Construction of a pBAD24 Vector Containing $\sigma^{32}$: Restoration of Function in $\sigma^{32}$ 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 $\sigma^{32}$. Studies have shown that the rpoH knockout mutant E. coli K165 exhibits inhibited growth at 37°C, a normal growth temperature for wild type E. coli. This suggests that $\sigma^{32}$, the transcription factor encoded by rpoH, 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 $\sigma^{32}$, we constructed a plasmid containing rpoH positively regulated through the arabinose induction system, pMWLD13w. We inserted full length rpoH 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 $\sigma^{32}$. $\sigma^{32}$ 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 $\sigma^{32}$ is encoded by the rpoH gene. Initial discovery of $\sigma^{32}$ revealed that overproduction of $\sigma^{32}$ 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 $\sigma^{32}$ 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 $\sigma^{32}$ leads to the increased expression of heat shock proteins and that the half-life of $\sigma^{32}$ is temperature dependent (6). Also, the E. coli $\Delta$rpoH 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 $\sigma^{32}$ 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 $\sigma^{32}$, we inserted the rpoH gene into pBAD24 and the resulting plasmid was named pMWLD13w. The expression vector pBAD24 has an arabinose inducible promoter which allows control of $\sigma^{32}$ expression. If functional $\sigma^{32}$ 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 $\sigma^{32}$ 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 $\sigma^{32}$ 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 DH5a 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 (ΔrpoH) have been previously described (5, 7). These two strains are identical except for an amber mutation in E. coli K165 which renders rpoH 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 DH5a-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 DH5a were prepared as previously described (3) with slight modifications. One milliliter 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 rpoH. 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'–TTAGCGAATTCCAGACGCTGCATGAGCACAAATTGCAGATTITTA–3' and reverse primer 5'–
AATTCA
TCTAGA
TTACGCTTCAATGGCAGCACG
–3’ were used for amplification of \(\text{rpoH}\). Underlined regions are restriction sites for EcoRI and XbaI, respectively. Italicized regions indicate bases complimentary to \(\text{rpoH}\) and bold bases indicate the start codon of \(\text{rpoH}\). 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 \(\text{rpoH}\) 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 \(\text{rpoH}\) 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 \(\text{rpoH}\) 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
DISCUSSION

Our results confirm that we have successfully cloned rpoH into the pBAD24 expression vector, and that it can restore the heat survival phenotype of the rpoH knockout. The full-length protein was expressed at levels similar to the wild-type strain, and the cells grew at the same rate whether or not they were induced with arabinose. The increased growth of E. coli MWLD13w-2 compared to E. coli K165 and E. coli SC122 was performed to determine if functional σ^H 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 SC122 was performed to determine if functional σ^H 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 SC122 was performed to determine if functional σ^H was being produced (Fig. 3).
knockout mutant *E. coli* K165 to a level comparable to wild type (Fig. 2). This suggests that pMWLD13w encodes a functional $\sigma^{32}$ that activates the heat shock response.

In a similar study investigating antibiotic stress induction of *rpoH* in *E. coli*, it was proposed that pretreatment with kanamycin mimics the heat response by enhancing accumulation of $\sigma^{32}$, but this did not correlate to higher heat tolerance (7). Our results contradict their conclusion as the expression of $\sigma^{32}$ in the *E. coli* K165 mutant was able to restore its ability to grow at 37°C, suggesting that $\sigma^{32}$ 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, $\sigma^{32}$ expression is essential for normal growth at elevated temperature (Fig. 3A). Therefore, we suspect that the positively regulated promoter that governs *rpoH* expression is being activated, allowing constitutive expression of $\sigma^{32}$. 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 (*rpoH*) into host genome. If integrated downstream of the start codon and upstream of the amber mutation, *rpoH* could be expressed within the bacteria at the same levels as *rpoH* strains because of the identical regulatory elements. Due to the ambiguity of the sequencing upstream of the *rpoH* 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, $\sigma^{32}$ 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 $\sigma^{32}$ 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 $\sigma^{32}$ by other proteins with similar molecular weights.

Our protein expression test results do not confidently show that $\sigma^{32}$ 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 $\sigma^{32}$. Also, in previous studies, $\sigma^{32}$ 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 *rpoH* 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 $\sigma^{32}$ bands are too low in quantity to be visible on the gel. This would need to be tested by measuring $\sigma^{32}$ via western blot.

Our results indicate that pMWLD13w contains the rpoH gene and produces functional $\sigma^{32}$ based on the restoration of function test. However, our results also indicate that rpoH 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 $\sigma^{32}$ in heat shock transformation, it is necessary to understand why there is constitutive expression of $\sigma^{32}$. In order to confirm that there are no mutations in the promoter or operator regions of the gene, sequencing upstream of the rpoH open reading frame can be performed.

Further assays can also test that the plasmid construct is expressing functional $\sigma^{32}$. 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 $\sigma^{32}$ to conclusively identify the presence of $\sigma^{32}$. This will also help to establish if there is any $\sigma^{32}$ 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 rpoH and accompanying regulatory regions.

Once induction of various levels of $\sigma^{32}$ can be controlled, other HSPs may also be probed to show that expressing $\sigma^{32}$ by pMWLD13w increases their expression. The presence or increased expression of downstream chaperone proteins, such as DnaK or GroES, and $\sigma^{32}$ regulation factors, such as RtsH, will provide cumulative evidence that the expressed $\sigma^{32}$ protein is functional. Using pMWLD13w, experiments can be done to study the downstream effects of expressing $\sigma^{32}$. 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 $\sigma^{32}$ above a normal physiological level.

Heat shock transformation is a common laboratory technique involving heat shock and overexpression of $\sigma^{32}$ during this procedure may help in increasing transformation efficiencies. $\sigma^{32}$ 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 $\sigma^{32}$ independent of the heat shock process can be studied. $\sigma^{32}$ expression may be largely responsible for the survival of E. coli following heat shock.

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