (p226) <i>recA::kan</i>	This study
CV9	NT580 Δ <i>ara174 leu</i> ⁺ <i>lon146::mtet</i>	This study
CV10	NT580 Δ <i>ara174 leu</i> ⁺	This study

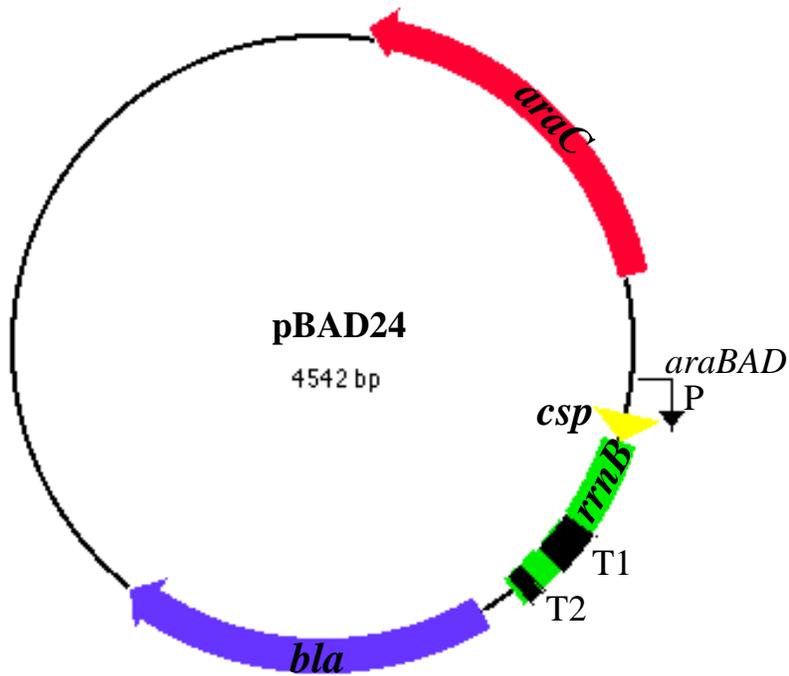
	<i>lon146::mtet recA::kan</i>	
GC7470 (P1CM)	<i>dadR trpE61 trpA62 tna-5 mukB106zcb::Tn10</i>	G. Phillips
MB100	MC4100 <i>ara</i> ⁺ <i>leuABCD::Tn10</i>	Silhavy, T.J., <i>et al.</i> , 1984
OS488	NT614 <i>λrcsA-lacZ</i>	O. Sand
CV12	OS488 <i>rcsC::kan</i>	This study
CV13	OS488 <i>rcsC::kan</i> <i>lon146::mtet</i>	This study
IF1	CV13 (pNT2)	I. Ferguson
IF2	CV13 (pBR322)	“
IF3	CV13 (pBAD24)	“
IF4	CV13 (pCH20)	“
IF5	CV13 (pCH21)	“
IF6	CV13 (pCH22)	“
IF7	CV13 (pCH24)	“
IF8	CV13 (pCH28)	“
IF9	CV13 (pCH25)	“
IF10	CV13 (pCH27)	“
IF11	CV13 (pCH26)	“

^a All strains are *Escherichia coli* K-12

Table 4. Plasmids

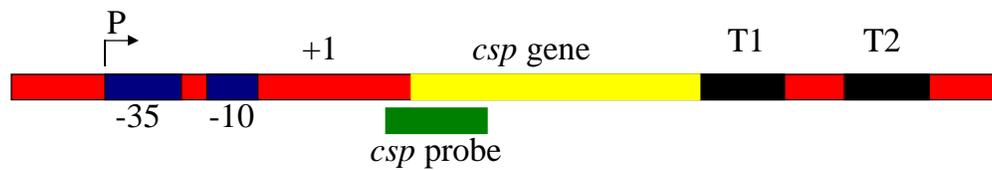
Plasmid	Description	Source or Reference
pBAD24	Arabinose inducible expression vector; derived from pDHB60; <i>bla</i>	Guzman, L-M. <i>et al.</i> , 1995 (Figure 7)
pCH20	pBAD24 with <i>cspA</i>	C. Hall
pCH21	pBAD24 with <i>cspB</i>	C. Hall
pCH22	pBAD24 with <i>cspC</i>	C. Hall
pCH24	pBAD24 with <i>cspE</i>	C. Hall
pCH25	pBAD24 with <i>cspG</i>	C. Hall
pCH26	pBAD24 with <i>cspI</i>	C. Hall
pCH27	pBAD24 with <i>cspH</i>	C. Hall
pCH28	pBAD24 with <i>cspF</i>	C. Hall
pNT2	pBR322 with chromosomal fragment containing <i>crcA cspE</i> and <i>crcB</i>	Hu, K <i>et al.</i> , 1996
pBR322	ColE1- derived plasmid	Bolivar & Rodriguez, 1977

A



B

pBAD24 DNA with *csp* *araBAD* promoter region



csp mRNA

SD ATG

Message 1

Message 2

Figure 7. (A) A diagram of the 4,452 bp pBAD24 expression vector with arabinose inducible promoter, *araBAD*, and *rrnB* terminators, T1 and T2. pBAD24 carries the *araC* gene, which encodes an inducer that binds to the *araBAD* promoter in the presence of arabinose. Ampicillin resistance is provided by the *bla* gene (B) Enlargement of *csp* promoter region with MCS (multiple cloning site) showing position of *csp* probe used for mRNA detection.

Plasmid DNA was harvested using Wizard Miniprep Kits (Promega, Madison, WI). Plasmids were transformed into *E. coli* by electroporation (Bullock, W., *et al.*, 1987; Hanahan, D., 1985). Cells were made competent for electroporation by subculturing an overnight culture into LB at a ratio of between 1:20-1:50 and growing at 37°C with aeration until an OD₆₀₀ 0.8-1.0 was reached. Cells were chilled on ice for 15 minutes. Cells were harvested by centrifugation at 10, 400 x g for 15 minutes at 4°C. The pellet was washed twice with 4°C sterile water and once with 4°C filter sterilized 10% glycerol before being resuspended in 10% glycerol. Electrocompetent cells were stored at -80°C. Prior to electroporation, competent cells were thawed on ice and were mixed with plasmid DNA. The mixture was transferred to a chilled 0.1 cm electroporation cuvette. The cuvette was placed in a Gene Pulser™ electroporator (BioRad, Hercules, CA) and 2.5 kV of electricity was administered. Immediately after electroporating the cells, 1 ml of sterile SOC media (20 g/L Bacto-tryptone, 5 g/L Bacto-yeast extract, 8 mM NaCl, 10 mM MgCl₂, 2.5 mM KCl, 20 mM glucose) (Sambrook, J. *et al.*, 1989) at room temperature was added. The mixture was transferred to a sterile Wasserman tube and incubated at 37°C with aeration for 1 hour. Cells were pelleted by centrifugation at 3,400 x g. The pellet was resuspended in 100 µl of SOC, plated on LB agar containing 50 µg/ml of ampicillin, and plates were incubated overnight at 37°C. Transformants were purified on LB-Amp agar.

Expression of Csp proteins in an *ftsH* mutant

Growth Conditions

Cells with the *ftsH* temperature sensitive mutation (BK1) containing individual *csp* clones were grown in LB with ampicillin at 30⁰C. Overnight cultures were diluted 1:20 into 10 ml of LB-Amp (uninduced) and LB-Amp supplemented with 0.25% L-arabinose (induced) and grown at 30⁰C with shaking to an OD₆₀₀ 0.1-0.2. Induced and uninduced cultures were shifted to 42⁰C with shaking for 4 hours.

Protein Precipitation

After induction, proteins were precipitated by mixing 50 µl of 100% trichloroacetic acid (TCA) with 1 ml of cells and incubating on ice for 10 minutes. Samples were centrifuged at 13, 500 x g in a microcentrifuge for 2 minutes and the pellet was resuspended in 1 ml of -20⁰C acetone. The sample was centrifuged for 2 minutes, the supernatant was removed, and the pellet was dried for 30 minutes at room temperature. The dry pellet was resuspended in 50 µl of 10mM Tris pH 7.5. A Bradford assay was used to determine protein concentration (BioRad, Hercules, CA).

SDS-PAGE gels

Proteins were separated on 10% Bis-Tris precast denaturing Nu-PAGE gels (Invitrogen, Carlsbad, CA). Samples were combined with 25 µl of 4X sample buffer (Invitrogen, Carlsbad, CA) and incubated for 5 minutes at 65⁰C. Each lane was loaded with 3 µg of total protein. Samples were electrophoresed in 1X MES (2-(N-morpholino

ethanesulfonic acid) buffer (Invitrogen, Carlsbad, CA) at 150 V for 45 minutes. Gels were stained in 30 ml of Microwave Blue using the manufacturer's protocol (Protiga, Frederick, MD) and were destained in 50 ml of distilled water. They were placed in 50 ml of 15% glycerol for 20 minutes and air-dried.

Determination of mRNA levels

RNA extraction

All glass wear and solutions were treated with 0.1% diethyl pyrocarbonate (DEPC) for 1 hour and autoclaved to eliminate nucleases. The electrophoresis tank was washed and rinsed with ethanol before it was soaked in 3% H₂O₂ for 10 minutes. The tank was rinsed with DEPC treated water (Sambrook, J. *et al.*, 1989). RNA was extracted from NT610 cells harboring each of the *csp*-containing plasmids with Trizol reagent (GibcoBRL, Grand Island, NY) according to the manufacturer's instructions. RNA was quantified spectrophotometrically at OD₂₆₀.

Sample preparation

Sample preparation was modified from that described (Sambrook, J. *et al.*, 1989). The following reagents were mixed in a sterile microcentrifuge tube: 2 µl of 5x formaldehyde gel running buffer (0.1 M MOPS pH7.0, 40 mM sodium acetate, 5 mM EDTA pH 8.0), 3.5 µl formaldehyde, 10 µl formamide, and sample. The mixture was incubated at 65°C for 15 minutes and chilled on ice before centrifuging at 13,500 x g for 5 seconds. 2 µl of gel loading dye (500 µl glycerol, 2 µl 0.5 M EDTA, 493 µl DEPC

treated water, 2.5 µl bromphenol blue (1 mg/ml), 2.5 µl xylene cyanol (1 mg/ml)) was added to each tube. The RNA 0.1-1 kb standard (Sigma, St Louis, MO) was prepared by adding 2 µl of gel loading dye before heating and chilling.

Electrophoresis on a 2% agarose formaldehyde gel

Agarose formaldehyde gels were run as described (Sambrook, J *et al.*, 1989), with several modifications. Ethidium bromide was added directly to the 2% agarose formaldehyde gel and 1X formaldehyde gel running buffer to a final concentration of 10 µg/ml. Each lane contained 10 µg of total RNA. The gel was run in 1X formaldehyde gel running buffer for 5 hours at 40 V with buffer recirculation. Following electrophoresis, the gel was photographed and soaked in 0.05 N NaOH for 20 minutes. Subsequently, the gel was rinsed in DEPC treated water and soaked in 25mM sodium phosphate pH 6.4 transfer buffer for 45 minutes (Amersham Pharmacia Biotech, Piscataway, NJ).

Blotting procedure

mRNA was transferred to a Hybond-XL nylon membrane (Amersham Pharmacia Biotech, Piscataway, NJ) via capillary action. Sodium phosphate pH 6.4 was used as the transfer buffer (Virca, G.D. *et al.*, 1990). Transfer of RNA to the membrane proceeded for 16-24 hours. After removal from the blotting apparatus, the membrane was dried for 30 minutes on 3 MM paper and UV-crosslinked in a FB-UVXL-1000 UV-crosslinker (Fisher, Pittsburgh, PA) at 70,000 µJ/cm² (Amersham Pharmacia Biotech, Piscataway, NJ).

Probe labeling

The 18mer and 25mer probes were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA) and were identical to the template (sense) strands of nucleotides 1298 through 1315 of the vector araBAD promoter and part of the *csp* gene (5' AAT TCC TCC TGC TAG CCC^{3'}) and 617 through 641 for *araC* (5' AGA ACC CCG TAT TGG CAA ATA TTG A^{3'}). Probes were heated at 95 °C for 5 minutes to eliminate hairpins and primer dimers before being 5' end labeled with [γ -³²P] ATP. Reaction mixtures containing 1 μ l probe, 1 μ l T₄ polynucleotide kinase, 5 μ l 1X T₄ polynucleotide kinase buffer (70 mM Tris-HCl pH 7.6, 10 mM MgCl₂, 5 mM dithiothreitol) 40 μ l water, and 3 μ l [γ -³²P] ATP were incubated for 30 minutes at 37°C. Following incubation, the reaction mixture was passed through a GS-50 column (Amersham Pharmacia Biotech, Piscataway, NJ) to remove any unincorporated [³²P-ATP]. Probe activity was determined with a TriCarb 2100TR scintillation counter (Perkin Elmer, Boston, MA). Probes were heated to 95°C for 5 minutes and immediately placed on ice.

Prehybridization and hybridization

Blots were placed into Seal-O-Meal bags with 10 ml of hybridization buffer (50 mM PIPES, 100 mM NaCl, 50 mM Na₂PO₄, 1 mM EDTA, 5% SDS) and were prehybridized for 15 minutes in a water bath at 50°C. Fresh hybridization buffer containing 10⁶ cpm/ml of the appropriate probe was added and blots were hybridized overnight at 50°C (Virca, G.D. *et al.*, 1990).

Washes and imaging

Blots were rinsed in 50 ml of 5% SDS in 1X SSC (3 M NaCl, 0.3 M C₆H₅Na₃O₇) at room temperature for 5 minutes. They were washed with 400 ml of 5% SDS in 1X SSC at 50⁰C for 20 minutes (Virca, G.D. *et al.*, 1990). After washing, blots were wrapped in plastic wrap and placed on a Biorad GS-525 phosphoimager sample loading dock to expose for 24 to 72 hours. Blots were analyzed and quantitated with a GS-525 Molecular Imager^R System phosphoimager (BioRad, Hercules, CA) and Molecular Analyst^R software (BioRad, Hercules, CA).

Physiological assays

Camphor resistance assays

Overnight cultures of NT785 strains containing each of the *csp*-containing plasmids were subcultured 1:100 into 125 ml flasks containing LB-Amp or LB-Amp with 0.05% L-arabinose and grown at 37⁰C with aeration to an OD₆₀₀ of approximately 0.15. A 1.5 ml sample was removed and 0.15 g of camphor was added to each flask. Samples were removed every 30 minutes for 120 minutes and were diluted into LB-Amp over a range of 1x10⁻¹ to 1x10⁻⁷. Cells were plated on LB-Amp plates. Plates with between 30 and 300 colonies were counted. Cell viability was assessed by using the following formula:

$$\text{Cell Viability at } t_x = (\# \text{ cells at } t_x / \# \text{ cells at } t_0).$$

β-galactosidase assays

β-galactosidase was assayed as described (Miller, J.H., 1992) with several modifications. Overnight cultures of IF1 through IF11 were grown in LB-Amp and subcultured 1:50 into 25 ml of LB-Amp until an OD₆₀₀ = 0.3 was reached. Samples were again subcultured 1:50 into LB-Amp (uninduced) and LB-Amp with 0.25% L-arabinose (induced). 1.5 ml samples were removed immediately after inoculation (lag phase) and at an OD₆₀₀ = 0.4- 0.6 (mid-log) and 0.7-1.0 (late log). The samples were incubated on ice for 20 minutes, and absorbance was measured at OD₆₀₀. Samples (0.5 ml) were combined with 0.5 ml of Z- buffer (60 mM Na₂HPO₄, 46 mM NaH₂PO₄, 10 mM KCl, 2.0 mM MgSO₄, 0.27% β-mercaptoethanol), 1 drop of 0.1% SDS, and 4 drops of chloroform. Samples were placed in a 28⁰ C water bath for 5 minutes before adding 0.2 ml of a 4 mg/ml solution of ONPG (o-nitrophenyl-β-D-galactopyranoside). After the reaction turned yellow, 0.5 ml of 1M Na₂CO₃ was added and the time for the ONPG reaction was recorded. The absorbance at OD₄₂₀ and OD₅₅₀ was recorded. Units of β-gal were determined with the following equation:

$$\beta\text{-Gal units} = 1000 \times \text{OD}_{420} - 1.75 (\text{OD}_{550}) / (t) (v) (\text{OD}_{600})$$

t = time of reaction (minutes), v = volume of culture for assay (ml)

DNA supercoiling

Growth conditions and plasmid DNA extraction

Overnight cultures of CV10 containing each of the *csp* plasmids were grown in LB-Amp. The cultures were diluted 1:200 in 100 ml of LB-Amp (uninduced) or LB-Amp with 0.25% L-arabinose (induced) and grown to an $OD_{600}=1$. Plasmid DNA was extracted from 100 ml of cells with a QiafilterTM plasmid maxi kit (Qiagen, Valencia, CA) according to the manufacture's instructions.

Electrophoresis on chloroquine gels

After adding 6x gel loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF, 30% glycerol in water) to each sample, 500 µg of plasmid DNA was electrophoresed on a 0.8% agarose chloroquine (10 µg/ml) gel at 4⁰ C. The samples were electrophoresed at 23 V in 0.5 x TPE (1 x: 0.09 M Tris-phosphate 0.002 M EDTA) for 72 hours with buffer recirculation at 24.5 V. The gel was washed four times in nanopure water with shaking for 1 hour. The gel was stained with 0.5 µg/ml ethidium bromide in nanopure water for 30 minutes and photographed. Less supercoiled DNA binds more chloroquine and migrates slower in 0.8% agarose gel, and is present in bands closer to the top of the gel. More supercoiled DNA binds less chloroquine and migrates faster, and is found closer to the bottom portion of the gel.

Monitoring Csp protein expression at various stages of cell growth in a *lon* mutant

Overnight cultures of NT785 (*lon*⁻) cells harboring individual *csp* clones were subcultured 1:50 into LB with ampicillin and LB with ampicillin supplemented with 0.25% L-arabinose. Samples (1 ml) were removed at OD_{600} 0.3, 0.6, and 1.0. Growth of

the cultures was continued overnight and 1 ml samples were removed. All samples were TCA precipitated and quantified using the Bradford assay as described previously. Proteins were separated using SDS-polyacrylamide gel electrophoresis as before. Gels were quantitated densitometrically using NIH Image 1.62 software.

RESULTS

While the regulation of the *csp* genes has been studied, little is known about their function(s). It is possible that while they are regulated differently, they have similar functions. It is equally possible that they have very different functions. In order to determine if Csp proteins functions are the same, different, or overlapping, each of the *csp* genes was cloned into a pBAD24 expression vector (Figure 7). The *csp* genes were expressed from the arabinose inducible promoter, *araBAD*, so that their wildtype regulation patterns were removed and their functions could be assessed.

Several different phenotypes for overexpression of *cspE* have been described. Over-expression of *cspE* has been shown to confer camphor resistance, and prevent the DNA decondensing effects of this compound (Hu, K., *et al.*, 1996). *cspE* has also been shown to upregulate expression of the *rcsA* gene, and to increase the number of supercoils in plasmid DNA (Sand, O., *et al.*, 2003). According to Yamanaka *et al.* and Hu *et al.*, *cspE* is a multicopy suppressor of the *mukB06* chromosomal partitioning mutant (1994). I will determine if overexpression of *cspA*, *cspB*, *cspC*, *cspF*, *cspG*, *cspH*, or *cspI* have the same phenotypes as overexpression of *cspE*. To ensure that lack

of phenotype is not merely the result of lack of production of the Csp protein, I will test each clone for mRNA production and protein expression.

Northern blots show that *cspA, B, C, E, G, and I* clones produce mRNA and *cspF* and *H* clones do not

Northern blots were performed in order to determine if *csp* mRNA is made from *csp* clones induced with arabinose. Blots of arabinose induced and uninduced samples electrophoresed on 2% agarose formaldehyde gels were hybridized with probes complementary to *araC* and *csp* mRNA. A general *csp* probe containing a complementary portion of DNA from the pBAD24 vector was designed to detect all of the *csp* mRNA transcripts produced from the arabinose inducible clones. NT610 is deleted for *araC*, so any detected *araC* gene expression was solely from the plasmid. The vector derived *araC* is not under the control of an arabinose inducible promoter, and as such *araC* mRNA was detected in both induced as well as uninduced samples of NT610 (Figure 8A, marked with an arrow). As shown in Figure 8B, *cspA*, *cspB*, *cspC*, *cspE*, *cspG*, and *cspI* mRNAs (900 base band marked with an asterisk) are present in arabinose induced NT610 strains harboring the respective *csp* clones. The ratio of *csp* mRNA as compared to the mRNA of the *araC* control (Table 4) shows that *cspF* and *cspH* mRNA were not present.

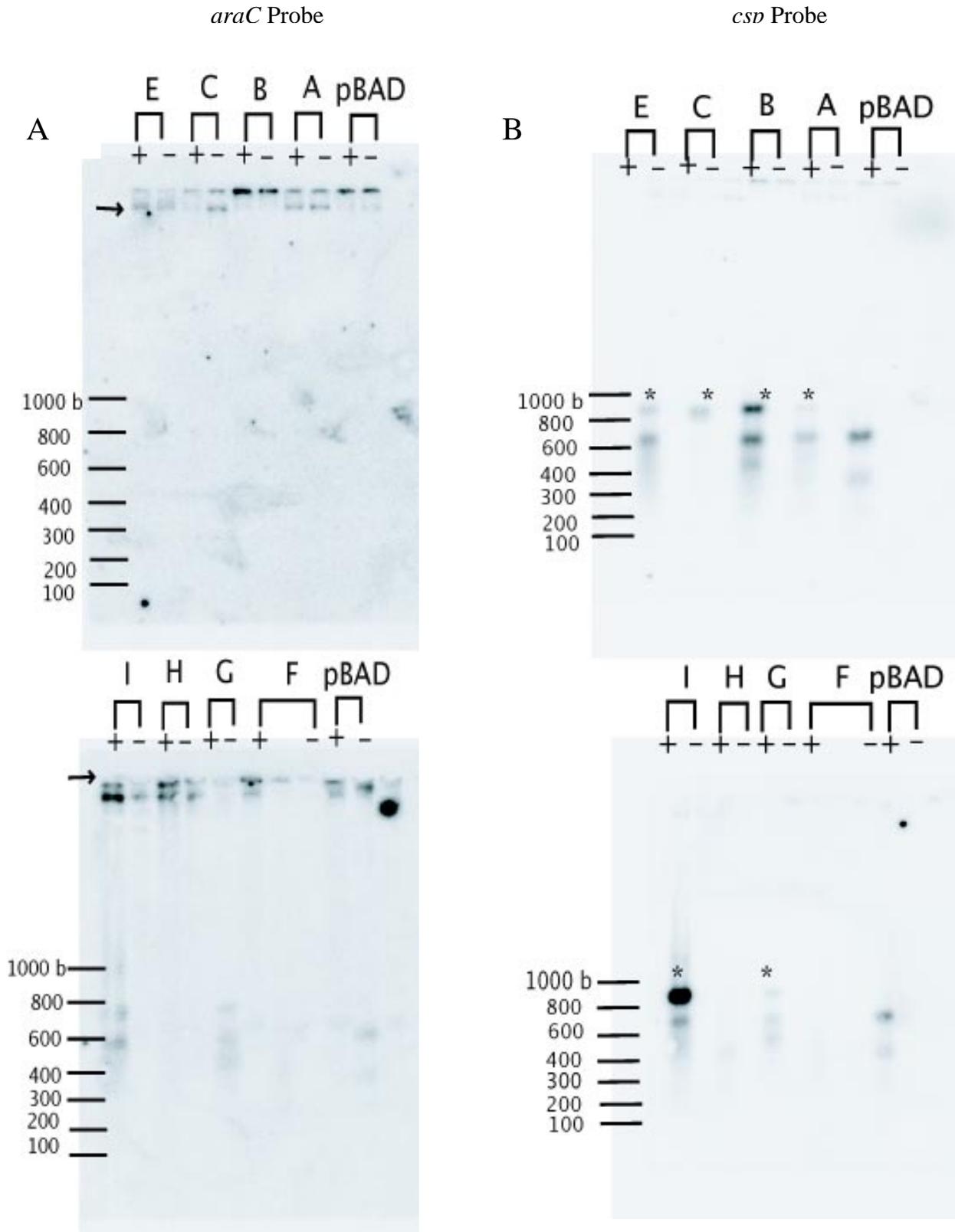


Figure 8. mRNA levels detected by northern blot with (A) *araC* and (B) *csp* probes. Numbers represent the size of each RNA transcript in bases. Both uninduced (-) and induced (+) samples were tested. Notice that *araC* mRNA was present in both arabinose induced and uninduced samples of NT610 (shown by arrows). *cspC*, *E*, *G* and *I* mRNAs, denoted by the asterisks, were present only in arabinose induced samples of NT610.

These *csp* mRNAs are significantly larger than their expected size of approximately 500 bases. Expected transcript sizes were determined by counting the total number of bases between the transcription start site of each *csp* gene and the bases at which transcription stops within the *rrnB* terminators. Two different sizes were obtained for each *csp* transcript, since termination could have occurred in either of two *rrnB* termination sites (t1 or t2). However, termination did not occur in either of these sites, but rather, transcription continued into the *bla* gene. *csp* mRNA was not present in arabinose induced samples with the pCH28 (*cspF*), pCH27 (*cspH*), and pBAD24 expression vectors. However, several smaller mRNAs (600 b and 350 b) bound by the *csp* probe were found in arabinose induced NT610 (pBAD24) samples.

Table 5. *csp* mRNA Expression

<i>csp</i>	Ratio of <i>csp</i> mRNA to <i>araC</i> mRNA	Expected Size of <i>csp</i> mRNA from +1 start site to termination sites within pBAD24 (bases)	Approx. Size of <i>csp</i> mRNA (bases) from northern blots
pBAD24	0	N/A	N/A
<i>cspA</i>	0.7	487-512	900
<i>cspB</i>	3.9	490-512	900
<i>cspC</i>	5.4	485-510	900
<i>cspE</i>	1.6	484-509	900
<i>cspF</i>	0	476-501	N/A
<i>cspG</i>	3.0	487-512	900
<i>cspH</i>	0	475-500	N/A
<i>cspI</i>	13.5	488-513	900

CspC, E, G, and I proteins were detected in *lon*⁻ mutants

Protein extracts prepared from both arabinose induced and uninduced samples of NT610 were electrophoresed on 10% Bis-Tris Nu-PAGE gels (Invitrogen, Carlsbad, CA) in order to determine if Csp protein is made by constructs that also produce stable *csp* mRNA. As shown in Figure 9A, there were no approximately 7 kDa Csp proteins present in induced samples of NT610 containing *csp* expression vectors. Since northern blots showed that *cspA*, *cspC*, *cspE*, *cspG*, and *cspI* mRNAs were produced from their respective expression vectors, it was suspected that cell proteases were degrading these proteins.

E. coli K-12 has several known multisubunit proteases responsible for degrading misfolded or abnormal proteins (Gottesman, S, 1996). Lon is one of the primary proteases responsible for the degradation of abnormal proteins. Lon protease is comprised of multiple identical subunits (Goldberg, A.A. *et al.*, 1994), each of which possesses an ATP-binding site and a protease site (Amerik, A.Y. *et al.*, 1991; Chin, D.T. *et al.*, 1988; Fisher, H. *et al.*, 1993). ClpXP and ClpAP are both comprised of two subunits. ClpP possesses protease activity (Katayama, Y. *et al.*, 1988; Woo, K.M. *et al.*, 1989), while ClpX or ClpA serve as the ATP-binding subunit (Krukltis, R. *et al.*, 1996; Levchenko, I. *et al.*, 1995; Wawrzynow, A. *et al.*, 1995). FtsH is the only essential protease in *E. coli*. FtsH is a membrane protein (Tomoyasu, T. *et al.*, 1993) that is responsible for the degradation of both cytoplasmic and membrane proteins (Herman, C. *et al.*, 1995; Kihara, A. *et al.*, 1995). ClpQY was the last of the four energy dependent proteases identified. The ClpQ subunit is thought to possess protease activity, while

ClpY is highly homologous to ClpX and believed to be the ATPase subunit (Gottesman, S. *et al.*, 1993).

lon, *clpP*, *clpQ*, and *ftsHts* protease mutants were obtained (S. Gottesman, pers. comm.) transformed with the *csp* clones, and protein expression was assessed. Csp proteins were not present in protein extracts prepared from arabinose induced strains with mutations in either *clp XP*, *clp QN*, or *ftsH* (Figure 9B, C, and D). FtsH, Clp XP, and Clp QN do not single handedly degrade the Csp proteins. CspC, G, E and I proteins were present in samples obtained from arabinose induced *lon* protease mutants containing each of the respective *csp* clones (Figure 9E). Lon protease is responsible for the degradation of these specific Csp proteins. A *lon clpP* double mutant was designed to determine if these proteases work in conjunction with one another. As shown in Figure 9F, CspC, CspE, CspG, and CspI proteins were present in extracts derived from arabinose induced *lon clpP* double mutants. This in combination with the data obtained from the single *lon* mutant suggests that the Lon protease does not require ClpQN to degrade these specific Csp proteins.

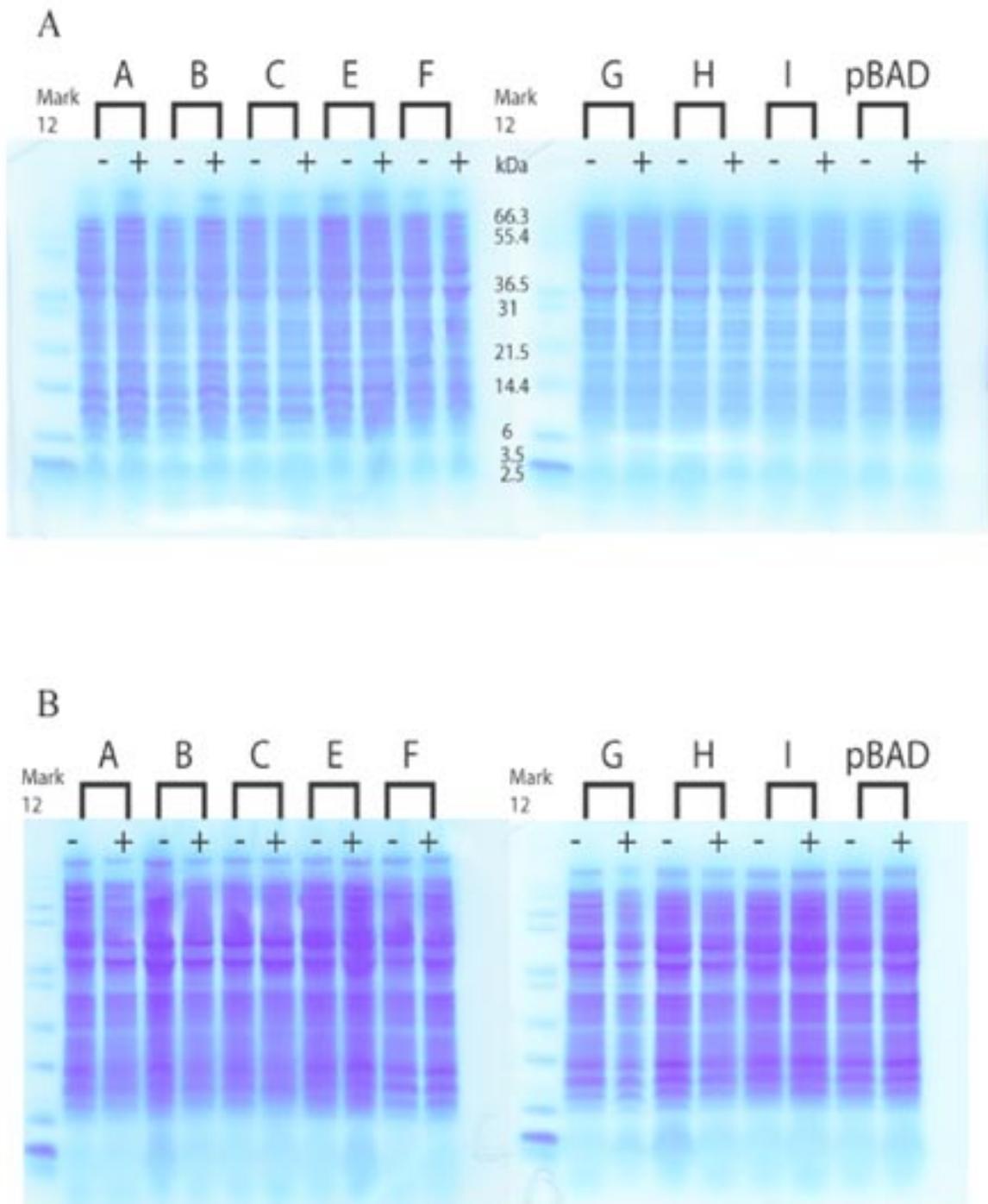
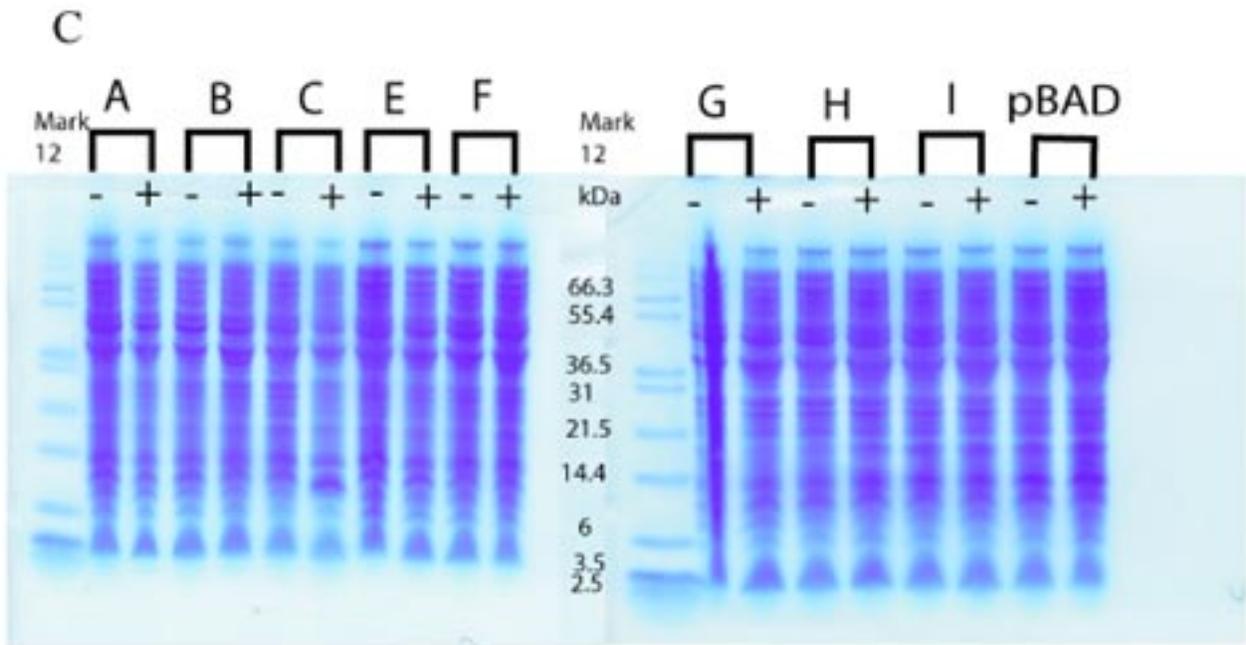
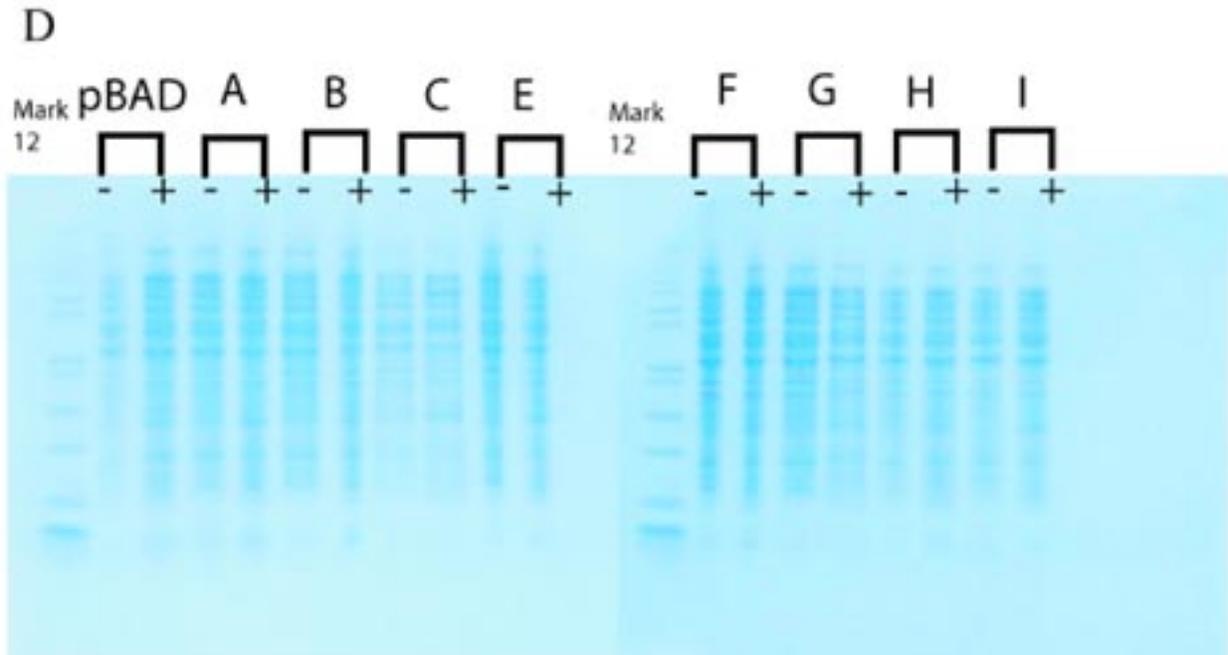


Figure 4. Expression of Csp proteins in cell protease mutants. Csp proteins were absent from arabinose induced samples (+) (uninduced is denoted by -) of (A) wildtype NT610, (B) *clpP* protease mutant, (C) *clpQ* protease mutant, and (D) *ftsH* protease mutant protein extracts. CspC, E, G, and I were present in arabinose induced samples (bands marked by an asterisk) of (E) *lon* protease mutant and (F) *lon clpQ* double protease mutant protein extracts.

clpQ

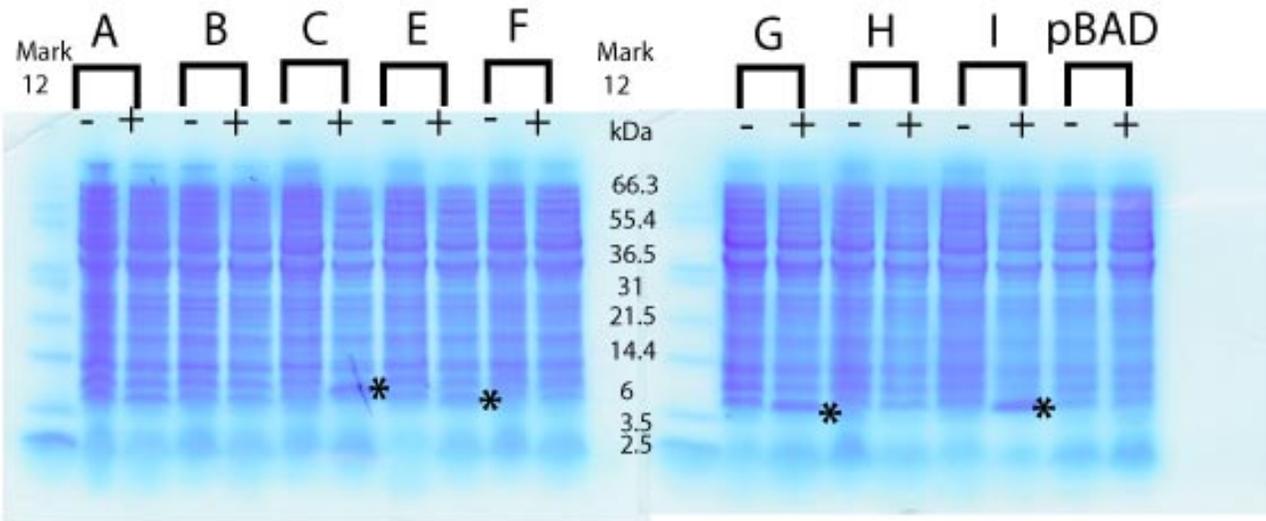


ftsH(ts) 42°C



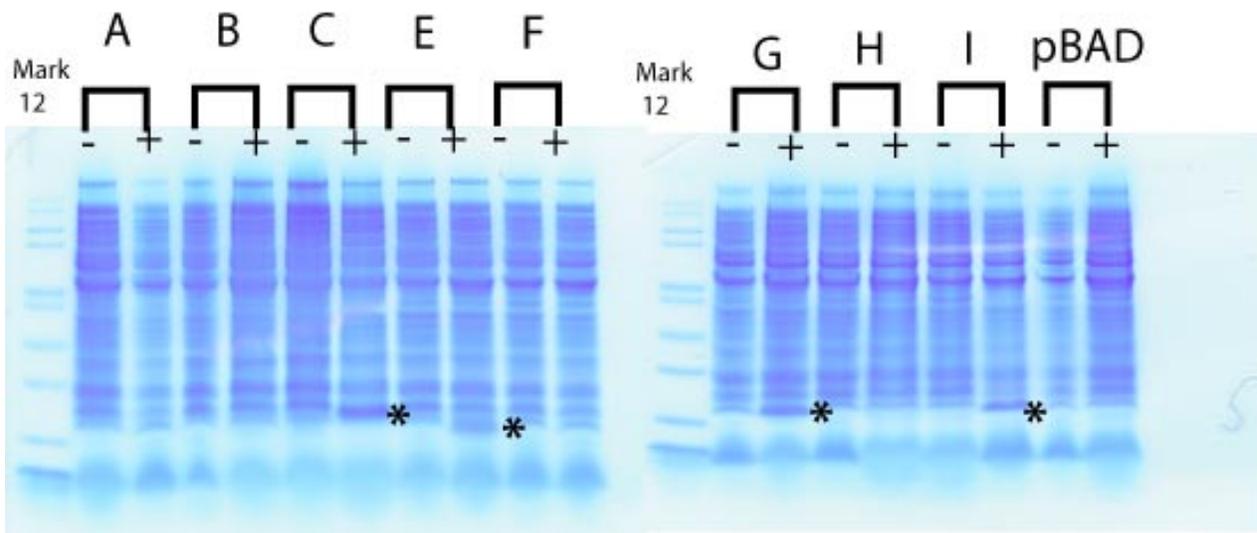
E

lon⁻



lon⁻ *clpQ*

F



Physiological Assays

Physiological assays were performed in order to determine if Csp protein overexpression produced phenotypes that were either the same or different from the known phenotypes demonstrated by CspE overexpression.

CspC, E, G, and I confer camphor resistance on *E. coli* K-12

Previous studies have shown that camphor is a DNA decondensing agent (Harrington and Trun, 1997), and that over-expression of *cspE*, confers camphor resistance in *E. coli* (Sand, O. *et al.*, 2003). Arabinose induced and uninduced cultures of NT785 with different *csp* containing plasmids were incubated with camphor for 120 minutes. Samples were removed just prior to the addition of camphor and every 30 minutes thereafter. Samples were titered and plated to assess cell viability (Actual cell counts and viabilities for all trials are provided in Tables 8 and 9 of the Appendix). Previous studies have demonstrated that the overexpression of *cspE*, confers a 10-fold increase in cell viability in the presence of camphor, and the overexpression of *cspE* with *crcA* and *crcB* increases viability by 1000-fold (Sand, O. *et al.*, 2003).

As shown in Figure 10 and Table 5, NT785 (pNT2 with *cspE*, *crcA*, and *crcB*) is just over 3 logs greater in viability than NT785 (pBR322) following 120 minutes of camphor exposure. Induced strains that were not found to produce stable Csp protein did not confer camphor resistance. NT785 strains containing, either *cspA*, *cspB*, *cspF*, or *cspH* on an arabinose inducible vector had viabilities equivalent to or 1 to 2 logs less than

NT785 with just the pBAD24 vector alone (Fig. 11A, Table 5). NT785 strains known to produce stable protein (CspC, E, G, and I) had viabilities that were 1 to 4 logs greater than the cell viability of the NT785 (pBAD24) strain after 120 minutes of camphor exposure (Figure 11B). Camphor resistance was conferred solely by protein producing *csp* clones.

Table 6. Cell Viabilities of Arabinose Induced NT785 (*lon*⁻) Strains Harboring *csp* Clones After 120 Minutes of Camphor Exposure

Sample	Fold Difference from pBAD24	Average Cell Viability
pNT2	*NA	$7.73 \times 10^{-3} \pm 5.00 \times 10^{-3}$
pBR322	NA	$5.67 \times 10^{-6} \pm 3.51 \times 10^{-6}$
pBAD24	NA	$3.03 \times 10^{-7} \pm 5.50 \times 10^{-8}$
A	1.5	$4.80 \times 10^{-7} \pm 4.01 \times 10^{-7}$
B	<1	Undetectable
C	172	$5.20 \times 10^{-5} \pm 4.16 \times 10^{-6}$
E	161	$4.87 \times 10^{-5} \pm 4.46 \times 10^{-5}$
F	<1	$5.00 \times 10^{-8} \pm 8.67 \times 10^{-8}$
G	249	$7.53 \times 10^{-5} \pm 4.27 \times 10^{-5}$
H	<1	$5.00 \times 10^{-8} \pm 8.66 \times 10^{-8}$
I	881	$2.67 \times 10^{-4} \pm 5.77 \times 10^{-5}$

*NA – Not Applicable

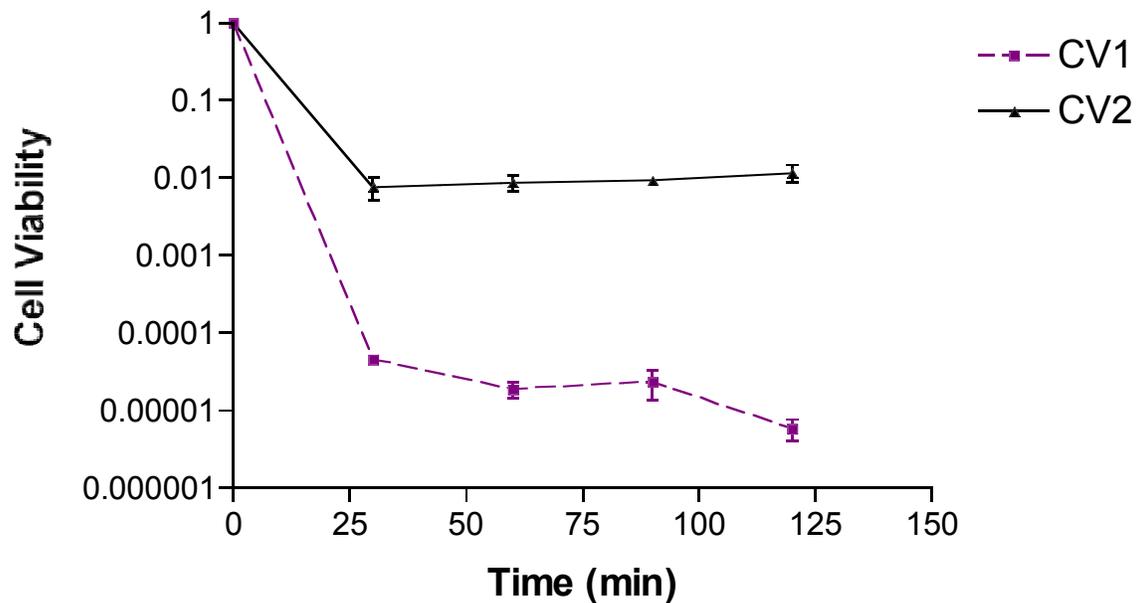
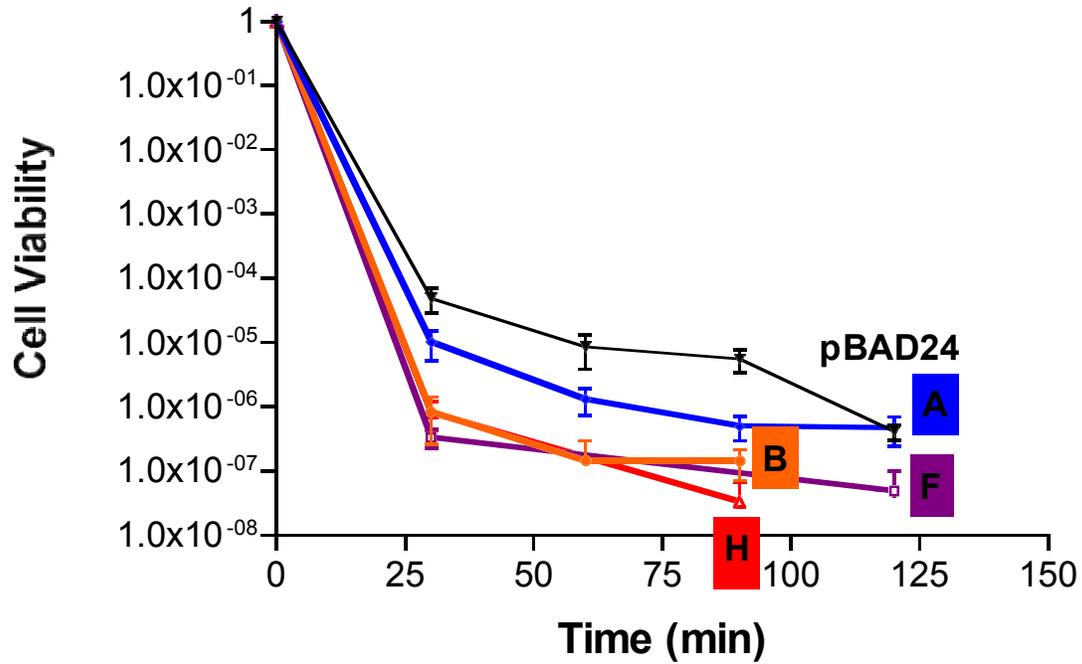


Figure 10. Camphor assay controls. CV2 cells harboring pNT2 demonstrated more than a three log increase in camphor resistance when compared to the pBR322 containing CV1 strain

A



B

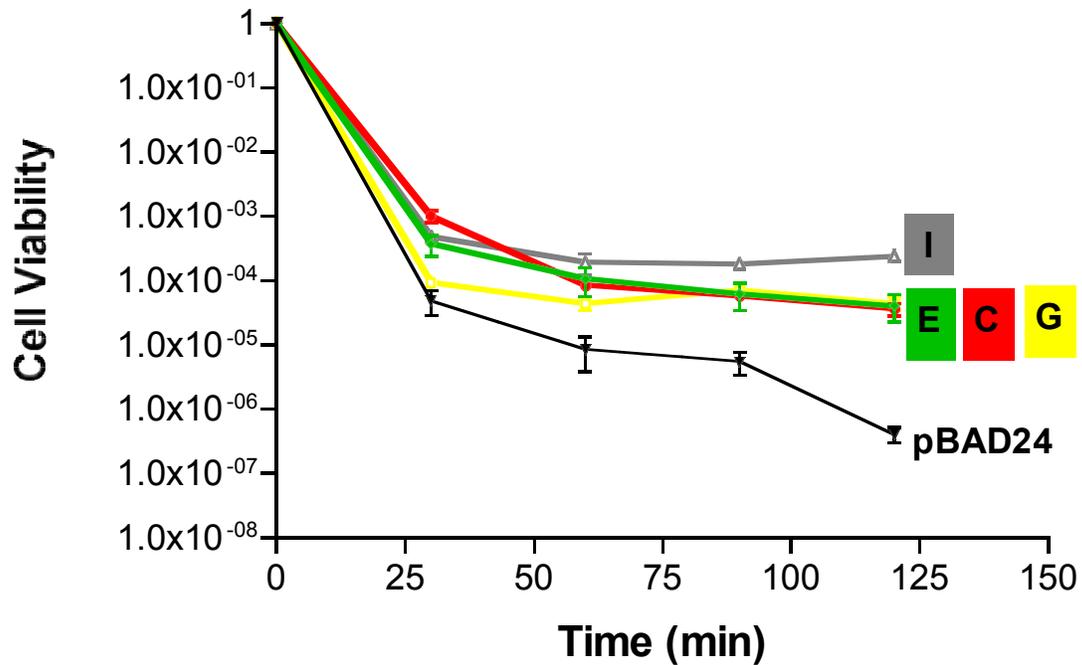


Figure 11. Arabinose induced samples exposed to camphor for various times. (A) NT785 strains with pBAD24 carrying *cspA*, *B*, *F*, or *H* displayed viabilities lower than NT785 (pBAD24). (B) NT785 strains with pBAD24 carrying *cspC*, *E*, *G*, or *I* displayed viabilities higher than NT785 (pBAD24). Note that cell viability was assigned a value of 1, at the addition of camphor.

***rcsA* upregulation**

cspE overexpression is capable of upregulating *rcsA* by 1.7-fold (Sand, O *et al.*, 2003). Using pNT2 (harboring *cspE*, *crcA*, and *crcB*) and pBR322 (vector) as controls, strains containing an *rcsA-lacZ* fusion in combination with one of the eight *csp* clones were tested for β -galactosidase activity. Induced and uninduced samples were removed at various stages of cell growth. Samples were removed from the same culture during the lag (OD_{600} 0-0.1), mid-log (OD_{600} 0.4-0.6), and late log (OD_{600} 0.7-1.0) phases. β -galactosidase activity was determined by measuring the cleavage of the β -galactosidase substrate, ONPG.

Measurements obtained for the controls of this study were somewhat higher than those obtained from previous studies (O, Sand *et al.*, 2003), which showed that β -galactosidase activity is 2.2-fold greater for cells containing pNT2 (*cspE*, *crcA*, and *crcB*) than those with the pBR322 vector. In this study cells containing pNT2 exhibited a 5-fold increase in comparison to the vector alone (Figures 12). β -galactosidase activity was 2.5-fold greater during late log for pNT2 containing strains than for the same strain during lag phase. Cells that possessed pBR322 exhibited relatively low and steady levels of β -galactosidase activity across all growth phases. β -galactosidase activity was greater for induced cultures than for uninduced cultures under most circumstances. However, β -galactosidase activity did not increase for induced samples harboring either pBAD24 or pBR322 in comparison to uninduced samples because neither of these vectors possessed *csp* genes under the control of the arabinose inducible promoter.

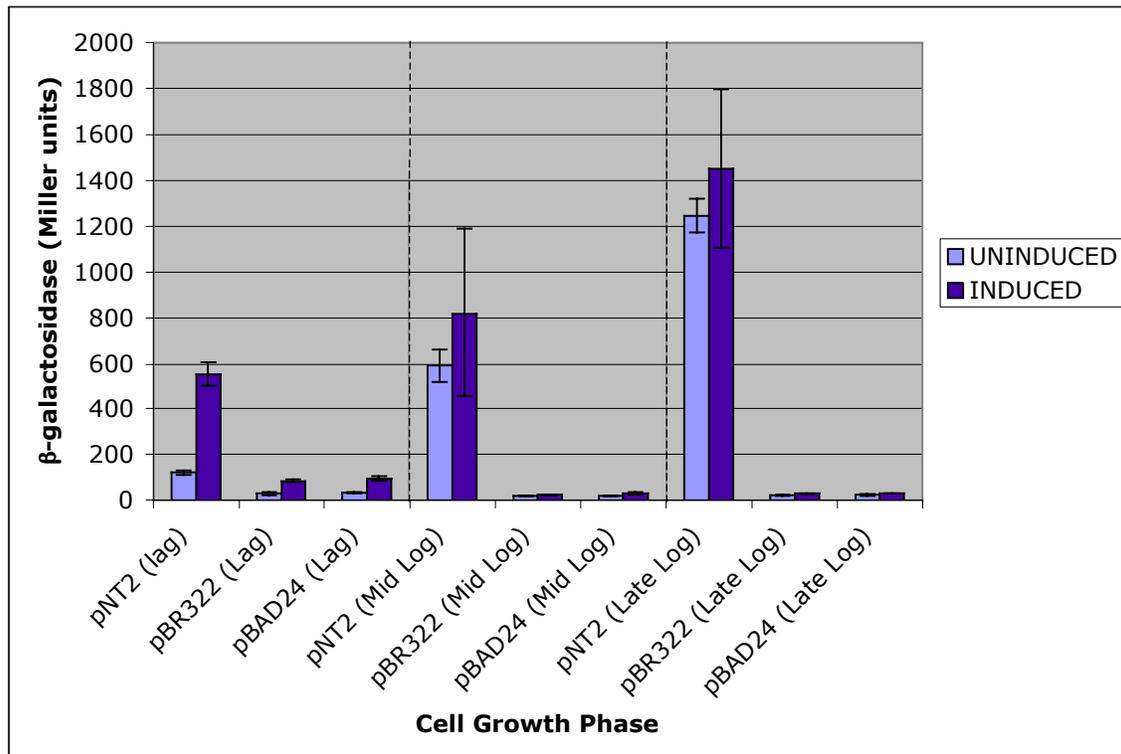


Figure 12. β -galactosidase activity for induced and uninduced strains carrying the *rcaA-lacZ* fusion and control vectors. Samples were taken at various phases of cell growth. Lag, mid log, and late log correspond to $OD_{600} = 0$ to 0.1, 0.4 to 0.6, and 0.7 to 1.0, respectively.

As shown in Figure 13, *cspC* and *cspG* containing clones produced 50-fold as much β -galactosidase as compared to the pBAD24 control during the lag phase of cell growth. However, *cspE* and *cspC* containing clones had the highest β -galactosidase activity during the mid-log stage with 35- and 65- fold the β -galactosidase activity as the vector, respectively. Amounts of β -galactosidase activity induced by these clones during the mid-log phase was significantly less in comparison to the activities obtained during

the late log phase. Also note that mid-log cells had lower amounts of β -galactosidase than the freshly diluted cells in lag phase. However, the amount of β -galactosidase increased once again during late log, and activities from all of the clones were greater than that displayed by pBAD24 alone.

Clones of *csp* genes that do not produce protein during early to mid stages of cell growth (OD_{600} 0.1-0.5) displayed an increase in β -galactosidase activity compared to pBAD24 during the late log phase (Figure 13). Cells containing *cspA* showed activity that was 50-fold greater than that displayed by the control. Cells with clones containing *cspB*, *F*, or *H* displayed 20-fold the amount of β -galactosidase possessed by the control during this same growth period.

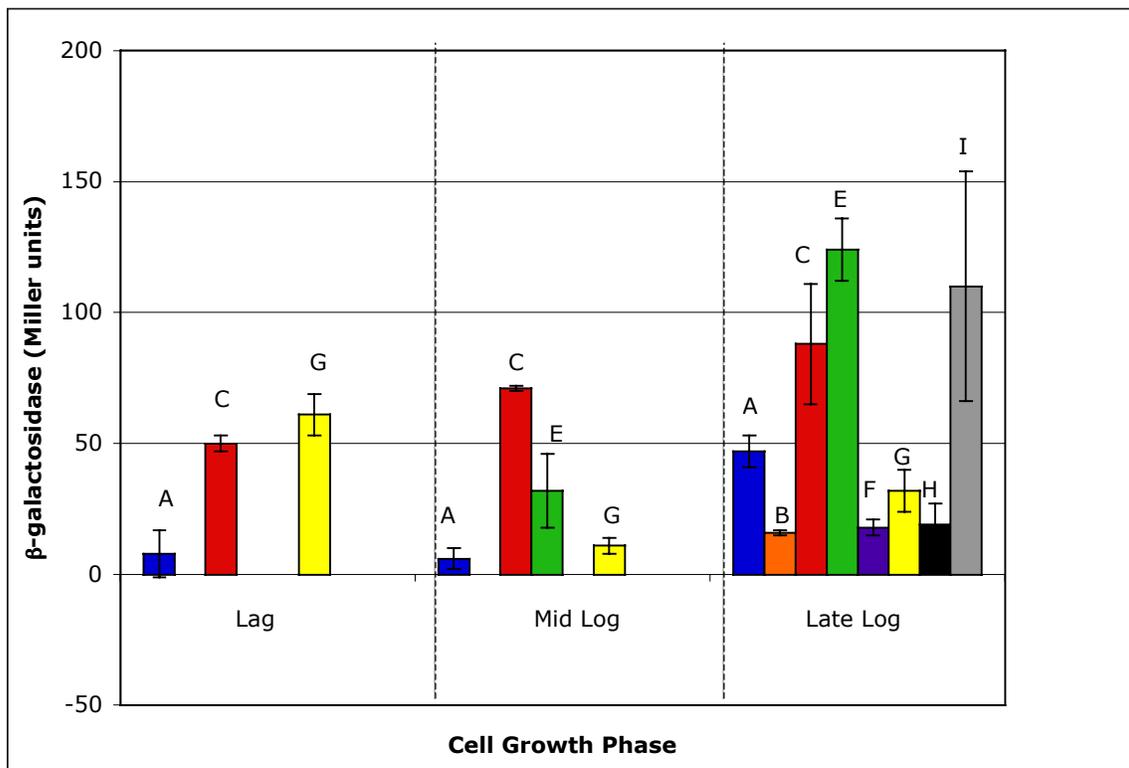


Figure 13. β -galactosidase activity for induced strains carrying the *rcaA-lacZ* fusion and designated *csp* clones. Samples were taken at various phases of cell growth. Lag, mid-log, and late log correspond to OD_{600} 0 to 0.1, 0.4 to 0.6, and 0.7 to 1.0, respectively. Background β -galactosidase activity from the pBAD24 vector has been subtracted from each of the values.

Plasmid DNA supercoiling on chloroquine gels

Previous studies have demonstrated that *cspE* overexpression leads to an increase in the number of plasmid DNA supercoils (Sand, O., *et al.*, 2003). In both this study and the aforementioned, gel electrophoresis was used to separate monomeric plasmid DNA on the basis of supercoil number. In this study plasmid DNA was extracted from *recA* mutant strains overexpressing one of each of the *csp* genes in order to determine if *cspE* paralogues are capable of supercoiling plasmid DNA.

As shown in Figure 14, induced and uninduced samples harboring pBAD24 produced 6 supercoils. Each sample, with the exception of induced samples containing *cspC* and *cspI*, shows between 7 and 9 supercoils (each band represents a different supercoil). Both induced and uninduced samples with *cspA* produced 9 supercoils. Induced and uninduced samples of *cspE* and *cspF* produced approximately 8 supercoils. The number of supercoils found in cells harboring *cspB*, *cspG*, and *cspH* were different between induced and uninduced samples, however, induced samples did not necessarily contain more supercoils. Induced samples obtained from clones containing *cspC* and *cspI* were unstable and were subsequently degraded upon electrophoresis.

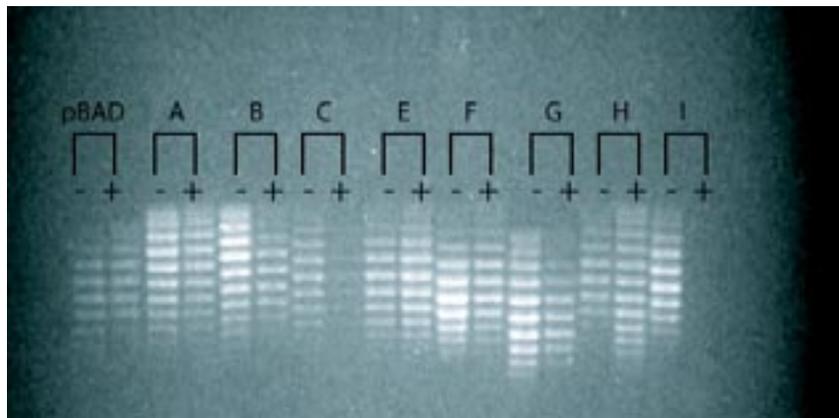


Figure 14. Monomeric pBAD24 plasmid DNA was extracted from a *recA*⁻ strain and electrophoresed on a 0.8% agarose gel with chloroquine. Both uninduced (-) and induced (+) samples are presented. Each band represents plasmid DNA with a different number of supercoils.

Csp proteins have different patterns of expression

Previous studies have demonstrated that *csp* genes have different means of induction. *cspC* and *cspE* are constitutively expressed (Bae, W., *et al.*, 1999), while *cspA*, *cspB*, *cspG*, and *cspI* are all induced upon cold shock (Etchegaray, J.P. *et al.*, 1996; Nakashima, K. *et al.*, 1996; Wang, N. *et al.*, 1999). *cspD* is the only *csp* gene induced by stationary phase (Yamanaka, K. & Inouye, M., 1997). Previous studies have addressed chromosomal *csp* expression and have shown that it is controlled at the posttranscriptional level. Posttranscriptional stability accounts for the increased presence of *cspA* mRNA (need numbers) upon cold shock (Brandi, A., *et al.*, 1996; Goldenberg, D., *et al.*, 1996; Fang, L., *et al.*, 1997).

This study utilized an arabinose inducible vector to monitor Csp protein expression, thereby, eliminating differences in transcriptional regulation. *lon*⁻ cells harboring individual *csp* genes were grown under induced and uninduced conditions. Samples were removed across four periods of growth: early log (OD₆₀₀ 0.3), mid-log (OD₆₀₀ 0.6), late log (OD₆₀₀ 1.0), and stationary phase (overnight). Protein extracts were prepared and electrophoresed on an SDS-PAGE gel, and were quantitated densitometrically with NIH-imager (Actual pixel densities for Csp protein are provided in Table 11 of the Appendix).

As shown in Table 6 and Figure 14, Csp protein was detected in the induced cells containing clones of *cspA*, *cspC*, *cspG*, and *cspI*. Levels of Csp protein varied between each phase of cell growth. For example, the highest levels of CspA expression occurred during stationary phase. CspC levels remained relatively high from early to late log;

however, levels peaked during mid-log. CspE levels were low by comparison to other Csp proteins, but levels remained steady. Cells containing *cspG* experienced an increase in CspG protein with the transition from late log to stationary phase. CspI increased during late log and remained high into the overnight stage. Csp protein was not detected for cells containing clones of *cspB*, *cspF*, and *cspH*.

Table 7. Ratio of Csp Protein to Total Protein Extracted from *lon*⁻ Cells Containing *csp* Clones Determined Using Densitometry

Csp Protein	Uninduced				Induced			
	Early log	Mid-log	Late log	Stationary phase	Early log	Mid-log	Late log	Stationary phase
pBAD24	0.012	0.016	0.013	0.018	0.012	0.011	0.013	0.007
A	0.023	0.023	0.023	0.024	0.021	0.026	0.023	0.053
B	0	0	0	0	0	0	0	0
C	0.009	0	0	0	0	0.073	0.048	0.023
E	0.004	0.005	0.011	0.007	0	0.009	0.018	0.014
F	0	0	0	0	0	0	0	0
G	0.002	0	0	0	0	0	0.009	0.086
H	0	0	0	0	0	0	0	0
I	0	0	0	0	0.004	0.007	0.086	0.089

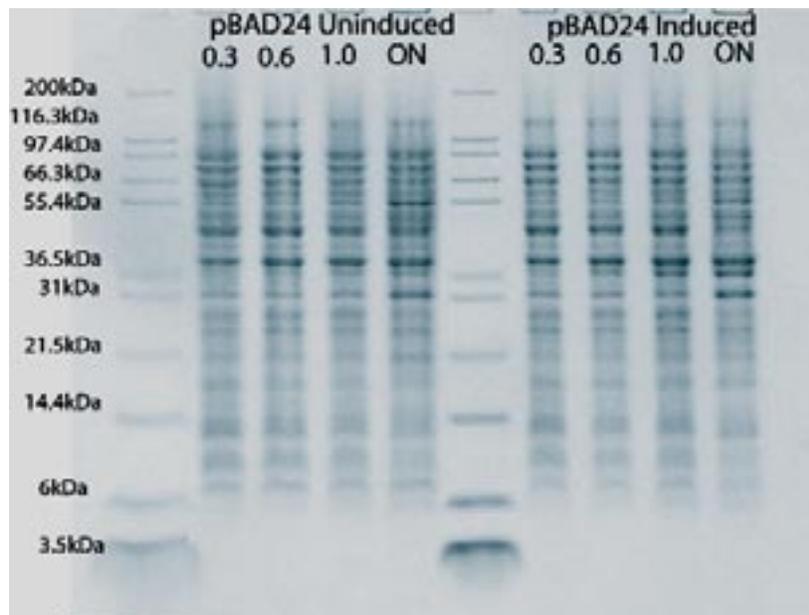
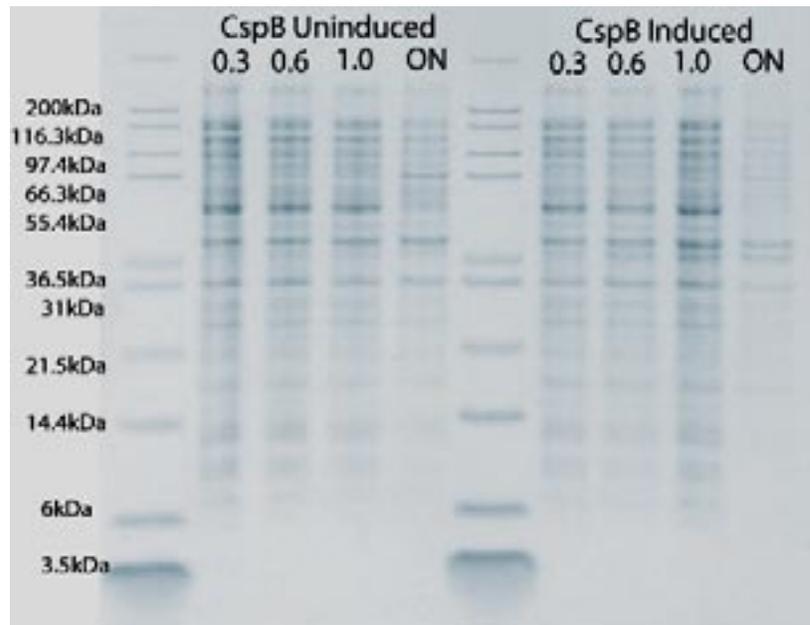
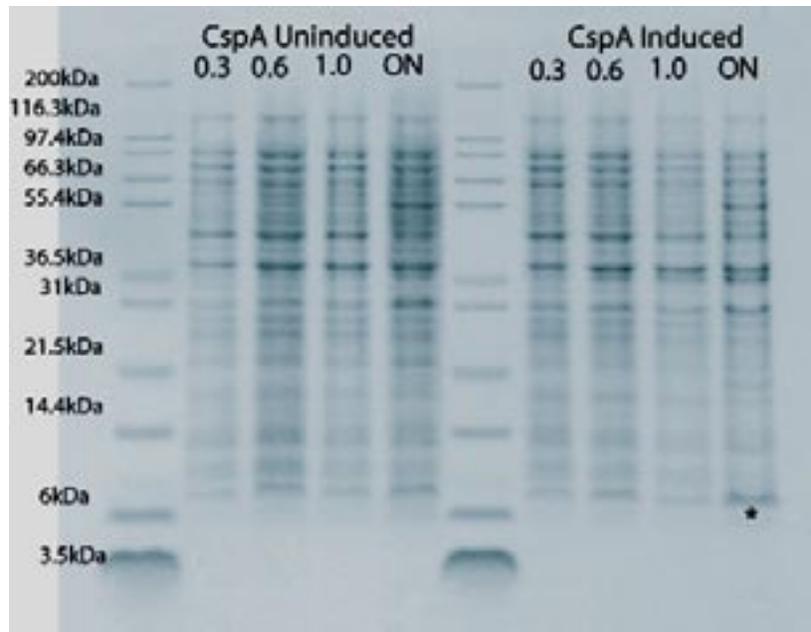
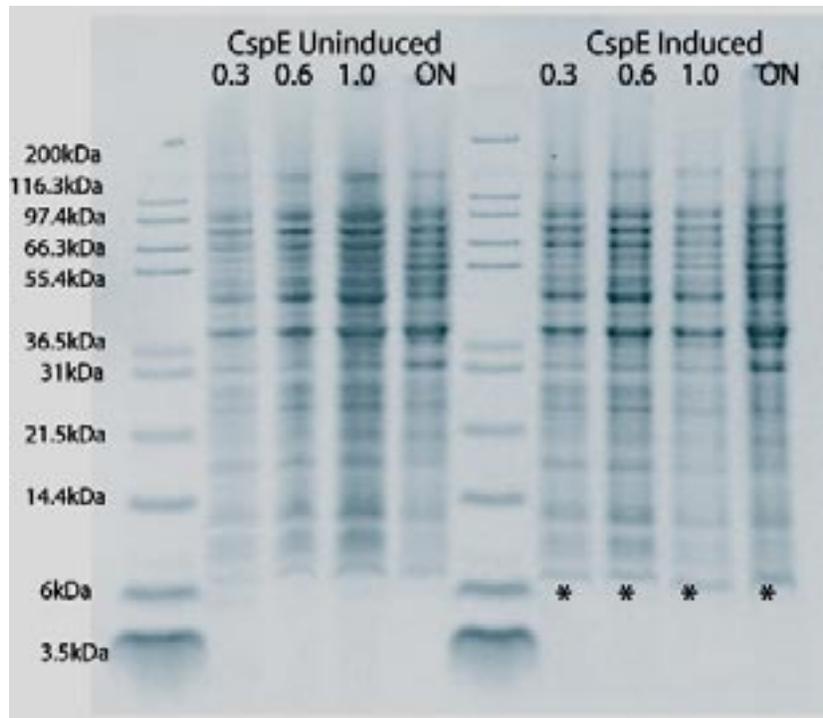
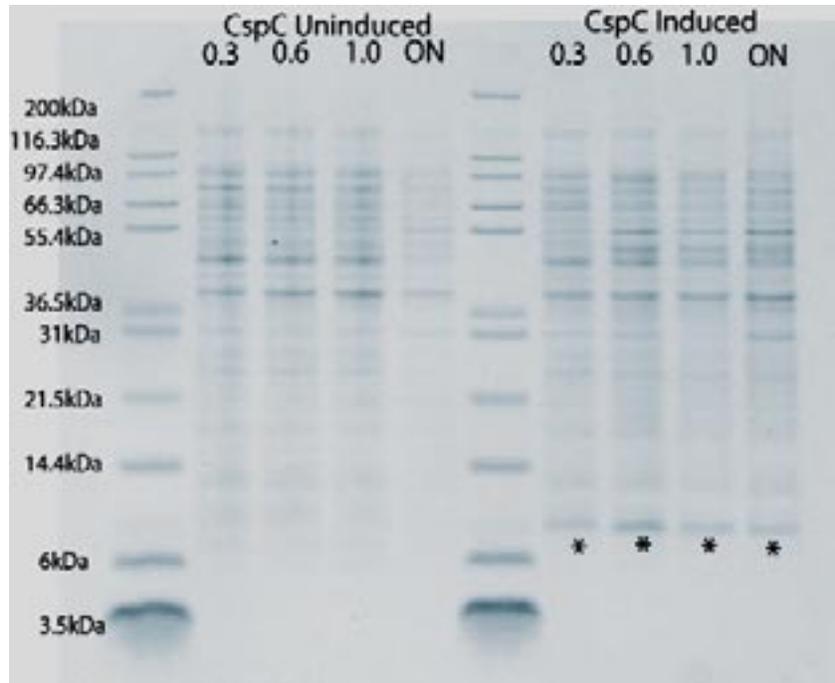
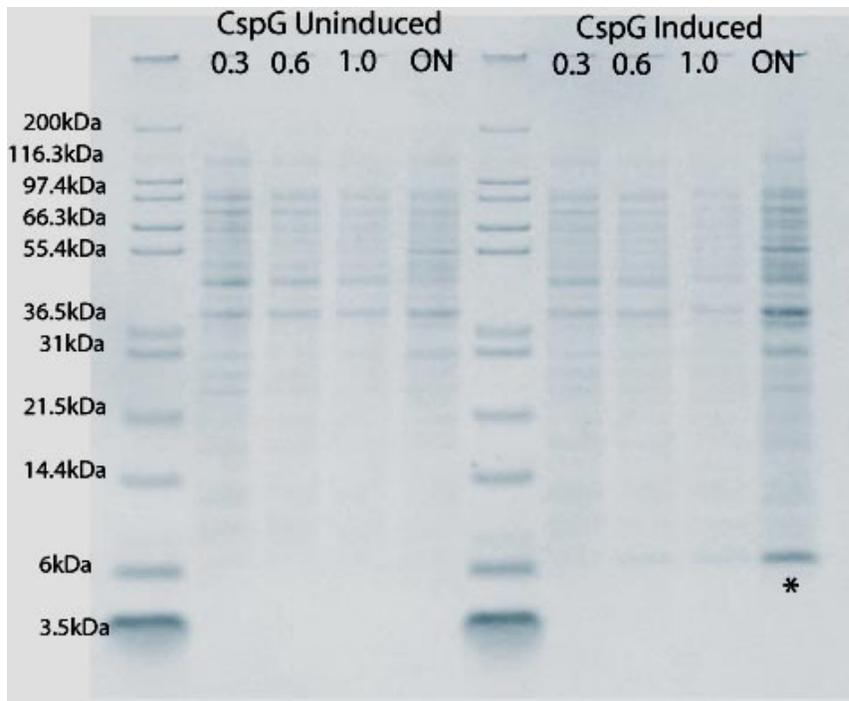
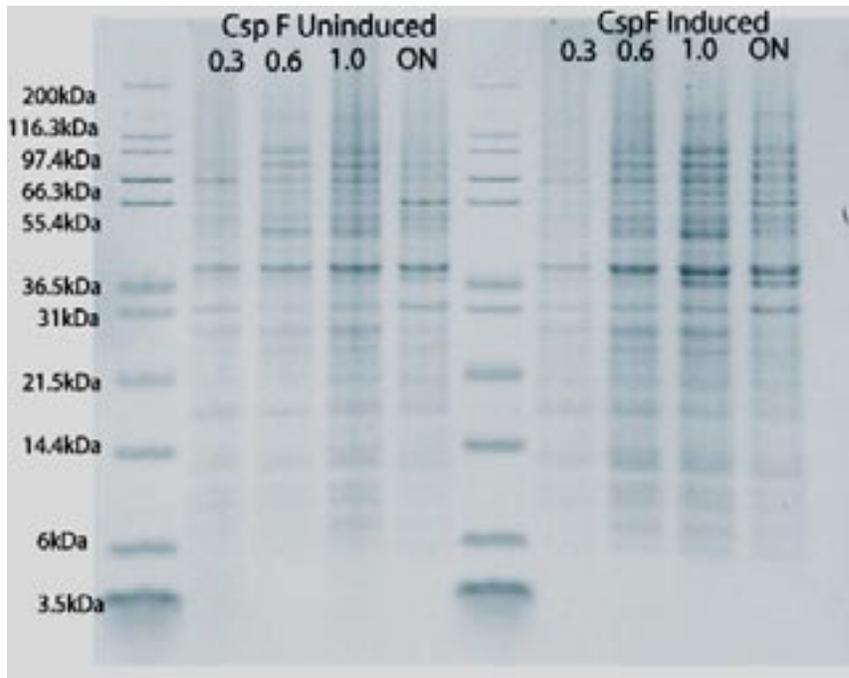
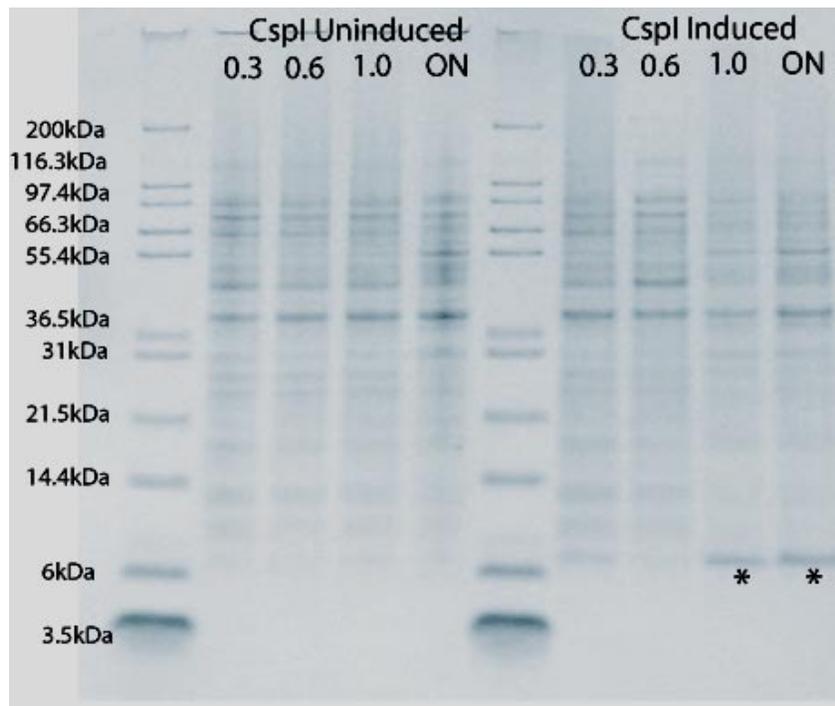
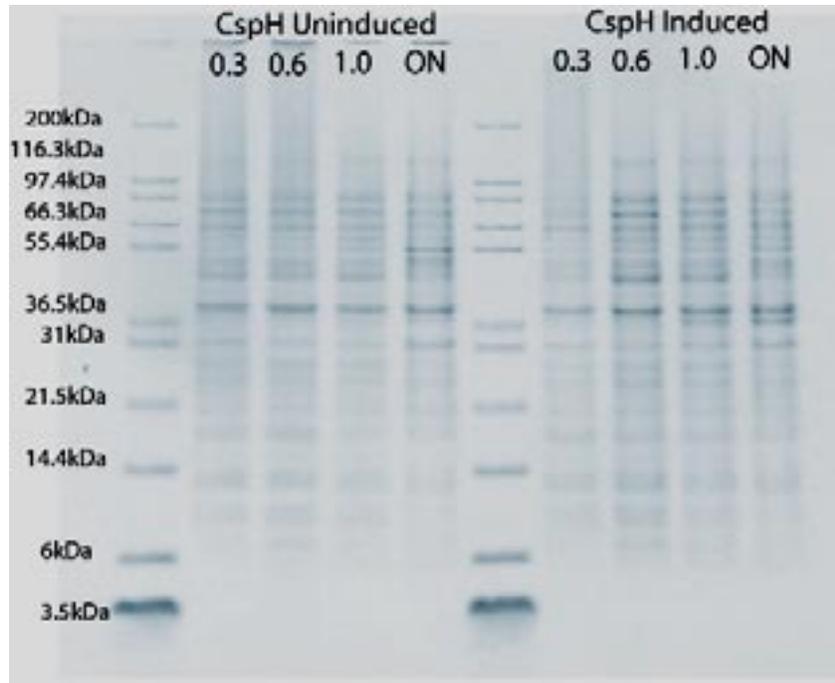


Figure 15, Csp protein expression across various stages of cell growth (Early log = OD₆₀₀ 0.3, Mid-log = OD₆₀₀ 0.6, Late log = OD₆₀₀ 1.0, and Stationary phase or Overnight) . Protein samples were obtained from both arabinose induced and uninduced *lon*⁻ mutant cells carrying individual *csp* clones and were electrophoresed on SDS-PAGE gels









DISCUSSION

CspA, CspB, CspG, and CspI are induced upon cold shock (Etchegaray, *et al.*, 1996; Nakashima, *et al.*, 1996; Wang, *et al.*, 1999), while CspC and CspE are constitutively expressed (Bae *et al.*, 1999). CspD is expressed in response to stationary phase (Yamanaka & Inouye, 1997). The regulation of CspF and CspH is not known. Previous studies have suggested several roles for CspE within the cell. CspE's ability to confer 10-fold camphor resistance and also to increase supercoiling of plasmid DNA suggests that it plays a role in DNA binding and chromosome condensation (Sand, O., *et al.*, 2003). Overexpression of *cspE* has also been shown to upregulate the *rcaA* gene by 1.7-fold, thereby suggesting a role in gene regulation.

Overall, phenotypic studies are in agreement with protein expression data. Those proteins present in *lon⁻* mutants (CspC, CspE, CspG, and CspI) all produce the same phenotypes, whereas those cells that do not overexpress stable Csp proteins (CspA, CspB, CspF, and CspH) do not exhibit these phenotypes. These results also suggest that the assays used for the phenotypic studies are not producing false positives. Moreover, CspC, CspG, and CspI confer the same phenotypes as CspE to varying extents. This data would suggest that these particular Csp proteins possess the same functions but different regulation.

Expression of *csp* mRNA and Csp protein

mRNA studies show that the expression of Csp proteins is not reliant on mRNA stability but rather protein stability, since mRNA was detected for most of the *csp* containing clones but protein was not. However, no mRNA was detected for CspF and CspH. The message itself may have been undetectable due to either extreme instability or it is possible that that message was never produced. The presence of 600 base mRNAs in samples obtained from pBAD24, *cspA*, *cspB*, *cspE*, and *cspI*-containing clones are most likely transcripts that contain the *araBAD* promoter region, and which terminate within the *bla* gene. The 350 base mRNAs found in the pBAD24 derived samples are thought to be mRNA degradation products.

As demonstrated in this study, CspC, CspE, CspG, and CspI are degraded by the Lon protease. On the other hand, CspA and CspB proteins were not present in the *lon*⁻ mutant, despite the detection of mRNA. Previous studies have shown that *cspA* mRNA is extremely unstable at 37^o C, but undergoes a 75-fold increase in stability upon a shift from 37^o C to 15^o C (Brandi, A., *et al.*, 1996; Fang, L., *et al.*, 1997; Goldenberg, D., *et al.*, 1996; Mitta, L., *et al.*, 1997). Consequently, *cspA* and *cspB* mRNA may possess certain characteristics that make them more susceptible to degradation at 37^o C. The other possibility for the absence of CspA and CspB protein may be that they are degraded by more than one protease. Experiments with various individual protease mutants (*lon*⁻, *ftsH*⁻, *clpQ*⁻, and *clpP*⁻) and a *lon*⁻ *clpP*⁻ double mutant suggest that these individual proteases are not solely responsible for the degradation of CspA and CspB. These

proteases may have overlapping specificities and could potentially compensate for the loss of one or more of their counterparts. Experiments with various combinations of double or triple mutants will determine if multiple proteases are responsible for the absence of these proteins.

Protein expression and resulting phenotypes

Studies performed in a *lon*⁻ mutant showed that CspC, CspE, CspG, and CspI overexpression resulted in various levels of camphor resistance. CspC, CspE, CspG, and CspI conferred a 100 fold increase in resistance to camphor in comparison to the vector. Overexpression of CspI increased cell viability by 1000 fold when compared to the vector. On the other hand, camphor resistance was not observed in strains containing clones of *cspA*, *cspB*, *cspF*, and *cspH*. This was not unexpected since none of these proteins were detected in *lon*⁻ mutants.

Results from gene regulation studies support those obtained from the studies on protein expression and camphor resistance. Although levels of β -galactosidase activity induced by each of the *csp* clones appeared to be growth phase dependent, those clones known to produce stable protein in *lon*⁻ mutants possessed the highest overall β -galactosidase activity. Cells containing CspC, CspE, CspG, and CspI showed substantial activation of the *rcsA-lacZ* fusion in comparison to those cells, which did not possess stable Csp proteins. The appearance of β -galactosidase activity for all of the non-protein-producing *csp* clones during late log may be due to accumulation of the *lacZ* enzyme as a result of continued basal level expression.

Results for DNA supercoiling show that in a wildtype cell DNA supercoiling levels do not change when Csp proteins are overproduced. Previous studies have shown that overexpression of *cspE* in combination with *crcA* and *crcB* increase supercoiling of plasmid DNA in wildtype cells (Sand, O., *et al.*, 2003). This study suggests that the overexpression of *cspE* or one of its paralogues by themselves do not have an effect on DNA supercoiling. Sand *et al.* also demonstrated that overexpression of *cspE*, *crcA*, and *crcB* can confer resistance to camphor in temperature sensitive DNA gyrase mutants incapable of supercoiling DNA. Future studies will reexamine the effect of *csp* overexpression in gyrase mutants that are defective in supercoiling to determine if DNA gyrase and CspE paralogues have overlapping functions.

Studies on levels of Csp protein accumulation across the growth curve have demonstrated that Csp proteins have different patterns of expression. CspA expression peaks during stationary phase. CspC and CspE proteins remain stable across all phases of cell growth. Levels of CspC are significantly increased during mid-log. Results obtained from β -galactosidase assays correlate with the findings on CspC protein stability. Cells overexpressing CspC had increased β -galactosidase activity during the mid-log phase, suggesting that the increase in CspC stability may contribute to a greater level of *rcaA* induction. Levels of CspG and CspI are highest during late log and stationary phase. These proteins may carry out most of their functions later in cell growth.

Overall, this study indicates that different Csps possess similar functions, yet are regulated differently. Different Csp proteins accumulate at different times during growth and suggest a testable hypothesis for why *E. coli* K-12 contains so many *csp* genes.

Further experiments on mutants deleted for specific *csp* genes will allow us to determine if certain Csps are required for viability under different conditions.

REFERENCES

1. **Amerik, A., V. K. Antonov, A. E. Gorbalenya, S. A. Kotova, T. V. Rotanova, and E. V. Shimbarevich.** 1991. Site-directed mutagenesis of La protease. A catalytically active serine residue. *FEBS Lett* **287**:211-4.
2. **Bader, A. G., K. A. Felts, N. Jiang, H. W. Chang, and P. K. Vogt.** 2003. Y box-binding protein 1 induces resistance to oncogenic transformation by the phosphatidylinositol 3-kinase pathway. *Proc Natl Acad Sci U S A* **100**:12384-9.
3. **Brandi, A., P. Pietroni, C. O. Gualerzi, and C. L. Pon.** 1996. Post-transcriptional regulation of CspA expression in *Escherichia coli*. *Mol Microbiol* **19**:231-40.
4. **Chin, D. T., S. A. Goff, T. Webster, T. Smith, and A. L. Goldberg.** 1988. Sequence of the lon gene in *Escherichia coli*. A heat-shock gene which encodes the ATP-dependent protease La. *J Biol Chem* **263**:11718-28.
5. **Choli, T., B. Wittmann-Liebold, and R. Reinhardt.** 1988. Microsequence analysis of DNA-binding proteins 7a, 7b, and 7e from the archaeobacterium *Sulfolobus acidocaldarius*. *J Biol Chem* **263**:7087-93.
6. **Dammel, C. S., and H. F. Noller.** 1995. Suppression of a cold-sensitive mutation in 16S rRNA by overexpression of a novel ribosome-binding factor, RbfA. *Genes Dev* **9**:626-37.
7. **Dersch, P., S. Kneip, and E. Bremer.** 1994. The nucleoid-associated DNA-binding protein H-NS is required for the efficient adaptation of *Escherichia coli* K-12 to a cold environment. *Mol Gen Genet* **245**:255-9.
8. **Didier, D. K., J. Schiffenbauer, S. L. Woulfe, M. Zacheis, and B. D. Schwartz.** 1988. Characterization of the cDNA encoding a protein binding to the major histocompatibility complex class II Y box. *Proc Natl Acad Sci U S A* **85**:7322-6.
9. **Dorn, A., B. Durand, C. Marfing, M. Le Meur, C. Benoist, and D. Mathis.** 1987. Conserved major histocompatibility complex class II boxes--X and Y--are transcriptional control elements and specifically bind nuclear proteins. *Proc Natl Acad Sci U S A* **84**:6249-53.
10. **Edmondson, S. P., L. Qiu, and J. W. Shriver.** 1995. Solution structure of the DNA-binding protein Sac7d from the hyperthermophile *Sulfolobus acidocaldarius*. *Biochemistry* **34**:13289-304.
11. **Etchegaray, J. P., and M. Inouye.** 1999. Translational enhancement by an element downstream of the initiation codon in *Escherichia coli*. *J Biol Chem* **274**:10079-85.
12. **Etchegaray, J. P., P. G. Jones, and M. Inouye.** 1996. Differential thermoregulation of two highly homologous cold-shock genes, *cspA* and *cspB*, of *Escherichia coli*. *Genes Cells* **1**:171-8.
13. **Fang, L., W. Jiang, W. Bae, and M. Inouye.** 1997. Promoter-independent cold-shock induction of *cspA* and its derepression at 37 degrees C by mRNA stabilization.

Mol Microbiol **23**:355-64.

14. **Fisher, H., and R. Glockshuber.** 1993. ATP hydrolysis is not stoichiometrically linked with proteolysis in the ATP-dependent protease La from *Escherichia coli*. J Biol Chem **268**:22502-22507.
15. **Friedman, D. F., E. Olsen, C. Georgopoulos, K. Tilly, I. Herskowitz, and F. Bannett.** 1984. Interactions of bacteriophage and host macromolecules in the growth of bacteriophage lambda. Microb Rev **48**:299-345.
16. **Fukada, T., and N. K. Tonks.** 2003. Identification of YB-1 as a regulator of PTP1B expression: implications for regulation of insulin and cytokine signaling. Embo J **22**:479-93.
17. **Fukada, T., and N. K. Tonks.** 2003. Identification of YB-1 as a regulator of PTP1B expression: implications for regulation of insulin and cytokine signaling. Embo J **22**:479-93.
18. **Goldberg, A. L., R. P. Moerschell, C. H. Chung, and M. R. Maurizi.** 1994. ATP-dependent protease La (lon) from *Escherichia coli*. Methods Enzymol **244**:350-75.
19. **Goldenberg, D., I. Azar, and A. B. Oppenheim.** 1996. Differential mRNA stability of the cspA gene in the cold-shock response of *Escherichia coli*. Mol Microbiol **19**:241-8.
20. **Goldenberg, D., I. Azar, A. B. Oppenheim, A. Brandi, C. L. Pon, and C. O. Gualerzi.** 1997. Role of *Escherichia coli* cspA promoter sequences and adaptation of translational apparatus in the cold shock response. Mol Gen Genet **256**:282-90.
21. **Goldstein, J., N. S. Pollitt, and M. Inouye.** 1990. Major cold shock protein of *Escherichia coli*. Proc Natl Acad Sci U S A **87**:283-7.
22. **Gottesman, S.** 1996. Proteases and their targets in *Escherichia coli*. Annu Rev Genet **30**:465-506.
23. **Gottesman, S., W. P. Clark, V. de Crecy-Lagard, and M. R. Maurizi.** 1993. ClpX, an alternative subunit for the ATP-dependent Clp protease of *Escherichia coli*. Sequence and in vivo activities. J Biol Chem **268**:22618-26.
24. **Gottesman, S., S. Wickner, and M. R. Maurizi.** 1997. Protein quality control: triage by chaperones and proteases. Genes Dev **11**:815-23.
25. **Graumann, P., and M. A. Marahiel.** 1996. Some like it cold: response of microorganisms to cold shock. Arch Microbiol **166**:293-300.
26. **Guzman, L. M., D. Belin, M. J. Carson, and J. Beckwith.** 1995. Tight regulation, modulation, and high-level expression by vectors containing the arabinose PBAD promoter. J Bacteriol **177**:4121-30.
27. **Hanna, M. M., and K. Liu.** 1998. Nascent RNA in transcription complexes interacts with CspE, a small protein in *E. coli* implicated in chromatin condensation. J Mol Biol **282**:227-39.
28. **Hebraud, M., E. Dubois, P. Potier, and J. Labadie.** 1995. Effect of growth temperatures on the protein levels in a psychrotrophic bacterium *Pseudomonas fragi*. J Bacteriol **176**:4017-4024.
29. **Hendrick, J. P., and F. U. Hartl.** 1993. Molecular chaperone functions of heat-shock proteins. Annu Rev Biochem **62**:349-84.
30. **Herendeen, S. L., R. A. VanBogelen, and F. C. Neidhardt.** 1979. Levels of major proteins of *Escherichia coli* during growth at different temperatures. J Bacteriol **139**:185-94.

31. **Herman, C., S. Lecat, R. D'Ari, and P. Bouloc.** 1995. Regulation of the heat-shock response depends on divalent metal ions in an *hflB* mutant of *Escherichia coli*. *Mol Microbiol* **18**:247-55.
32. **Hu, K. H., E. Liu, K. Dean, M. Gingras, W. DeGraff, and N. J. Trun.** 1996. Overproduction of three genes leads to camphor resistance and chromosome condensation in *Escherichia coli*. *Genetics* **143**:1521-32.
33. **Jiang, W., L. Fang, and M. Inouye.** 1996. The role of the 5'-end untranslated region of the mRNA for CspA, the major cold-shock protein of *Escherichia coli*, in cold-shock adaptation. *J Bacteriol* **178**:4919-25.
34. **Jiang, W., Y. Hou, and M. Inouye.** 1997. CspA, the major cold-shock protein of *Escherichia coli*, is an RNA chaperone. *J Biol Chem* **272**:196-202.
35. **Jones, P. G., R. Krah, S. R. Tafuri, and A. P. Wolffe.** 1992. DNA gyrase, CS7.4, and the cold shock response in *Escherichia coli*. *J Bacteriol* **174**:5798-802.
36. **Jones, P. G., R. Krah, S. R. Tafuri, and A. P. Wolffe.** 1992. DNA gyrase, CS7.4, and the cold shock response in *Escherichia coli*. *J Bacteriol* **174**:5798-802.
37. **Jones, P. G., M. Mitta, Y. Kim, W. Jiang, and M. Inouye.** 1996. Cold shock induces a major ribosomal-associated protein that unwinds double-stranded RNA in *Escherichia coli*. *Proc Natl Acad Sci U S A* **93**:76-80.
38. **Jones, P. G., R. A. VanBogelen, and F. C. Neidhardt.** 1987. Induction of proteins in response to low temperature in *Escherichia coli*. *J Bacteriol* **169**:2092-5.
39. **Julseth, C., and W. Inniss.** 1990. Induction of protein synthesis in response to cold-shock in psychrotrophic yeast *Trichosporan pullans*. *Can J Microbiol* **36**:519-524.
40. **Katayama, Y., S. Gottesman, J. Pumphrey, S. Rudikoff, W. P. Clark, and M. R. Maurizi.** 1988. The two-component, ATP-dependent Clp protease of *Escherichia coli*. Purification, cloning, and mutational analysis of the ATP-binding component. *J Biol Chem* **263**:15226-36.
41. **Kihara, A., Y. Akiyama, and K. Ito.** 1995. FtsH is required for proteolytic elimination of uncomplexed forms of SecY, an essential protein translocase subunit. *Proc Natl Acad Sci U S A* **92**:4532-6.
42. **Koike, K., T. Uchiumi, T. Ohga, S. Toh, M. Wada, K. Kohno, and M. Kuwano.** 1997. Nuclear translocation of the Y-box binding protein by ultraviolet irradiation. *FEBS Lett* **417**:390-4.
43. **Krukltis, R., D. J. Welty, and H. Nakai.** 1996. ClpX protein of *Escherichia coli* activates bacteriophage Mu transposase in the strand transfer complex for initiation of Mu DNA synthesis. *Embo J* **15**:935-44.
44. **La Teana, A., A. Brandi, M. Falconi, R. Spurio, C. L. Pon, and C. O. Gualerzi.** 1991. Identification of a cold shock transcriptional enhancer of the *Escherichia coli* gene encoding nucleoid protein H-NS. *Proc Natl Acad Sci U S A* **88**:10907-11.
45. **Levchenko, I., L. Luo, and T. A. Baker.** 1995. Disassembly of the Mu transposase tetramer by the ClpX chaperone. *Genes Dev* **9**:2399-408.
46. **Maniak, M., and W. Nellen.** 1988. A developmentally regulated membrane protein gene in *Dictyostelium discoideum* is also induced by heat shock and cold shock. *Mol Cell Biol* **8**:153-9.
47. **McAfee, J. G., S. P. Edmondson, P. K. Datta, J. W. Shriver, and R. Gupta.** 1995. Gene cloning, expression, and characterization of the Sac7 proteins from the hyperthermophile *Sulfolobus acidocaldarius*. *Biochemistry* **34**:10063-77.

48. **Menzel, R., and M. Gellert.** 1983. Regulation of the genes for *E. coli* DNA gyrase: homeostatic control of DNA supercoiling. *Cell* **34**:105-13.
49. **Mertens, P. R., S. Harendza, A. S. Pollock, and D. H. Lovett.** 1997. Glomerular mesangial cell-specific transactivation of matrix metalloproteinase 2 transcription is mediated by YB-1. *J Biol Chem* **272**:22905-12.
50. **Michel, V., I. Lehoux, G. Depret, P. Anglade, J. Labadie, and M. Hebraud.** 1997. The cold shock response of the psychrotrophic bacterium *Pseudomonas fragi* involves four low-molecular-mass nucleic acid-binding proteins. *J Bacteriol* **179**:7331-42.
51. **Miller, J. H.** 1992. *A Short Course in Bacterial Genetics*. Cold Spring Harbor Laboratory, New York.
52. **Mitta, M., L. Fang, and M. Inouye.** 1997. Deletion analysis of *cspA* of *Escherichia coli*: requirement of the AT-rich UP element for *cspA* transcription and the downstream box in the coding region for its cold shock induction. *Mol Microbiol* **26**:321-35.
53. **Nakashima, K., K. Kanamaru, T. Mizuno, and K. Horikoshi.** 1996. A novel member of the *cspA* family of genes that is induced by cold shock in *Escherichia coli*. *J Bacteriol* **178**:2994-7.
54. **Newkirk, K., W. Feng, W. Jiang, R. Tejero, S. D. Emerson, M. Inouye, and G. T. Montelione.** 1994. Solution NMR structure of the major cold shock protein (*CspA*) from *Escherichia coli*: identification of a binding epitope for DNA. *Proc Natl Acad Sci U S A* **91**:5114-8.
55. **Ng, H., J. L. Ingraham, and A. G. Marr.** 1962. Damage and depression in *Escherichia coli* resulting from growth at low temperature. *J Bacteriol* **84**:331-339.
56. **Niki, H., A. Jaffe, R. Imamura, T. Ogura, and S. Hiraga.** 1991. The new gene *mukB* codes for a 177 kd protein with coiled-coil domains involved in chromosome partitioning of *E. coli*. *Embo J* **10**:183-93.
57. **Ohga, T., T. Uchiumi, Y. Makino, K. Koike, M. Wada, M. Kuwano, and K. Kohno.** 1998. Direct involvement of the Y-box binding protein YB-1 in genotoxic stress-induced activation of the human multidrug resistance 1 gene. *J Biol Chem* **273**:5997-6000.
58. **Plumbridge, J. A., F. Deville, C. Sacerdot, H. U. Petersen, Y. Cenatiempo, A. Cozzone, M. Grunberg-Manago, and J. W. Hershey.** 1985. Two translational initiation sites in the *infB* gene are used to express initiation factor IF2 alpha and IF2 beta in *Escherichia coli*. *Embo J* **4**:223-9.
59. **Robinson, H., Y.-G. Gao, B. S. McCrary, S. P. Edmondson, J. W. Shriver, and A. Wang.** 1998. The hyperthermophile chromosomal protein Sac7d sharply kinks DNA. *Letters to Nature* **392**:202-205.
60. **Ross, W., K. K. Gosink, J. Salomon, K. Igarashi, C. Zou, A. Ishihama, K. Severinov, and R. L. Gourse.** 1993. A third recognition element in bacterial promoters: DNA binding by the alpha subunit of RNA polymerase. *Science* **262**:1407-13.
61. **Rudd, K. E.** 1998. Linkage map of *Escherichia coli* K-12, edition 10: the physical map. *Microbiol Mol Biol Rev* **62**:985-1019.
62. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. *Molecular Cloning: A laboratory manual*. Cold Spring Harbor Laboratory, New York.
63. **Sand, O., M. Gingras, N. Beck, C. Hall, and N. Trun.** 2003. Phenotypic

- characterization of overexpression or deletion of the *Escherichia coli* *crcA*, *cspE* and *crcB* genes. *Microbiology* **149**:2107-17.
64. **Schindelin, H., W. Jiang, M. Inouye, and U. Heinemann.** 1994. Crystal structure of CspA, the major cold shock protein of *Escherichia coli*. *Proc Natl Acad Sci U S A* **91**:5119-23.
 65. **Schmid, M. B.** 1990. More than just "histone-like" proteins. *Cell* **63**:451-3.
 66. **Silhavy, T. J., M. L. Berman, and L. W. Enquist.** 1984. *Experiments with Gene Fusions*. Cold Spring Harbor Laboratory, New York.
 67. **Sugino, A., C. L. Peebles, K. N. Kreuzer, and N. R. Cozzarelli.** 1977. Mechanism of action of nalidixic acid: purification of *Escherichia coli* *nalA* gene product and its relationship to DNA gyrase and a novel nicking-closing enzyme. *Proc Natl Acad Sci U S A* **74**:4767-71.
 68. **Tafari, S. R., and A. P. Wolffe.** 1992. DNA binding, multimerization, and transcription stimulation by the *Xenopus* Y box proteins in vitro. *New Biol* **4**:349-59.
 69. **Tiku, P. E., A. Y. Gracey, A. I. Macartney, R. J. Beynon, and A. R. Cossins.** 1996. Cold-induced expression of delta 9-desaturase in carp by transcriptional and posttranslational mechanisms. *Science* **271**:815-8.
 70. **Tomoyasu, T., T. Yuki, S. Morimura, H. Mori, K. Yamanaka, H. Niki, S. Hiraga, and T. Ogura.** 1993. The *Escherichia coli* FtsH protein is a prokaryotic member of a protein family of putative ATPases involved in membrane functions, cell cycle control, and gene expression. *J Bacteriol* **175**:1344-51.
 71. **Trun, N., and D. Johnston.** 2003. Folding chromosomes in bacteria: Examining the role of Csp proteins and other small nucleic acid binding proteins. *Curr Topics Develop Biol* **55**:173-201.
 72. **Virca, C. D., W. Northemann, B. R. Shiels, G. Widera, and S. Broome.** 1990. Simplified northern blot hybridization using 5% sodium dodecyl sulfate. *Biotechniques* **8**:370-371.
 73. **Walker, G. C.** 1984. Mutagenesis and inducible responses to deoxyribonucleic acid damages in *Escherichia coli*. *Microb Rev* **48**:60-93.
 74. **Wang, N., K. Yamanaka, and M. Inouye.** 1999. CspI, the ninth member of the CspA family of *Escherichia coli*, is induced upon cold shock. *J Bacteriol* **181**:1603-9.
 75. **Wawrzynow, A., D. Wojtkowiak, J. Marszalek, B. Banecki, M. Jonsen, B. Graves, C. Georgopoulos, and M. Zylicz.** 1995. The ClpX heat-shock protein of *Escherichia coli*, the ATP-dependent substrate specificity component of the ClpP-ClpX protease, is a novel molecular chaperone. *Embo J* **14**:1867-77.
 76. **Willmsky, G., H. Bang, G. Fischer, and M. A. Marahiel.** 1992. Characterization of *cspB*, a *Bacillus subtilis* inducible cold shock gene affecting cell viability at low temperatures. *J Bacteriol* **174**:6326-35.
 77. **Wistow, G.** 1990. Cold shock and DNA binding. *Nature* **344**:823-4.
 78. **Wolffe, A. P.** 1994. Structural and functional properties of the evolutionarily ancient Y-box family of nucleic acid binding proteins. *Bioessays* **16**:245-51.
 79. **Wolffe, A. P.** 1994. Structural and functional properties of the evolutionarily ancient Y-box family of nucleic acid binding proteins. *Bioessays* **16**:245-51.
 80. **Wolffe, A. P., S. Tafari, M. Ranjan, and M. Familiari.** 1992. The Y-box factors: a family of nucleic acid binding proteins conserved from *Escherichia coli* to man. *New Biol* **4**:290-8.

81. **Woo, K. M., W. J. Chung, D. B. Ha, A. L. Goldberg, and C. H. Chung.** 1989. Protease Ti from *Escherichia coli* requires ATP hydrolysis for protein breakdown but not for hydrolysis of small peptides. *J Biol Chem* **264**:2088-91.
82. **Yamamoto, Y., H. Aiba, T. Baba, K. Hayashi, T. Inada, K. Isono, T. Itoh, S. Kimura, M. Kitagawa, K. Makino, T. Miki, N. Mitsuhashi, K. Mizobuchi, H. Mori, S. Nakade, Y. Nakamura, H. Nashimoto, T. Oshima, S. Oyama, N. Saito, G. Sampei, Y. Satoh, S. Sivasundaram, H. Tagami, T. Horiuchi, and et al.** 1997. Construction of a contiguous 874-kb sequence of the *Escherichia coli* K-12 genome corresponding to the 50.0-68.8 min on the linkage map and analysis of its sequence features (supplement). *DNA Res* **4**:169-78.
83. **Yamanaka, K.** 1999. Cold shock response in *Escherichia coli*. *J Mol Microbiol Biotechnol* **1**:193-202.
84. **Yamanaka, K., L. Fang, and M. Inouye.** 1998. The CspA family in *Escherichia coli*: multiple gene duplication for stress adaptation. *Mol Microbiol* **27**:247-55.
85. **Yamanaka, K., L. Fang, and M. Inouye.** 1998. The CspA family in *Escherichia coli*: multiple gene duplication for stress adaptation. *Mol Microbiol* **27**:247-55.
86. **Yamanaka, K., and M. Inouye.** 1997. Growth-phase-dependent expression of *cspD*, encoding a member of the CspA family in *Escherichia coli*. *J Bacteriol* **179**:5126-30.
87. **Yamanaka, K., T. Mitani, T. Ogura, H. Niki, and S. Hiraga.** 1994. Cloning, sequencing, and characterization of multicopy suppressors of a *mukB* mutation in *Escherichia coli*. *Mol Microbiol* **13**:301-12.

APPENDIX

Table 8. Cell counts obtained for *csp*-containing clones after exposure to camphor

Time (min) after addition of camphor	CV1 (pBR322)			CV2 (pNT2)			pBAD24 Induced		
	Cells/ ml of LB			Cells/ ml LB			Cells / ml LB		
	Trial 1	Trial 2	Trial 3	Trial 1	Trial2	Trial 3	Trial 1	Trial 2	Trial 3
0	5.60E+11	8.50E+07	6.30E+07	1.24E+07	1.69E+07	1.97E+07	8.30E+07	7.90E+07	1.13E+07
30	2.80E+03	3.10E+03	3.20E+03	7.00E+04	7.70E+04	2.58E+05	5.30E+03	6.70E+02	8.50E+03
60	1.04E+03	9.40E+02	1.68E+03	1.19E+05	7.90E+04	2.37E+05	4.00E+02	2.30E+02	2.00E+03
90	9.00E+02	1.00E+03	2.70E+03	1.09E+05	1.12E+05	1.93E+05	5.80E+02	1.00E+02	9.30E+02
120	3.60E+02	2.10E+02	5.40E+02	1.61E+05	1.06E+05	3.12E+05	3.00E+01	2.00E+01	7.00E+01

Time (min) after addition of camphor	CspA Induced			CspB Induced			Csp C Induced		
	Cells / ml LB			Cells / ml LB			Cells / ml LB		
	Trial 1	Trial2	Trial 3	Trial 1	Trial 2	Trial 3	Trial 1	Trial 2	Trial 3
0	1.00E+08	1.06E+08	9.60E+07	4.80E+07	4.50E+07	6.30E+07	1.07E+08	1.51E+08	1.00E+08
30	3.00E+02	8.40E+02	1.88E+03	1.00E+01	9.00E+01	2.00E+01	6.90E+04	2.13E+05	1.04E+05
60	8.00E+01	7.00E+01	2.40E+02	0.00E+00	2.00E+01	0.00E+00	9.30E+03	1.29E+04	7.80E+03
90	3.00E+01	3.00E+01	9.00E+01	1.00E+01	1.00E+01	0.00E+00	6.00E+03	8.40E+03	6.10E+03
120	3.00E+01	2.00E+01	9.00E+01	0.00E+00	0.00E+00	0.00E+00	5.70E+03	4.10E+03	3.00E+03

Time (min) after addition of camphor	CspE Induced			CspF Induced			CspG Induced		
	Cells / ml LB			Cell/ ml LB			Cell/ ml LB		
	Trial 1	Trial 2	Trial 3	Trial 1	Trial 2	Trial 3	Trial 1	Trial 2	Trial 3
0	7.80E+07	1.61E+08	7.70E+07	6.70E+07	5.80E+07	3.00E+07	6.00E+07	7.80E+07	6.00E+07
30	2.18E+04	3.40E+04	4.90E+04	1.00E+01	3.00E+01	1.00E+01	5.60E+03	5.30E+03	7.40E+03
60	4.60E+03	8.70E+03	1.58E+04	0.00E+00	0.00E+00	0.00E+00	3.50E+03	2.08E+03	2.89E+03
90	3.10E+03	4.70E+03	9.30E+03	0.00E+00	0.00E+00	0.00E+00	4.80E+03	3.30E+03	5.70E+03
120	2.12E+03	3.12E+03	6.10E+03	0.00E+00	0.00E+00	0.00E+00	3.20E+03	2.06E+03	3.37E+03

Time (min) after addition of camphor	CspH Induced			CspI Induced		
	Cells / ml LB			Cells / ml LB		
	Trial 1	Trial 2	Trial 3	Trial 1	Trial 2	Trial 3
0	6.80E+07	1.25E+08	1.02E+08	3.60E+07	4.20E+07	4.80E+07
30	1.00E+01	1.00E+02	1.50E+02	1.62E+04	2.33E+04	2.32E+04
60	0.00E+00	0.00E+00	0.00E+00	9.90E+03	1.03E+04	2.70E+03

Table 9. Cell viabilities calculated for *csp*-containing clones exposed to camphor

Time (min)	CV1 Induced			CV2 Induced			pBAD24 Induced		
	Trial 1	Trial 2	Trial 3	Trial 1	Trial 2	Trial 3	Trial 1	Trial 2	Trial 3
0	1.00E+00	1.00E+00	1.00E+00	1.00E+00	1.00E+00	1.00E+00	1.00E+00	1.00E+00	1.00E+00
30	5.00E-05	3.60E-05	5.10E-05	5.60E-03	4.60E-03	1.30E-02	6.40E-05	8.50E-06	7.50E-05
60	1.90E-05	1.10E-05	2.70E-05	9.60E-03	4.70E-03	1.20E-02	4.80E-06	2.90E-06	1.80E-05
90	1.60E-05	1.20E-05	4.30E-05	9.00E-03	9.00E-03	1.00E-02	7.00E-06	1.30E-06	8.20E-06
120	6.40E-06	2.50E-06	8.60E-06	1.30E-02	6.00E-03	1.60E-02	3.60E-07	2.50E-07	6.20E-07
Time (min)	CspA Induced			CspB Induced			CspC Induced		
	Trial 1	Trial 2	Trial 3	Trial 1	Trial 2	Trial 3	Trial 1	Trial 2	Trial 3
0	1.00E+00	1.00E+00	1.00E+00	1.00E+00	1.00E+00	1.00E+00	1.00E+00	1.00E+00	1.00E+00
30	3.00E-06	7.90E-06	2.00E-05	2.10E-07	1/E-6	3.20E-07	6.40E-04	1.40E-03	1.00E-03
60	8.00E-07	6.60E-07	2.50E-06	0.00E+00	4.40E-07	0.00E+00	8.70E-05	8.50E-05	7.80E-05
90	3.00E-07	2.80E-07	9.40E-07	2.10E-07	2.20E-07	0.00E+00	5.60E-05	5.60E-05	6.10E-05
120	3.00E-07	1.90E-07	9.40E-07	0.00E+00	0.00E+00	0.00E+00	5.30E-05	2.70E-05	2.90E-05
Time (min)	CspE Induced			CspF Induced			CspG Induced		
	Trial 1	Trial 2	Trial 3	Trial 1	Trial 2	Trial 3	Trial 1	Trial 2	Trial 3
0	1.00E+00	1.00E+00	1.00E+00	1.00E+00	1.00E+00	1.00E+00	1.00E+00	1.00E+00	1.00E+00
30	2.80E-04	2.10E-04	6.40E-04	1.50E-07	5.20E-07	3.30E-07	9.30E-05	6.80E-05	1.20E-04
60	5.90E-05	5.40E-05	2.10E-04	0.00E+00	0.00E+00	0.00E+00	5.80E-05	2.70E-05	4.80E-05
90	4.00E-05	2.90E-05	1.20E-04	0.00E+00	0.00E+00	0.00E+00	8.00E-05	4.20E-05	9.50E-05
120	2.70E-05	1.90E-05	7.90E-05	1.50E-07	0.00E+00	0.00E+00	5.30E-05	2.60E-05	5.60E-05
Time (min)	CspH Induced			CspI Induced					
	Trial 1	Trial 2	Trial 3	Trial 1	Trial 2	Trial 3			
0	1.00E+00	1.00E+00	1.00E+00	1.00E+00	1.00E+00	1.00E+00			
30	1.50E-07	7/E-7	1.50E-06	4.50E-04	5.50E-04	4.80E-04			
60	0.00E+00	0.00E+00	0.00E+00	2.80E-04	2.50E-04	5.60E-05			
90	0.00E+00	0.00E+00	9.80E-08	1.60E-04	2.00E-04	1.90E-04			
120	0.00E+00	0.00E+00	0.00E+00	2.60E-04	2.50E-04	2.10E-04			

Table 10. Average cell viability determined for *csp*-containing clones after 120 minutes of camphor exposure

Sample	Fold Difference from pBAD24	Average Cell Viability
pNT2	*NA	$7.73 \times 10^{-3} \pm 5.00 \times 10^{-3}$
pBR322	NA	$5.67 \times 10^{-6} \pm 3.51 \times 10^{-6}$
pBAD24	NA	$3.03 \times 10^{-7} \pm 5.50 \times 10^{-8}$
A	1.5	$4.80 \times 10^{-7} \pm 4.01 \times 10^{-7}$
B	<1	Undetectable
C	172	$5.20 \times 10^{-5} \pm 4.16 \times 10^{-6}$
E	161	$4.87 \times 10^{-5} \pm 4.46 \times 10^{-5}$
F	<1	$5.00 \times 10^{-8} \pm 8.67 \times 10^{-8}$
G	249	$7.53 \times 10^{-5} \pm 4.27 \times 10^{-5}$
H	<1	$5.00 \times 10^{-8} \pm 8.66 \times 10^{-8}$
I	881	$2.67 \times 10^{-4} \pm 5.77 \times 10^{-5}$

*N/A – Not applicable

Averages were obtained by averaging the individual viabilities determined for each of the three trials

Table 11. Pixel densities obtained from densitometry of protein extracts obtained across various phases of cell growth and electrophoresed on SDS-PAGE gels

Mean Pixel Density of Entire Lane Obtained from Densitometry

Time (Growth Phase)	pBAD24 Uninduced	pBAD24 Induced	CspA Uninduced	CspA Induced	CspB Uninduced	CspB Induced	CspC Uninduced	CspC Induced
Early Log	48755	43756	39242	46335	26541	15348	25466	35923
Mid-Log	41375	37765	50518	38369	22556	21700	18245	23961
Late Log	40926	42982	40668	29972	19271	25568	14034	21423
Stationary	44288	35910	49598	36001	11177	9994	10069	16696

Mean Pixel Density of Csp Protein Obtained from Densitometry

Time (Growth Phase)	pBAD24 Uninduced	pBAD24 Induced	CspA Uninduced	CspA Induced	CspB Uninduced	CspB Induced	CspC Uninduced	CspC Induced
Early Log	603	536	920	988	0	0	240	0
Mid-Log	644	428	1172	994	0	0	0	1752
Late Log	546	547	937	677	0	0	0	1023
Stationary	795	268	1188	1904	0	0	0	387

Ratio of Csp Protein to Total Protein Determined by Densitometry

Time (Growth Phase)	pBAD24 Uninduced	pBAD24 Induced	CspA Uninduced	CspA Induced	CspB Uninduced	CspB Induced	CspC Uninduced	CspC Induced
Early Log	0.012	0.012	0.023	0.021	0	0	0.009	0
Mid-Log	0.016	0.011	0.023	0.026	0	0	0	0.073
Late Log	0.013	0.013	0.023	0.023	0	0	0	0.048
Stationary	0.018	0.007	0.024	0.053	0	0	0	0.023

Mean Pixel Density of Entire Lane Obtained from Densitometry

Time (Growth Phase)	CspE Uninduced	CspE Induced	CspF Uninduced	CspF Induced	CspG Uninduced	CspG Induced	CspH Uninduced	CspH Induced	CspI Uninduced	CspI Induced
Early Log	34680	36578	6330	41192	18159	9265	15690	5730	20669	15730
Mid-Log	45426	23718	15623	16715	11608	11685	12089	21605	13347	29230
Late Log	30064	26683	24232	28424	11232	8231	23642	19787	15897	18650
Stationary	26006	36518	22227	19846	14665	17360	16114	19390	17791	28350

Mean Pixel Density of Csp Protein Obtained from Densitometry

Time (Growth Phase)	CspE Uninduced	CspE Induced	CspF Uninduced	CspF Induced	CspG Uninduced	CspG Induced	CspH Uninduced	CspH Induced	CspI Uninduced	CspI Induced
Early Log	259	344	0	0	33	0	0	0	0	60
Mid-Log	485	187	0	0	0	0	0	0	0	190
Late Log	165	478	0	0	0	74	0	0	0	1610
Stationary	99	517	0	0	0	1686	0	0	0	2510

Ratio of Csp Protein to Total Protein Determined by Densitometry

Time (Growth Phase)	CspE Uninduced	CspE Induced	CspF Uninduced	CspF Induced	CspG Uninduced	CspG Induced	CspH Uninduced	CspH Induced	CspI Uninduced	CspI Induced
Early Log	0.004	0.008	0	0	0.002	0	0	0	0	0.004
Mid-Log	0.005	0.009	0	0	0	0	0	0	0	0.007
Late Log	0.011	0.018	0	0	0	0.009	0	0	0	0.080
Stationary	0.007	0.014	0	0	0	0.086	0	0	0	0.080