Journal of Experimental Microbiology and Immunology (JEMI) Vol. 18: 129 – 133

Construction of a pBAD24 Vector Containing σ^{32}: Restoration of Function in σ^{32} Knockout *Escherichia coli*

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Many regulatory elements in *Escherichia coli* that mediate a wide array of responses when subjected to external stress have been progressively discovered and characterized. One of many external stresses that induce a specific response in *E. coli* is heat shock. This response is largely controlled by σ^{32}. Studies have shown that the *rpo*H knockout mutant *E. coli* K165 exhibits inhibited growth at 37°C, a normal growth temperature for wild type *E. coli*. This suggests that σ^{32}, the transcription factor encoded by *rpo*H, is responsible for the downstream activation of various heat shock response genes that allow normal cellular function and growth at elevated temperatures. To study the effects of σ^{32}, we constructed a plasmid containing *rpo*H positively regulated through the arabinose induction system, pMWLD13w. We inserted full length *rpo*H into a conventional pBAD24 cloning expression vector. Phenotype restoration testing showed that *E. coli* K165 transformed with pMWLD13w had a comparable growth rate to the wild type *E. coli* SC122.

When cells are subjected to environmental stress, such as a sudden increase in temperature, heat shock genes express a set of proteins that are collectively known as the heat shock proteins (HSPs). HSPs exhibit sophisticated mechanisms to protect and maintain normal cellular functions, which include preventing non-specific protein aggregation as well as repairing misfolded proteins (1). In *E. coli*, the heat shock genes are functionally divided into two regulons, one of which is controlled by σ^{32}. σ^{32} has been established to be essential for growth at elevated temperatures (2), but the role it plays at normal physiological temperature remains debatable.

The *E. coli* transcription factor σ^{32} is encoded by the *rpo*H gene. Initial discovery of σ^{32} revealed that overproduction of σ^{32} upon temperature upshift results in overexpression of heat shock proteins, including molecular chaperones and ATP-dependent proteases (3, 4). The heat shock response chaperone system is a consequence of an increase in intracellular σ^{32} levels and activity (5) and acts to alleviate stress-induced damage caused by misfolded or unfolded proteins, allowing an increase in membrane fluidity while maintaining cellular functions (4).

It has been previously noted that overproduction of σ^{32} leads to the increased expression of heat shock proteins and that the half-life of σ^{32} is temperature dependent (6). Also, the *E. coli* Δ*rpo*H mutant strain K165 exhibits significant reduction in viability compared to wild-type when subjected to heat stress upon transfer to a higher temperature (7). These observations suggest that σ^{32} plays a role in the heat stress response in order to maintain normal cellular function; however, as observation of the system involves additional complicating factors, including temperature, the exact mechanism is unclear. In order to study the effects of σ^{32}, we inserted the *rpo*H gene into pBAD24 and the resulting plasmid was named pMWLD13w. The expression vector pBAD24 has an arabinose inducible promoter which allows control of σ^{32} expression. If functional σ^{32} can be produced by pMWLD13w, then the growth rate and viability of these transformed cells should be comparable to that of *E. coli* SC122. An increase in the 32 kDa σ^{32} protein should also be visible on an SDS-PAGE gel. The introduction of pMWLD13w will be used to develop a controllable system to investigate expression of σ^{32} and its effects on transformation efficiency independent of the heat shock response.

MATERIALS AND METHODS

Bacterial strains and culture conditions used. *E. coli* SC122, K165, and DH5α strains were obtained from the MICB 421 culture collection in the Department of Microbiology and Immunology at the University of British Columbia. *E. coli* SC122 and K165 (Δ*rpo*H) have been previously described (5, 7). These two strains are identical except for an amber mutation in *E. coli* K165 which renders *rpo*H inactive. All strains were grown at 30°C in Luria-Bertani (LB) medium.

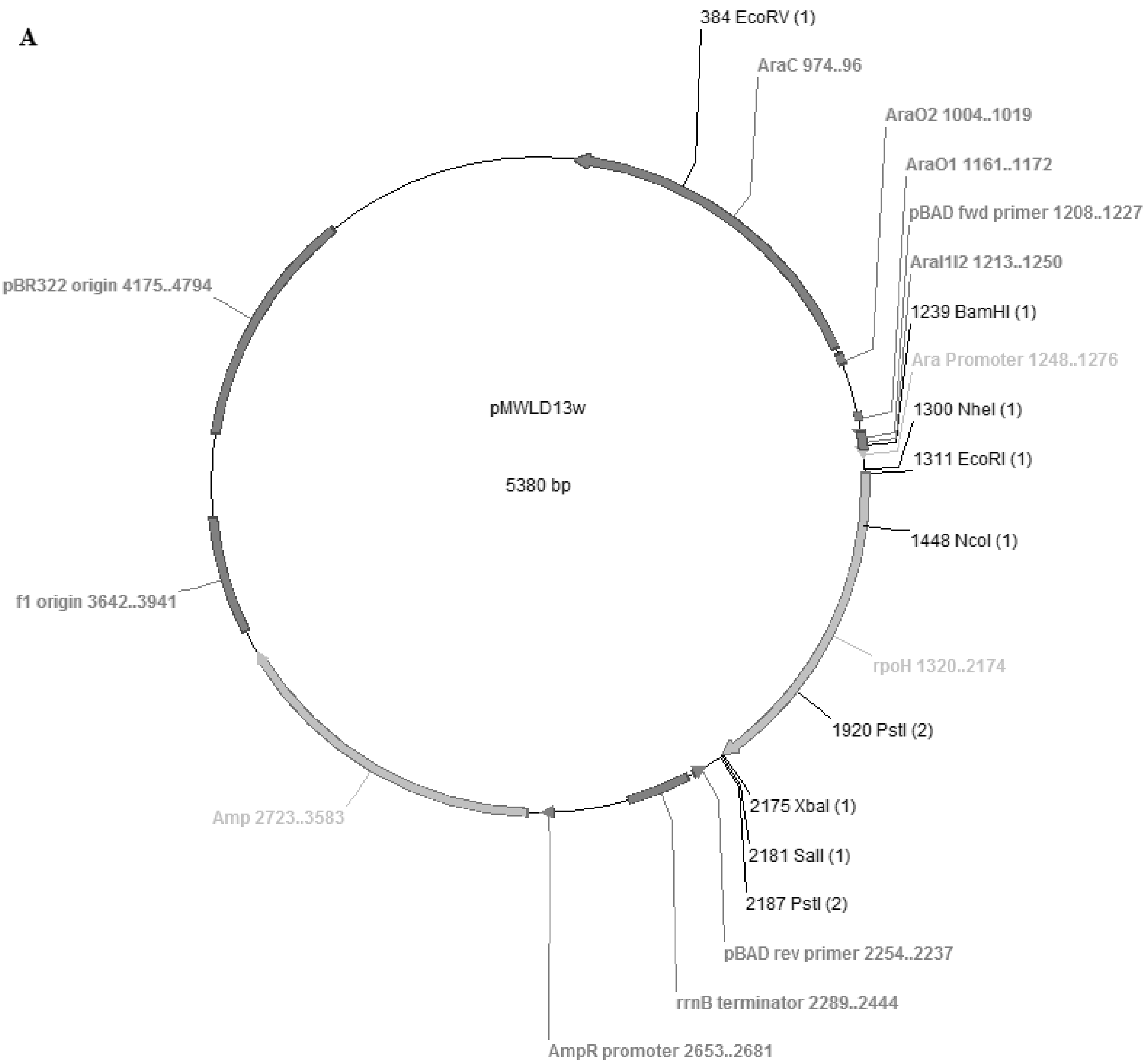
Antibiotic stock solution preparation. Stock solutions of ampicillin (Sigma-Aldrich) were prepared in distilled water at a final concentration of 100 mg/ml and then filter sterilized. Stock solutions were diluted to working concentrations of 100 μg/ml.

pBAD24 plasmid purification. *E. coli* DH5α-containing pBAD24 were provided by the MICB 421 culture collection in the Department of Microbiology and Immunology at the University of British Columbia. These cultures were grown overnight in 5 ml of LB at 37°C containing 100 μg/ml ampicillin. Plasmid purification was performed using GeneJET Plasmid Miniprep Kit (Thermo Scientific).

Preparation of competent cells. Competent *E. coli* SC122, K165, and DH5α were prepared as previously described (3) with slight modifications. One millilitre of an overnight culture was inoculated into 29 ml of LB broth. The strains were grown until they reached an OD_{600} of 0.4, and then pelleted by centrifugation at 3000 rpm for 10 minutes. Cells were then resuspended in 4 ml of sterile 0.1 M $CaCl_2$, incubated on ice for 30 minutes, re-pelleted, and resuspended in 2 ml 0.1 M $CaCl_2$ with 10% (w/v) glycerol.

PCR amplification and purification of *rpo*H. *E. coli* SC122 was grown overnight at 30°C on LB agar. A colony was picked, added to a PCR mix of 1X *Pfu* Polymerase Buffer (Fermentas), 200 μM dNTP and 200 nM of both forward and reverse primers. Designed primers with EcoRI and XbaI restriction sites were used for cloning into the pBAD24 vector. The forward primer, 5'–TTAGGCGAATTCACC***ATG***ACTGACAAAATGCAAAGTTTA–3' and reverse primer 5'–

A



B

5’ terminal of *rpo*H

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t t g g g c t a g c a g g a g g a a t t c a c c A T G A C T G A C A A A A T G C A A A G T T
| | | | | | | | | | | | | | | | | | | | | # | | | | | | | | | | | | | | | | | | | | | | | |
t t g g g c t a g c a g g a g g a a t t c n c c A T G A C T G A C A A A A T G C A A A G T T
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3’ terminal of *rpo*H

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G C G T G C T G C C A T T G A A G C G T A A t c t a g a g t c g a c c t g c a g g c a t g c
| | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
G C G T G C T G C C A T T G A A G C G T A A t c t a g a g t c g a c c t g c a g g c a t g c
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FIG. 1 **Plasmid map and sequence identity of *rpo*H and pBAD24. (A)** *rpo*H was inserted into the MCS of pBAD24, EcoRI and XbaI restriction sites are depicted at the ends of the inserted gene. **(B)** Results of sequencing for *rpo*H from GENEWIZ. Displayed regions are at the 5’ and 3’ terminals of *rpo*H. Capitalized base pairs are part of the *rpo*H gene and number signs indicate unknown sequence identities due to poor base quality scoring at that position. Top lines are that of the reference sequence and bottom lines are sequencing results.

AATTCA<u>TCTAGA</u>*TTACGCTTCAATGGCAGCACG*–3’ were used for amplification of *rpo*H. Underlined regions are restriction sites for EcoRI and XbaI, respectively, italicized regions indicate bases complimentary to *rpo*H and bold bases indicate the start codon of *rpo*H. PCR reactions were performed in the Bio-Rad® Gene Cycler™ Thermal Cycler (Cat #170-6700). Initial denaturation was performed for 10 minutes at 92°C, followed by 36 cycles of denaturation at 92°C for 30 seconds, annealing at 61.5°C for 30 seconds, extension at 72°C for 3 minutes, and a final extension at 72°C for 7 minutes. Amplification was confirmed by visualization of an 855-bp *rpo*H gene on agarose gel. The PCR product was purified using GeneJET PCR Purification Kit (Thermo Scientific).

Construction of pMWLD13w. A double digest was performed on pBAD24 and the *rpo*H PCR product using EcoRI (Invitrogen) and XbaI (Invitrogen) restriction endonucleases in Buffer E (Promega) and BSA (Promega) at 1 mg/ml. The digest was carried out at 37°C for 2 hours and the digested product was verified on an agarose gel. Digested products were purified using GenElute Gel Extraction Kit (Sigma). Digested pBAD24 and *rpo*H were directionally ligated in T4 ligase buffer with T4 DNA ligase (Fermentas) at a 5:1 insert-to-vector molar ratio. The constructed plasmid was named pMWLD13w. Positive clones

were screened for by restriction enzyme digestion using EcoRV (Promega) and NcoI (Promega). Insert sequence identity was verified by sequencing (GENEWIZ, Cal, USA).

Transformation of DH5α, SC122, and K165 with pMWLD13w. 200 µl of competent *E. coli* cells were incubated on ice with 30 ng of plasmid DNA or 50 ng of ligated product for 30 minutes. Cells were then quickly transferred to 42°C for 45 seconds and then placed back on ice for 2 minutes. 300 µl of fresh LB was added and cells were recovered at 30°C for 1 hour. Cells were plated on LB agar with 100 mg/ml ampicillin, colonies were picked and streak-plated the following day. *E. coli* DH5α, K165, and SC122 transformed with pMWLD13w were named *E. coli* MWLD13w-1, MWLD13w-2, and MWLD13w-3, respectively.

Protein expression test. MWLD13w-2 was grown at 30°C to an OD_{600} of 0.2, then either induced with 0.1% (w/v) arabinose or not induced. A time course experiment was performed with arabinose stimulation for 0 minutes, 30 minutes, and 60 minutes. Cells were centrifuged at 10,000 rpm for 2 minutes and the supernatant removed. Cells were then resuspended in 50 µl of SDS sample buffer and lysed by heating at 95°C for 8 minutes. 15 µl of the cell lysate was loaded onto a 10% SDS-PAGE gel. Electrophoresis was carried out at 200 V for 50 minutes, then the gel was stained in Coomassie blue solution.

Growth rate analysis. A half a millilitre of overnight culture was added to 29.5 ml fresh LB, either with or without 0.1% (w/v) arabinose. *E. coli* strains were grown at 37°C. OD_{600} was measured by the Ultrospec 2000 UV/Visible (Biochrom) every 30 minutes for the duration of the experiment. When 3 consecutive time points were approximately equivalent or the second dropped below the first, the measurements were no longer taken.

RESULTS

Sequencing of *rpo*H in pMWLD13w showed sequence identity and proper insertion. Sequencing results from GENEWIZ indicated that the digestion and ligation of *rpo*H and pBAD24 was successful and that no mutations were introduced during the cloning procedure. The full constructed plasmid is shown in Fig. 1A. *rpo*H was successfully cloned into the multiple cloning site (MCS) of pBAD24 between the EcoRI and XbaI restriction sites. Fig. 2A shows an excerpt of the sequencing results of *rpo*H. The sequencing primer, pBAD reverse primer, was provided by GENEWIZ and its location is indicated on Fig. 1A. The regions corresponding to the ligation point between *rpo*H and pBAD24 are displayed. One unidentified base was observed in the sequencing results. Analysis of the chromatograph indicated that the peak in question corresponded to a shoulder of the following peak which identified the same base. The only increase in signal observed at that position was for the correct base, and all other signals were at baseline levels. This suggests that proper inducible expression of *rpo*H can be expected. However, the sequencing results also showed poor consensus between the promoter and operator regions of pBAD24 and the sequenced fragment. Because these bases are near the end of the sequence run, poor base qualities were expected and no mutations in these areas were assumed.

***E. coli* σ^{32} knockouts complemented with pMWLD13w with arabinose-inducible promoter showed putative σ^{32} protein expression.** *E. coli* MWLD13w-2 was induced with arabinose and samples

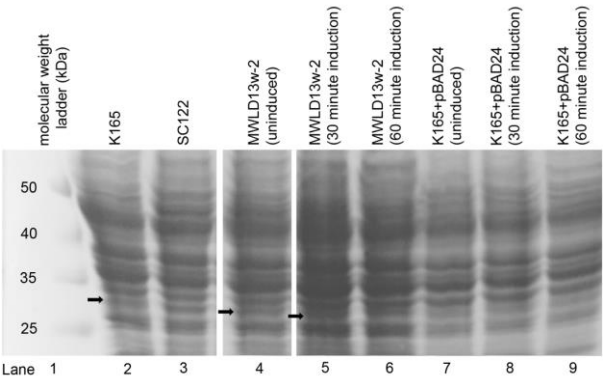

FIG. 2 **Time course of σ^{32} expression visualized on SDS-PAGE.** Putative σ^{32} protein appears to be present at similar levels in strain SC122 and strain MWLD13w-2 regardless of induction time. A faint band is observed at the same position in strain K165 and strain K165 + pBAD24 samples. Cell lysate proteins were stained using Coomassie blue solution. Arrows indicate locations of putative σ^{32} protein.

were taken at three time points. We detected a band in all of the time points that was not present in the protein samples from *E. coli* K165 with the empty control vector pBAD24 (Fig. 2A). The band size is consistent with the 32 kDa size of σ^{32}. However, the method of Coomassie blue staining is not specific for σ^{32}. The difference in band density across the *E. coli* MWLD13w-3 cell lysate induced for various durations is minimal, suggesting that the observed band is contributed by constitutive expression of σ^{32} and that production of σ^{32} occurs with or without induction with arabinose. Quantification of band density showed no significant differences across all samples (data not shown).

***E. coli* MWLD13w-2 grew successfully at 37°C.** A functional assay measuring the growth rate at 37°C of *E. coli* MWLD13w-2 compared to *E. coli* K165 and *E. coli* SC122 was performed to determine if functional σ^{32} was being produced (Fig. 3). Previously, *E. coli* K165 was shown to have reduced growth and viability at higher temperatures (8). Unexpectedly, *E. coli* MWLD13W-2 grew at the same rate whether or not they were induced with arabinose (data not shown). The increased growth of *E. coli* MWLD13W-2 compared to *E. coli* K165 and *E. coli* K165 with empty pBAD24 (Fig. 3A and B) suggests that pMWLD13w was able to successfully restore the phenotype observed in *E. coli* SC122. A slight increase in the initial growth of both wild-type *E. coli* K165 and that transformed with pBAD24 is observed and may have been due to their growth in rich media and the lack of misfolded protein in the initial stages of growth following transfer from 30°C. Another explanation for this observation could be the time it takes for the culture to go from 30°C to 37°C.

DISCUSSION

Our results confirm that we have successfully cloned *rpo*H into the pBAD24 expression vector, and that it can restore the heat survival phenotype of the *rpo*H

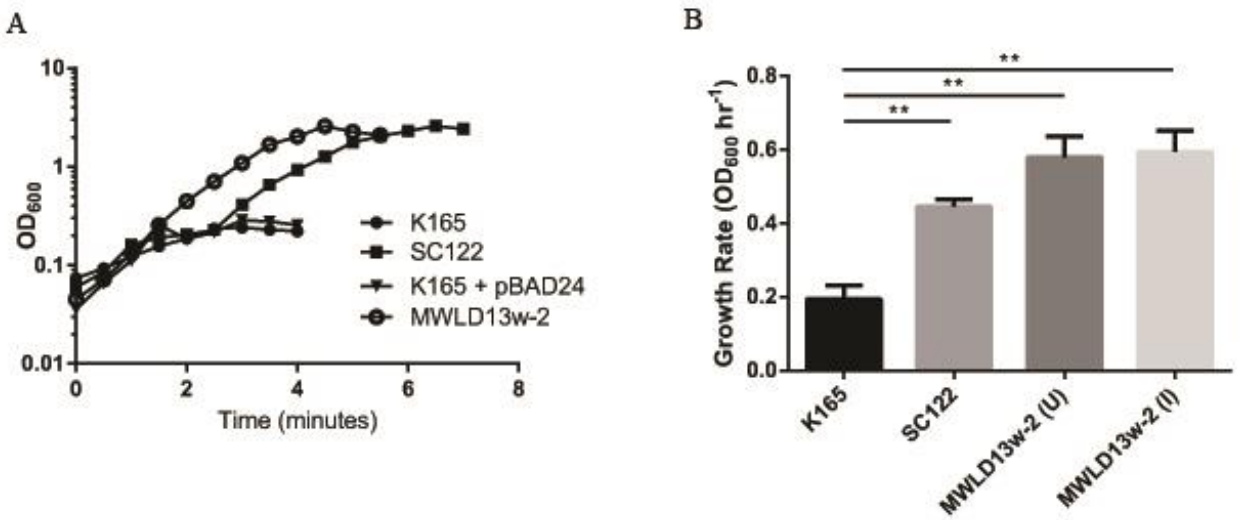

FIG. 3 **Growth of different *E. coli* strains at 37°C. (A)** *E. coli* MWLD13w-2 grew at a comparable rate to that of *E. coli* SC122, suggesting that the reintroduction of *rpo*H allowed *E. coli* K165 to restore its ability to grow at higher temperatures. **(B)** The growth rates of each wild-type *E. coli* strain K165 was compared to *E. coli* strains SC122, MWLD13w-2 induced (I) and uninduced (U). A significant difference in growth rates of those strains containing functional σ^{32} relative to *E. coli* K165 was observed using one-tailed student's t-test. No difference was observed between *E. coli* SC122 and MWLD13w-2 strains. Growth rate values are averaged between single replicates of two separate experiments +/- SD, statistical significance is denoted as ** $p < 0.005$.

knockout mutant *E. coli* K165 to a level comparable to wild type (Fig. 2). This suggests that pMWLD13w encodes a functional σ^{32} that activates the heat shock response.

In a similar study investigating antibiotic stress induction of *rpo*H in *E. coli*, it was proposed that pre-treatment with kanamycin mimics the heat response by enhancing accumulation of σ^{32}, but this did not correlate to higher heat tolerance (7). Our results contradict their conclusion as the expression of σ^{32} in the *E. coli* K165 mutant was able to restore its ability to grow at 37°C, suggesting that σ^{32} is associated with higher heat tolerance of *E. coli*. However, this may be true only to a certain temperature threshold because our results do not encompass temperatures higher than 37°C, while the previously mentioned paper (7) performed their experiment at 45°C, a temperature generally intolerable by wild-type *E. coli*.

Unexpectedly, the growth rate of uninduced *E. coli* MWLD13w-2 was comparable to that of the *E. coli* MWLD13w-2 induced with arabinose (Fig. 3B). Based on the results from the negative controls, σ^{32} expression is essential for normal growth at elevated temperature (Fig. 3A). Therefore, we suspect that the positively regulated promoter that governs *rpo*H expression is being activated, allowing constitutive expression of σ^{32}. Potential mechanisms that bypass the designed positive regulation system may include plasmid integration, where a single homologous recombination event can integrate segments of the transformed plasmid (*rpo*H) into host genome. If integrated downstream of the start codon and upstream of the amber mutation, *rpo*H could be expressed within the bacteria at the same levels as *rpo*H$^+$ strains because of the identical regulatory elements. Due to the ambiguity of the sequencing upstream of the *rpo*H gene, there may also have been mutations that altered the arabinose operator that resulted in stronger affinity for AraC (Fig. 1A). Based on the analysis of Coomassie blue-stained SDS-PAGE, σ^{32} protein band increases in intensity with treatment of arabinose. However, due to the non-specific nature of Coomassie blue staining technique, we cannot conclude the presence of σ^{32} solely based on the presence of the visible band at 32 kDa. In addition, the non-specificity of Coomassie blue results in the staining of other intracellular proteins, leading to potential masking of σ^{32} by other proteins with similar molecular weights.

Our protein expression test results do not confidently show that σ^{32} is being produced by cells containing pMWLD13w. There are a number of different proteins in *E. coli* that are 32 kDa, such as succinyl-CoA synthetase, that may be confused with σ^{32}. Also, in previous studies, σ^{32} has been shown to have a half-life of 1 to 4 minutes and decreases with an increase in temperature (10, 11, 12). This temperature-sensitive protein may have degraded during the lysis and denaturation steps of this experiment. Overloading the gel with sample may have also contributed to the difficulty in interpreting the results. Another limitation that may have contributed to the ambiguity of our results is the nature of the *rpo*H mutants. The proteins within these temperature-sensitive mutants have extensive aggregation, leading to inclusion body formation at the non-stress temperature of 30°C (13).

This characteristic may have detrimental effects on cellular function. It is possible that the system might be under control of arabinose, but the σ^{32} bands are too low in quantity to be visible on the gel. This would need to be tested by measuring σ^{32} via western blot.

Our results indicate that pMWLD13w contains the *rpo*H gene and produces functional σ^{32} based on the restoration of function test. However, our results also indicate that *rpo*H may not be under the control of an inducible arabinose promoter.

FUTURE DIRECTIONS

Before pMWLD13w is used in further experiments, such as looking at the role of σ^{32} in heat shock transformation, it is necessary to understand why there is constitutive expression of σ^{32}. In order to confirm that there are no mutations in the promoter or operator regions of the gene, sequencing upstream of the *rpo*H open reading frame can be performed.

Further assays can also test that the plasmid construct is expressing functional σ^{32}. The protein expression data reported here shows some ambiguity in interpretation due to the high level of background present in all of the samples. This test should be repeated, this time followed by western blot analysis with an antibody to σ^{32} to conclusively identify the presence of σ^{32}. This will also help to establish if there is any σ^{32} escaping from pBAD regulation. An alternative strain can be made to eliminate some confounding variables. Amber mutations are often unstable and stop codons will still result in truncated proteins. Transposition mutagenesis can be created to remove the remaining *rpo*H and accompanying regulatory regions.

Once induction of various levels of σ^{32} can be controlled, other HSPs may also be probed to show that expressing σ^{32} by pMWLD13w increases their expression. The presence or increased expression of downstream chaperone proteins, such as DnaK or GroES, and σ^{32} regulation factors, such as FtsH, will provide cumulative evidence that the expressed σ^{32} protein is functional. Using pMWLD13w, experiments can be done to study the downstream effects of expressing σ^{32}. Many proteins are expressed as a result of heat shock, yet the function of many of these proteins remains poorly characterized. Additional studies can investigate the effect of expressing σ^{32} above a normal physiological level.

Heat shock transformation is a common laboratory technique involving heat shock and overexpression of σ^{32} during this procedure may help in increasing transformation efficiencies. σ^{32} expression is rapid in response to increases in temperature; however, its involvement in the heat shock response is unclear. By using pMWLD13w, the effects of σ^{32} independent of the heat shock process can be studied. σ^{32} expression may be largely responsible for the survival of *E. coli* following heat shock.

ACKNOWLEDGEMENTS

This study was supported by the Department of Microbiology and Immunology, University of British Columbia, Vancouver, Canada. We wish to thank Dr. William Ramey and Kirstin Brown for their invaluable guidance and support throughout the course of this project. We would also like to thank Nick Cheng, Lando Robillo, and group 3γ for their technical support.

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