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ACTIVATION OF *Escherichia coli*’s STATIONARY PHASE GENE *rpos*

by

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Abstract

ACTIVATION OF *Escherichia coli*'s STATIONARY PHASE GENE *proS*
by Derek W. Sykes

The *rpoS* gene encodes a stationary phase specific sigma factor of RNA polymerase and is a key regulator of *Escherichia coli*'s stationary phase responses. It has been observed under laboratory conditions that gene expression is induced by stressful environmental conditions and certain metabolic intermediates. Batch and continuous cultures were employed to investigate the effect of environmental conditions and high reactor acetate levels on the transcriptional activation of the *rpoS* promoter. In complex media, the *rpoS* gene was induced in the late exponential phase of batch growth. Chemostat experiments were then conducted in complex media to examine the effects of acetate, glucose, and variations in cell density due to media quality on gene expression. Residual acetate and glucose concentrations were found to modulate *rpoS* behavior. However, it was observed that under these particular experimental conditions, cell density had no appreciable effect on the transcriptional activation of the *rpoS* promoter.
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Background

Chapter 1

1.1 Introduction

In the last few decades, *E. coli* has been the model organism for the exploration of genetics. Due to the wealth of knowledge available on this host, genetic engineers have the necessary tools and opportunities available to custom design recombinant protein production systems, control metabolic fluxes, and selectively manipulate organisms to produce high yields of desired products under laboratory conditions. However, the information available on the unique genetic and physiological conditions associated with scaled-up production is scarce and a matter of practical concern. Particularly important is the development of high yield fermentation processes for the production of heterologous proteins. This often requires the optimization of growth conditions and host strain physiology for the production of both high biomass concentrations and high specific yields of the heterologous gene product.

Organisms employed in the large scale production of recombinant proteins are very likely to experience stressful culture conditions. These cells are often exposed to nutrient starvation, high cell densities, shifts in osmotic pressure, growth inhibitors, metabolic byproducts, and many other physical conditions deleterious to cell growth. Microorganisms are able to maintain their viability during environmentally stressful conditions through activation of specific genomic regions designed to protect the
organism from harm. This concept is clearly illustrated by the starvation induced entry of the cell into the stationary phase of growth. The enzyme products of stationary phase-induced genes mediate stress responses in the cell that ultimately produce phenotypical changes in the cell membrane, decrease the overall metabolic rate, increase protein turnover, and generally produce a more resistant cellular state. These phenotypical and metabolic shifts may critically affect recombinant protein yields.

The current study focuses on the factors affecting the transcriptional activation of the rpoS gene in E. coli. rpoS encodes a stationary phase specific sigma factor of RNA polymerase. The gene product itself has been recognized as a major regulator in the starvation response network of E. coli. Previous studies conducted in this laboratory indicated that deletion of rpoS and other stationary phase regulatory genes produce an expression-system dependent improvement of recombinant protein yields under characteristic scale-up conditions (Chou et al., 1995).

The goal of this research is to extend the knowledge base on the physiological and genetic triggers for this stationary phase response regulator in E. coli. The effects of several parameters on the induction of a rpoS::lacZ+ mutant strain of E. coli were investigated. Chief among these parameters is acetate, a molecule known to be a potent inhibitor of growth and cellular productivity. The industrially significant effects of cell density, glucose levels, and slow growth rates on the expression of the gene fusion were also examined. These results may be useful for developing optimal host/gene expression
conditions for large scale recombinant protein production. Chemostats where used extensively throughout this work since they provide a convenient means to fix the cellular growth rate and thus the internal transcriptional and translational "machinery" of the cell.

1.2 The Metabolic Production and Physiological Effects of Acetate

1.2.1 Formation of Acetate

*E. coli* like all other living organisms utilizes a central metabolic pathway to provide itself with the biosynthetic building blocks and energy required for growth. Formation of new cellular material involves the anabolism of a specific set of intracellular, monomeric precursors most of which are utilized for the synthesis of more complex polymeric structures. Under aerobic conditions, the inputs for this process of cellular growth are oxygen, inorganic ions, and carbon sources while the outputs are carbon dioxide, water, energy, and biosynthetic building blocks. *E. coli* can make use of a variety of compounds as its sole carbon source. These sources may include but are not limited to glucose, ethanol, acetate, pyruvate, and methanol.
Figure 1. Central metabolic pathway for glucose metabolism. Depicted here are the major pathways for glucose utilization and energy production including glycolysis, the pentose phosphate cycle, the TCA cycle, the electron transport chain, and the anaerobic fermentation pathway.

Under carbon limiting conditions like those found in a chemostat with low dilution rates, the carbon flux to the cells is balanced by biosynthesis and energy generation. However, when carbon flux exceeds the capacity of the central metabolic pathway the cell balances carbon overflow through other pathways. This concept is best illustrated in the case of metabolism with glucose as the main carbon source. Glucose is a particularly attractive substrate for most microbial fermentations by virtue of its relative low cost, and efficient energetic and cellular yields. However, the control of glucose uptake by *E. coli* is not well regulated and can easily exceed the capacity of the central metabolic pathway (Han...
et al., 1992; Chou et al., 1994). An overabundance of glucose has also been linked to the enhancement of glycolysis, suppression of the TCA cycle, and a decrease in both the activity of the pentose cycle and cytochrome content of facultative anaerobes (Doelle et al., 1981). When saturation of the central metabolic pathway occurs, the cell is forced to redirect the flux of carbon away from the central metabolic pathway. *E. coli* accomplishes this redirection through three major mechanisms.

1. Excess intermediates are redirected to storage polymers, for example, glycogen (Dawes and Senior, 1973; Dietzler et al. 1979).

2. The excess carbon is oxidized to carbon dioxide and the excess energy given off as heat (Neijssel and Tempest, 1979; Stouthamer, 1979; Roels, 1980; Tempest and Neijssel 1984).

3. The surplus intermediates of the central metabolic pathways are converted into excretable compounds that are then discharged into the extracellular space (Demain, 1972; Meyer et al.; 1984; Holms, 1986).

The mechanism of interest in this study is the balancing of carbon overflow by the excretion of acetate (also known as glucose mediated acidogenesis or the Crabtree effect).

According to Han et al. (1992), the central metabolic pathway can be further simplified by subdividing it into two major segments: the oxidative and the fermentative.
Figure 2. Simplification of intracellular carbon flux. Cellular carbon flux can be divided into two major pathways, the oxidative and the fermentative. Due to loose regulation of glucose uptake, carbon tends to overflow into the fermentative pathway and is excreted as acetate, a potent inhibitor of growth and cellular productivity.

The oxidative pathway provides the major anabolic and catabolic functions of the cell. During slow growth (low dilution rates in chemostats), the oxidative pathway meets the catabolic and anabolic demands for the cell. The production of acetate is virtually absent. Above a critical growth rate, the oxidative pathway is no longer able to satisfy the cellular requirements for biosynthesis. This limitation of capacity is most probably due to the intracellular accumulation of NADH in the presence of excess carbon sources. This hypothesis is supported by the fact that NADH inhibits some of the key enzymes of the TCA cycle and the electron transport chain (Doelle et al., 1981; Ko et al., 1993) The inhibition by NADH therefore results in inherent bottlenecks for carbon flux through the
TCA Cycle. At higher growth rates, the oxidative pathway approaches saturation and the cell is forced to allocate a portion of its carbon flux through the fermentative, acetic acid-formation pathway. When this shift in metabolism occurs, acetyl CoA is diverted from the TCA cycle to acetyl phosphate and then to acetate. This results in the production of one mole of ATP per mole of acetate (Ko, Bentley, and Weigand, 1993). The realigning of carbon fluxes allows the cell to generate the second highest amount of ATP and NADH for a given flux of carbon.

The excretion of acetate by *E. coli* under aerobic conditions has been studied in continuous (El- Mansi and Holms, 1989; Han *et al.*, 1992; Meyer *et al.*, 1984), batch (Adams and Hall, 1988), and fed-batch cultures (Macdonald and Neway, 1990; Shimizu *et al.*, 1988). Both batch and semi-batch (Heather *et al.*, 1990; Jensen and Carlsen, 1990; Shimizu *et al.*, 1988) experiments consistently indicate that acetate continuously accumulates along with biomass in the cell cultures. Meyer *et al.* (1984) found that in a continuous chemostat culture of *E. coli* K12 D1, acetate formation was initiated at a dilution rate of approximately 0.32 h\(^{-1}\) on defined media and 0.2 h\(^{-1}\) on complex media and increased dramatically with dilution rate until washout. Han *et al.* (1992) observed a similar trend for acetate flux in *E. coli* K12 for a variety of both defined and complex media.

In chemostat experiments, complex media initiates a larger shift to acetate formation at lower dilution rates compared to cells grown on defined media (Meyer *et al.*, 1984).
Complex media contain a variety of preassembled biosynthetic precursors that can be readily utilized by the cells. Cells grown aerobically on complex media divert a smaller portion of cellular energy and material flow to biosynthesis than cells grown on defined media. Consequently, cells grown on complex media experience higher levels of carbon flux through their central metabolic pathway and balance the overflow through acetate excretion.

1.2.2 Acetate and the Proton Motive Force

One phenomenon associated with acetate accumulation in culture media is the destruction of the cell’s proton motive force. The proton motive force is an intricate component of the bioenergetics of aerobically grown cells. E. coli like all other organisms generates intracellular energy equivalents as ATP and reducing equivalents as NADH from the glycolysis and metabolism of energy rich carbon substrates. Under aerobic conditions, NADH produced from this process is oxidized and recycled in the cell through oxidative phosphorylation. Here the cell utilizes a set of membrane-bound protein pumps collectively known as the electron transfer chain to oxidize NADH and subsequently reduce molecular oxygen. The transfer of electrons from NADH to oxygen provides the membrane-bound proteins with free energy for pumping protons from the interior to the exterior of the membrane. The difference in electrochemical potential of hydrogen ions across the membrane is known as the proton motive force (ΔP) - originally proposed by Mitchell (1976). The two contributions to the proton motive force are the difference in
hydrogen ion concentration across the membrane (ΔpH) and the electrical potential across the membrane (Δψ). The proton motive force can be exploited to do work in the form of active metabolite transport or ATP synthesis. The synthesis of ATP is catalyzed by a membrane-bound proton pumping ATP synthetase (ATPase). ATPase utilizes the free energy of the proton motive force to couple proton translocation back into the membrane to the endergonic generation of ATP.

When metabolites overflow the central metabolic pathway, acetate is readily produced by the cell. In its undissociated form, acetic acid and other short chained fatty acids are able to freely permeate the cellular membrane and diffuse into the environment. Accumulation of extracellular acetic acid forces the undissociated species to diffuse back into the cell. Acetic acid then dissociates and releases hydrogen ions into the cytoplasm. Thus acetic acid effectively acts as a proton conductor and destroys the ΔpH portion of the proton motive force (Slonzewski et al., 1981; Diaz-Ricci et al., 1990; Aristidou, 1994). The short circuit of hydrogen ions back into the cell via acetate reduces the energy yield and efficiency of cellular metabolism. This phenomenon is logically more pronounced at external pH’s lower than the pK value of acetic acid (4.75) where the majority of acetate present is in its protonated form.
Figure 3. Depiction of the electron transport chain with acetate short circuit. A membrane bound electron transport chain utilizes a proton motive force to couple NADH oxidation to ATP synthesis. The proton motive force is created from membrane bound proteins that pump hydrogen ions across the cellular membrane. Acetate molecules bind to the hydrogen ions and thus provide a short circuit for hydrogen ions across the membrane.

1.2.3 The Effect of Acetate on Growth and Product Formation

One of the key consequences of acetate accumulation in culture medium is the inhibition and in some cases the cessation of growth entirely. In fact acetic acid has been traditionally used as an antimicrobial agent in food storage and packing (Smuldens et al., 1986). Adams and Hall (1988) observed that for batch cultures of E. coli grown at pH 6, acetate concentrations as low as 2.1 g/l reduced the relative growth rate by over 50%. Koh et al. (1992) demonstrated that acetate addition to shake flask cultures not only inhibited host strains of E. coli K12 but also significantly reduced the growth rate of recombinant strains. Acetate's ability to inhibit microbial growth can best be appreciated in dense cell cultures. Here acetate accumulates in the medium more rapidly and to a higher extent due to the higher cell mass (Macdonald and Neway, 1990; Pan et al., 1987).
Luli and Stohl (1990) reported that in glucose-feedback-controlled fed batch cultures, acetate logarithmically inhibits the growth rates of both wild type and recombinant strains.

The second problem most often identified with acetate is the reduction of cellular productivity (Bauer et al., 1990; George et al. 1992). From a process point of view, acetate is a useless compound whose excretion decreases the process yield of recombinant *E. coli* (Konstantinov, *et al.*, 1990). In the case of shake flask cultures, acetate levels of 2 g/l have been known to reduce the overall yield of fusion protein TGFα-PE40 by as much as 38% (Sun *et al.*, 1993). Jensen and Carlsen (1990) investigated the effect of externally added sodium acetate on human growth hormone (hGH) production in recombinant *E. coli* MC1061 (a derivative of K12). The X-hGH gene used in this set of experiments contained a synthetic constitutive promoter and was harbored on the vector pAT153. Acetate concentration of 2.4 g/l during continuous runs produced no significant effect on reactor cellular concentrations, however specific hGH production rates dropped by 35%. A reactor acetate concentration of 6 g/l, the steady state bacteria concentration was reduced by 75% and the specific production rate of hGH decreased by more than 50%.
1.3 The Role of *rpoS* and the *rpoS* regulon in *E. coli*

1.3.1 Starvation in Bacteria

In nature, *E. coli* is commonly found in two environments. First, it has adapted to live symbiotically in the intestinal tracks of mammals. The maintenance of constant pH, temperature, and other physical conditions by the animal's body provides a nearly optimum environment for bacterial growth. However, even under these well-regulated conditions, the bacterium typically experiences short periods of substrate abundant growth followed by longer durations of extremely limited growth due to substrate starvation. Second, *E. coli* can exist as a free living organism. In this habitat, both environmental and substrate levels are apt to fluctuate uncontrollably. The survival of the bacterium is dependent on its ability to maintain viability during these periods of nutrient limitation that it often encounters. *E. coli* has evolved an inducible program designed to lower the cellular metabolism and produce a state more resistance to starvation and stresses in general (Siegele and Kolter, 1992).

Although *E. coli* does not in the strictest sense produce spores, starvation does induce characteristic changes in both the cell's physiology and morphology. The envelopes of starved cells show increased cross linking and distinct variation in phospholipid composition (Huisman and Kolter, 1994). The cytoplasm is condensed and the periplasm is increased (Reeve *et al.*, 1984). It has also been observed that under light microscopy,
starved *E. coli* become smaller and almost spherical (Ingraham *et al.*, 1983). Starved cells also tend to accumulate intracellular polymeric substances such as glycogen, polysulphurs, and polyphosphates (Steinbuckel and Schlegel, 1991).

One of biggest consequence of the starvation response is the redirection of cellular metabolism from maintenance of exponential growth to sustainment of stationary phase viability (Huisman and Kolter, 1994b). In other words, starvation generated signals initiate stationary phase specific responses in the cell. These responses create a metabolically less active but more resistant state. The cell does preserve an extremely reduced rate of endogenous metabolism to maintain some level of energy reserve and proton motive force. These reserves allow the cell to make use of alternative nutrient scavenging systems and give the cell the ability to resume growth once substrate levels become more favorable (Siegele and Kolter, 1992). It has been suggested that *E. coli* fuels this low level metabolism through the turnover of endogenous proteins and excess ribosomes. This view is supported by the observation that starved *E. coli* increases protein turnover by a factor of five compared to exponentially growing cells (Mandelstam, 1960). Reeves *et al.* (1984) using peptidase mutants, showed that protein degradation is required for survival of prolonged starvation. Increased protein turnover and reduced cellular metabolism are often detrimental to recombinant protein production in genetically engineered organisms due to the metabolic burden imposed on the cell by the cloned genes.
While both bulk protein and recombinant protein synthesis are slowed down in the cell in response to starvation, the synthesis of at least 50 identified proteins is induced (Davis et al., 1986; Groat et al., 1986). These induced proteins, including RpoS, GSH, DnaK, to name a few, mediate the above mentioned changes in cellular metabolism and physiology while also protecting the cell against chemical attack, heat shock, pH extremes, and a host of other challenges to cell viability.

The specific consequences, mechanisms, and means of control of these starvation-induced responses are an important topic of investigation in the field of microbiology and industrial protein production. From a pragmatic point of view, elucidation of stationary phase and starvation specific responses shed light upon recombinant protein production under fed-batch, nutrient limiting, and other slow growth conditions. Information on these types of responses may be useful in the design of optimal host/vector physiology. Furthermore, many antibiotics and industrially important secondary metabolites are produced by organisms in the stationary phase, including Streptomyces (Huisman and Kolter, 1994). Investigation into these mechanisms also provides insight on the complexities of gene regulation and on the incredible ability of prokaryotes to survive and flourish in every habitat on earth and under the harshest environmental conditions imaginable.

1.3.2 *rpoS* and the *rpoS* Regulation
The survival of *E. coli* and similar prokaryotes depend upon their ability to rapidly sense sudden environmental changes and respond appropriately. This feat is accomplished through rapid and efficient control of gene expression. Prokaryotes have thus evolved the ability to coordinate related gene expression into highly organized networks known as gene regulons. Here, genes in the regulon are activated in response to particular conditions sensed by the cell. These regulatory networks are favored over a single operon since the number of genes involved are often too large to be accommodated in a single operon. Furthermore, a single operon does not offer the robustness and flexibility that a regulatory network can provide (Nystrom, 1995). One of the better known regulatory network for *E. coli* includes the catabolically repressed enzymes and operons engaged in carbon source metabolism and energy synthesis (Nystrom, 1995). Regulons commonly function as stress induced responses to a variety of cellular challenges. Different regulatory networks have been identified for stresses due to temperature shifts (Neidhardt, 1987), DNA damage, oxidative stresses (Christman *et al.*, 1985), and nutrient starvation (Gottesman and Neidhardt, 1983). In the case of nutrient starvation, the regulons of *E. coli* do not appear to be exclusive to one particular stress signal and are typically interrelated through shared member genes.

A common motif in stress induced regulation of genes is the utilization of alternative σ subunits of RNA polymerase. These σ factors compete with the vegetative sigma subunit, σ^30 (gene product of *rpoD*), for a limited amount of RNA polymerase core enzyme in the cell. Once these alternative σ factors bind to the core enzyme, they
enhance cooperative binding of the complex to promoters involved in their particular regulon. For instance, during nitrogen limiting conditions the Ntr regulon of the cell makes use of alternative sigma factor $\sigma^{54}$ (gene product of rpoN) to transcribe glutamine synthetase for the assimilation of ammonia and to transcribe related protein products designed to facilitate survival under nitrogen starvation (Feng et al., 1992). In a related manner, the gene rpoH encodes the heat shock -specific sigma factor involved in transcription during the heat shock response and also associated with bulk protein synthesis during starvation (Jenkins et al., 1990).

Similarly, the sigma protein product of the rpoS gene in E. coli is a major regulator of the general starvation and oxidative stress response (Lange and Hengge-Aronis, 1991b; McCann et al., 1991). A comparison between parent and $\Delta rpoS$ mutants reveal that the gene product is responsible for the synthesis of at least 32 carbon starvation proteins and the stationary phase repression of approximately 10 proteins (McCann et al., 1991). Historically, the rpoS gene was first identified as katF, a regulatory gene for katE which encodes HPII Catalase (Loewen and Briggs, 1984). Further study of the katF gene revealed extensive homology with rpoD, E. coli's vegetative $\sigma$ subunit of RNA polymerase (Mulvey and Lowen, 1989). In vitro experiments have shown that the gene product KatF does indeed act as a $\sigma$ factor with the core RNA polymerase (Ishiharna et al., 1993). Thus, the gene has been deemed rpoS and the gene product RpoS has been designated $\sigma^5$, S for stationary.
The importance of the rpoS gene in the survival of E. coli has been clearly illustrated through deletion experiments. rpoS mutants exhibit extremely reduced starvation survival and lower plating efficiencies (Lange and Hengge-Aronis, 1991b). Inactivation of E. coli's rpoS sequence also produces a remarkable inability of stationary phase cells to develop resistance against heat shock (Jenkins et al., 1988), hydrogen peroxide (Jenkins et al., 1988), pH extremes (Small et al., 1994), near UV irradiation (Sak et al., 1989), and osmotic challenge (McCann et al., 1991). There is also evidence suggesting that rpoS plays a role in protein protection during the stationary phase (Hengge-Aronis, 1993). Under starvation conditions, the cell maintains a balance between the protection of essential proteins and the degradation of dispensable proteins. rpoS may play a role in the catabolic degradation of proteins and the maintenance of precursor pools for de novo protein synthesis. Recent studies by Aviv et al. (1994) found that the expression of the genes coding for the E. coli integration host factor (IHF) is controlled by rpoS and ppGpp. IHF is involved in the expression of over 100 different proteins and operons including the isoleucine, valine, and the biodegradative threonine deaminase operons (Freundlich et al., 1992). The study conducted by Chou et al. (1995) also indicates that inactivation of rpoS has a significant effect on recombinant protein production at slow growth rates. Chemostat experiments demonstrated that inactivation of rpoS resulted in a 30% (4 fold) increase in recombinant β-Galactosidase expression at a dilution rate of 0.13 h⁻¹, a growth rate typical of large scale fed-batch cultures.
The \(rpoS\) gene along with the genes whose expression depends on \(\sigma^S\) make up the \(\sigma^S\) regulon. Some member genes and their associated functions are shown in table 1. Preliminary data on the \(rpoS\) regulon indicates that the identified genes can be grouped into three distinct classes (Huisman and Kolter, 1994). The first class includes those genes that encode proteins for general starvation-induced stress resistance. The members of this class include the \(katE\), \(xthA\), \(dps\), \(appA\), and \(mcc\) genes. The proteins encoded by these genes are designed to offer starved cells protection against such stress as pH, oxidative, and heat shock. The second class of genes are involved in osmotic protection. The genes \(cfa\), \(treA\), \(otsA\), \(otsB\), \(osmB\), \(osmE\), and \(osmY\) provide osmotic protection through enhancement of membrane permeability and physical stability. \(\sigma^S\) dependent genes involved in cell morphology and cell division constitute the last class in the \(rpoS\) regulon. This class includes \(bolA\) which is associated with the rod-to-spherical phenotype change in stationary phase cells. Another gene in this category is the \(ftsQ\) that has been linked with cell division and cellular septation. The data provided by Avis et al. (1994) and Chou et al. (1994) alludes to a possible fourth class of genes (including the \(himA\) and \(himD/hip\) genes that encode the structural subunits for IHF) responsible for maintaining precursors for protein synthesis and involved in protein degradation and protection.

Recently, it has been discovered that a few of the member genes, including \(bolA\) and \(ftsQ\), in the \(rpoS\) regulon display a characteristic sequence in the -10 region of their promoters. Promoters with this homology are known as “gearbox” promoters and are typically induced by the cellular shift from exponential to stationary growth phase.
(Bohannon et al., 1991). Genes with this characteristic promotor sequence are often regulated at the transcription level with activity inversely proportional to growth rate (Aldeci et al., 1990).

**Table 1.** Identified members of the *rpoS* regulon.

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<tr>
<th>Gene</th>
<th>Function</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>appA</em></td>
<td>encodes a periplasmic acid phosphatase</td>
<td>Lange and Hengge-Aronis, 1991b</td>
</tr>
<tr>
<td><em>bolA</em></td>
<td>involved in morphological changes in cell size and sphericity</td>
<td>Bohannon et al., 1991;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Lange and Hengge-Aronis, 1991</td>
</tr>
<tr>
<td><em>cfa</em></td>
<td>encodes the enzyme Cyclopropane Fatty Acid Synthase, involved in the stationary phase modification of the lipid bi-layer</td>
<td>Wang and Cronan, 1994</td>
</tr>
<tr>
<td><em>dps</em></td>
<td>DNA binding protein involved in $H_2O_2$ protection</td>
<td>Almiron et al., 1992</td>
</tr>
<tr>
<td><em>glgS</em></td>
<td>gene essential in the glycogen biosynthesis pathway</td>
<td>Hengge-Aronis and Fischer, 1992</td>
</tr>
<tr>
<td><em>katE</em></td>
<td>Catalase HPII</td>
<td>Loewen and Triggs, 1984;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sak et al., 1989</td>
</tr>
<tr>
<td><em>himA</em></td>
<td>encodes subunits of the integration host factor protein-a DNA binding protein and an important regulator of gene expression.</td>
<td>Freundlich et al., 1992;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Avis et al., 1994</td>
</tr>
<tr>
<td><em>mcc</em></td>
<td>produces MccC7, a peptide antibiotic inhibiting protein synthesis</td>
<td>Diaz-Guerra et al., 1989</td>
</tr>
<tr>
<td><em>osmB</em></td>
<td>involved in osmotic protection</td>
<td>Huisman and Kolter, 1994</td>
</tr>
<tr>
<td><em>osmE</em></td>
<td>exponential phase osmotic protection</td>
<td>Hengge-Aronis et al., 1991</td>
</tr>
<tr>
<td><em>osmY</em></td>
<td>exponential phase osmotic protection</td>
<td></td>
</tr>
<tr>
<td><em>otsA</em></td>
<td>periplasmic trehalase, involved in thermal and osmotic protection</td>
<td>Boos et al., 1987</td>
</tr>
<tr>
<td><em>treA</em></td>
<td>encodes Exonuclease III, an essential DNA repair enzyme, protecting against $H_2O_2$ and UV</td>
<td>Sak et al., 1989</td>
</tr>
<tr>
<td><em>xthA</em></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
1.3.3 Regulation of rpoS

The genes of the rpoS regulon have been shown to be activated at the onset of starvation-induced stationary phase (Huisman and Kolter, 1994). Batch experiments performed with transcriptional fusions of rpoS::lacZ' indicate that rpoS transcription initiates during the late exponential phase and increases until the stationary phase is reached (Lange and Hengge-Aronis, 1991b; Schellhorn and Stones, 1992). The cell does maintain a basal expression rate of rpoS during early exponential growth, however the transition from exponential to stationary phase produces at least a five-fold increase in intracellular protein concentration in rich media. The sudden increase in rpoS transcription at the onset of the stationary phase strongly suggests the existence of intracellular sensing mechanisms that activate the transcription of rpoS in response to particular metabolic or environmental triggers.

Several metabolic and environmental factors have been linked with the activation of the rpoS gene. Gentry et al. (1993) demonstrated that the synthesis of $\sigma^S$ is positively regulated by the phosphonucleotide ppGpp. This compound along with AppppA plays a critical role in the stringent response to amino acid starvation and is regarded as a modulator of general stress responses. Huisman and Kolter (1994) note that elongation factor Tu (EF-Tu) may also play a role in rpoS regulation since this membrane bound molecule acts as a nutrient sensor and intracellular signal. The addition of cAMP to culture medium also has been shown to reduce rpoS expression (Lange and Hengge-
Aronis, 1991). This effect may be associated with the fact that cellular cAMP synthesis and extracellular excretion are drastically increased by glucose-starvation induced entry into the stationary phase.

![Diagram showing the rpoS regulon](image)

**Figure 4.** A preliminary representation of the *rpoS* regulon. (adapted from Huisman and Kolter, 1994). The *rpoS* gene encodes a stationary phase specific sigma factor denoted by $\sigma^S$. Stressful environmental and certain intracellular signals induce the expression of the gene and the production of the sigma factor. The sigma factor then initiates the transcription of the genes included in the *rpoS* regulon. Initial studies have indicated that the regulon itself consists of four families of genes.

Recent studies also imply that *rpoS* activation may be linked to high cell density.

Huisman and Kolter (1994) demonstrated that *rpoS* was activated by homoserine lactone, a metabolite synthesized from intermediates in the threonine biosynthesis pathway.

Homoserine lactone and its derivatives are known to accumulate in the culture media due to their ppGpp-dependent production during starvation-induced stationary phase.
Homoserine lactones have also been known to act as inducers in several other cell density dependent systems including bioluminescence (Swift et al., 1994) and the synthesis of exozymes and antibiotics (Pirhonen et al., 1993).

Batch experiments conducted by Schellhorn and Stones (1992) indicate that acetate along with other weak acids such as benzoate and propionate induces rpoS transcription in complex media. As mentioned before, acetate is a metabolic byproduct that is excreted by growing cells in the late exponential phase of batch growth. This compound continuously accumulates in the extracellular media and acts as a potent inhibitor of cell growth and protein synthesis. Furthermore, the activation of rpoS by acetate implies that acetate may play a role as an intercellular signal molecule much like the homoserine lactones. E. coli cells may interpret high acetate concentrations as an indicator of high cell density, harsh environmental conditions, or incipient starvation. This conjecture is supported by the fact that the acetyl moiety of acetate, acetyl phosphate, and acetyl-CoA is involved in the control of the phosphate regulon (Wanner and Wilmes-Riesenber, 1992) and may act as a general intracellular signal molecule. However, this interpretation is a matter of debate since the studies by Melvey et al. (1990) found no effect of acetate on rpoS.

The current study focuses on three possible triggers for rpoS induction. The role of glucose exhaustion as a possible activator is examined. This set of experiments provides additional evidence linking rpoS expression to carbon limitation and starvation in
general. Chemostat experiments are employed to determine the effect of externally added acetate on \textit{rpoS} expression. Since acetate's effect on gene expression is a matter of current debate, the study attempts to establish acetate as a definite activator of \textit{rpoS} and a possible cellular signal molecule. This in turn would provide a genetic insight into acetate's inhibitory nature. The effect of cell density on gene activation was also investigated using batch and continuos experiments. These experiments were undertaken to narrow the critical parameters involved in the cell density dependent activation of \textit{rpoS} indicated by the homoserine and homoserine lactone dependent pathways.

\subsection*{1.4 A Precautionary Note Reguarding Biological Systems}

Biological systems occupy a level of complexity that Science and Engineering has only begun to comprehend. Due to the complexities and widespread variability involved in the experimentation and the subsequent interpretation of biological data, the results obtained in this and any other study conducted on a biological system hold strictly to those particular experimental condition. Although much insight can be gained from experimentation on such inherently variable systems, prudence should be exercised whenever one attempts to interpolate results and observations outside the realm of a particular experiment.
Materials and Methods

Chapter 2

2.1 Bacterial Strains

The two bacterial strains used throughout this study are listed in table 2. Both HS143 and HS143m are kanomyacin resistant derivatives of *E. coli* K-12 containing a chromosomal *rpoS::lacZ'* transcriptional fusion. HS143m is a merodiploid strain that contains an additional wild type *rpoS* chromosomal sequence.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>HS143</td>
<td>$F^\Delta$lacU169 $\Phi(rpoS::lacZ')143$</td>
<td>Schellhorn and Stone, 1992</td>
</tr>
<tr>
<td>HS143m</td>
<td>$F^\Delta$lacU169$rpoS^+$ $\Phi(rpoS::lacZ')143$</td>
<td>Schellhorn and Stone, 1992</td>
</tr>
</tbody>
</table>

Table 2. List of strains used in this study.

2.2 Media

2.2.1 Culture Media

Complex media were extensively used throughout this study. Luria-Bertani (LB) (Miller, 1972) medium contains 5 g/l NaCl, 10 g/l tryptone (Difco, Detroit, MI) and 5 g/l yeast extract (Difco). Enriched medium contains 5 g/l NaCl, 32 g/l tryptone (Difco, Detroit,
MI), 20 g/l yeast extract (Difco), and 55.4 mM Glucose. The reduced medium used in this study contains 5 g/l NaCl, 2 g/l tryptone (Difco, Detroit, MI), 1 g/l yeast extract (Difco), and 28 mM Glucose. When appropriate, certain media were also supplemented with known quantities of glucose or sodium acetate. Antifoam (Sigma) was also used in the amount of 50 µl/l for chemostat and batch runs.

Table 3. Compositions of the complex media used for cultures.

<table>
<thead>
<tr>
<th>Medium</th>
<th>NaCl (g/l)</th>
<th>Tryptone (g/l)</th>
<th>Yeast Extract (g/l)</th>
<th>Glucose (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LB</td>
<td>5</td>
<td>10</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>Enriched</td>
<td>5</td>
<td>32</td>
<td>20</td>
<td>55</td>
</tr>
<tr>
<td>Reduced</td>
<td>5</td>
<td>2</td>
<td>1</td>
<td>28</td>
</tr>
</tbody>
</table>

2.2.2 Storage Media

Glycerol stocks of particular strains were prepared for long term storage. These stocks were prepared by transferring 0.5 ml of LB-grown cells into sterile vials containing 0.5 ml of glycerol. The vials were then vortexed and allowed to sit at room temperature for 2 hours. The vials were then transferred to freezers at -20°C for relatively short term (several months to a year) or at -70°C for long term (several years to indefinite) storage.
2.3 Cell Cultivation

2.3.1 Shake Flasks

Shake flasks were used to provide adequate inoculum for chemostat and batch experiments. The shake flasks themselves were 250 ml Erlenmeyer flasks covered with foam plugs. Each flask contained 50 ml LB medium. During a typical experimental startup sequence, 5 μl of glycerol stock was used to inoculate 15 ml vials containing 5 ml of LB. These vials were then grown for 6-12 hours in an orbital shaker maintained at 37°C and 250 rpm. 5 μl of the above vial culture was then used to inoculate a flask.

Each shake flask contained the appropriate antibiotic to provide selective pressure on the culture and help prevent foreign contamination. Shake flask cultures were then grown aerobically for 6-12 hours in a rotary shaker maintained at 37°C and 250 rpm. Aliquots of approximately 10 ml of shake flask culture were then used to inoculate chemostat cultures. For a batch run, 10 μl of exponentially growing shake flask culture was used as the inoculum.

2.3.2 Chemostats

Chemostat experiments were conducted in a 1 liter fermentor with a working volume of approximately 800 ml. The pH in the reactor was monitored by a glass electrode (Phoenix) and controlled at 7.0 ± 0.1 by addition of either HCl or NaOH solutions. The
temperature control was provided by a thermocouple that was linked to a heating element. The set point for the temperature in all experiments was 30°C. The agitation speed of the mixer was set at a constant 600 rpm. All experiments were conducted aerobically with an air sparging rate of at least 2 L/min. Sparger air was filter sterilized before entering the fermentor through three 0.22 μm membrane filters connected in series. Effluent gas was bubbled through a 1 M CuSO₄ solution to prevent back contamination and bacterial escape.

2.3.3 Batch Cultures

Batch studies were conducted in a 1 liter fermentor with a volume of approximately 900 ml. The pH in the reactor was monitored by a glass electrode (Phoenix) and was maintained at 7.0 ± 0.1 by addition of HCl or NaOH solutions. The temperature control was provided by a thermocouple that was linked to a heating element contained in the fermentor. The set point for the temperature in all experiments was 30°C. The agitation speed of the mixer was set at a constant 600 rpm. All experiments were conducted aerobically with an air sparging rate of at least 2 L/min. Sparger air was filter sterilized before entering the fermentor through three 0.22 μm membrane filters connected in series. Effluent gas was bubbled through a 1 M CuSO₄ solution to prevent back contamination and bacterial escape.

2.4 Analytical Procedures
2.4.1 Acetate Concentration Measurements

Extracellular acetate concentration was determined from acidified, cell-free medium. A Beckman microfuge was used to centrifuge 1 ml of culture sample for 5 minutes. 0.5 ml of the cell free supernatant was then acidified and thoroughly mixed with 10 μl of 50% sulfuric acid. 5 μl of the acidified, cell free medium was injected into a gas chromatograph equipped with a 6 ft 100/120 Chromosorb 101 packed column (Alltech) and a Flame Ionization Detector. Acetate concentration was determined from the resulting chromatogram using experimentally obtained calibration curves.

2.4.2 Glucose Concentration Measurements

Glucose (HK) reagent (Sigma, MO) was used for the quantitative enzymatic determination of glucose concentration in extracellular media. Exactly 1 ml of reconstituted glucose hexokinase reagent was added to a quartz cuvette and the UV absorbance at 340 nm was recorded. 0.5 ml of culture sample was then centrifuged for 5 minutes to remove cells and cell debris from the media. The cell free supernatant (2 μl) was then transferred to the quartz cuvette containing the enzymatic reagents. The cuvette was then inverted three times to ensure sufficient mixing. The glucose in the cuvette undergoes the following chemical reactions in the presence of the hexokinase reagents:
\[
\text{Glucose} + \text{ATP} \xrightarrow{\text{HK}} \text{G6P} + \text{ADP} \\
\text{G6P} + \text{NAD} \xrightarrow{\text{G6PDH}} \text{6PG} + \text{NADH}
\] (1)

The change in UV absorbance after ten minutes was then recorded. The increase in absorbance of the sample is due to the reduction of the NAD to NADH. Since the reaction stoichiometry requires equimolar elimination of glucose with subsequent oxidation of NADH, the change in absorbance thus is directly proportional to glucose concentration.

The following empirical relationship between sample glucose concentration and the increase in absorbance at 340 nm was provided by the manufacturer:

\[
\text{Glucose}(C_g) = 2.93 \times (\text{OD}_{340}(\text{sample}) - \text{OD}_{340}(\text{blank}))
\] (2)

Where \(C_g\) is the extracellular glucose concentration in g/l, \(\text{OD}_{340}(\text{sample})\) is the absorbance of the cuvette containing the 2 \(\mu\)l sample after ten minutes, and \(\text{OD}_{340}(\text{blank})\) is the absorbance of the sample free cuvette.

2.4.3 \(\beta\)-Galactosidase Assay

\(\beta\)-Galactosidase assays were performed as outlined in Aristidou (1994) which is modified from the original protocol suggested by Miller (1972). This particular assay takes
advantage of the reaction catalyzed by the enzyme β-Galactosidase that converts the 
substrate o-nitrophenol-β-D-Galactopyranoside (ONPG) into a yellow product (o-
nitrophenol) that can be spectrophotometrically detected at 420 nm. In this assay, a cell 
culture was first lysed by sonification. A microliter sample volume (up to 400 µl) of the 
lysed cell culture (plus one blank containing an equal amount of water) was added to 
effectively 1 ml aliquots of reaction buffer (Z buffer) and vortexed. The reaction buffer (Z-
Buffer) used in this procedure contained the following: 60 mM Na₂HPO₄, 40 mM 
NaH₂PO₄, 10 mM KCl, 1 mM MgCl₂, pH 7 and also contained a final concentration of 
50 mM of β-mercaptoethanol that was added separately. The above solution was allowed 
to thermally equilibrate in a water bath at 28°C for 5-10 minutes. The reaction was then 
initiated by the addition of 200 µl of the ONPG solution (4 mg/ml ONPG, 100 mM PO₄²⁻; 
buffer stored at -20°C). After ten minutes, the reaction was arrested by the injection of 
500 µl of 1 M Na₂CO₃ solution. The optical density at 420 nm (OD₁₉₀) of the reaction 
mixture was then measured against the blank. Assays on cell culture samples were 
conducted in triplicate to insure accuracy. Specific enzymatic activity was reported in 
terms of Miller Units as defined below.

\[
MillerUnit(MU) = \left( \frac{1000 \times OD_{420}}{time \times V_s \times OD_{600}} \right)
\]

(3)

where \( V_s \) is the sample volume of the cell culture measured in ml, \( time \) is the reaction 
time measured in minutes (in this case ten), and \( OD_{600} \) is the optical density at 600 nm of 
the cell culture.
Volumetric Activity of the enzyme can be calculated from the following equation:

\[ Volumetric \text{Activity} (U/\text{ml}) = OD_{600} \times MU \]  

(4)

2.4.4 Cell Density Measurement

Cell growth was monitored off line by measuring the optical density of culture samples at 600 nm using a Spectronic 1001 spectrophotometer (Baush & Lomb). When appropriate, a culture sample was diluted using 0.15 M NaCl to maintain the instrument reading below a value of 0.4 OD\(_{600}\) units. These dilutions were necessary to ensure that the optical density measurements were well within the linear response range of the instrument.
Results and Discussion

Chapter 3

3.1 Batch Experiments

3.1.1 Results Using HS143

Batch experiments were conducted in complex media (LB supplemented with glucose) to establish the activation profiles of the HS143 strain with respect to time and cell density. In the HS143 strain the wild type sequence of the rpoS gene is replaced by a chromosomal rpoS::lacZ' transcriptional fusion. Here, a fully functional promoter sequence of the rpoS gene is fused to a lacZ cassette employed as the reporter gene. The transcriptional activation of the promotor sequence could then be monitored by assaying the β-galactosidase activity - the protein product of the lacZ cassette. The existence of a time profile itself demonstrated that rpoS transcription is not under the control of the stationary phase sigma factor, RpoS, since the specific phenotype of the HS143 strain is rpoS (Shellhorn and Stone, 1992)
**Figure 5.** *rpoS* induction for HS143 (rpoS::lacZ<sup>+</sup>) grown aerobically in LB media supplemented with glucose. Batch culture was inoculated with 10μl of exponentially growing cells. Fermentor conditions were controlled at 30°C and pH 7. Gene activity was monitored by β-galactosidase assay.

The time profile shown in Figure 5 indicated that gene expression maintained a baseline level of approximately 440 MU for the early to middle exponential phase culture. Basal expression of the *rpoS* gene was expected since a number of exponentially active genes including *otsA* and *otsB* (Hengge-Aronis *et al.*, 1991) are dependent upon the *rpoS* sigma factor for their transcription. Activation of the gene originated in the late exponential phase of growth and increased into the stationary phase. This result confirmed similar trends observed by other laboratories (Shellhorn and Stone, 1992; Huisman and Kolter, 1994). A value of approximately 1950 MU was obtained after more than 21 hours of the study, indicating a 4.5 fold increase in gene expression from early exponential to middle stationary phase growth.
Figure 6. Summary for batch culture of HS143 (rpoS::lacZ') Extracellular acetate and glucose along with intracellular activity of β-galactosidase are plotted against cell density.

Cell density profiles for acetate, glucose, β-galactosidase activity are shown in figure 6.

The β-galactosidase profile indicated that gene activation occurred at an OD$_{600}$ of approximately 1.2 and increased along with cell densities into the stationary phase.

Glucose and acetate levels were also monitored in the culture and are shown as functions of cell density. As expected, extracellular glucose concentration declined with increasing cell density until exhaustion at cell densities above 6.8. Acetate accumulation in the extracellular media was concurrent with glucose consumption throughout the exponential phase and paralleled a similar increase in specific gene expression. The link between acetate production and glucose consumption by exponentially growing cultures may originate from the loose regulation of glucose uptake system in E. coli which results in carbon overflow of the central metabolic pathway into the acetic acid formation pathway.

(Demain, 1972; Meyer et al., 1984; Holms, 1986)
3.1.2 Results Using HS143m

Additional batch experiments were conducted in complex media (LB supplemented with glucose) to characterize the activation profiles of the HS143m merodiploid strain and to observe the effect of the additional wild type copy of the *rpoS* gene. The HS143m strain contains a wild type copy of the *rpoS* gene along with a chromosomal *rpoS::lacZ*\(^+\) transcriptional fusion. As in the HS143 strain, the transcriptional activation of the *rpoS::lacZ*\(^+\) transcriptional fusion could be monitored by the \(\beta\)-galactosidase assay. However the phenotype of the HS143m strain is *rpoS*\(^+\) which implies a fully functional RpoS sigma factor and *rpoS* regulon.

![Graph](image)

**Figure 7.** *rpoS* induction for HS143m (*rpoS*\(^+\)(*rpoS::lacZ*\(^+\))) grown aerobically in LB media supplemented with glucose. Batch culture was inoculated with 10\(\mu\)l of exponentially growing cells. Fermentor conditions were controlled at 30\(^\circ\)C and pH 7. Gene activity was monitored by \(\beta\)-galactosidase assay.
The time profile of HS134m shown in figure 7 was similar to those obtained for the HS143 strain. In this case, gene expression maintained a baseline level of approximately 365 MU for the early to middle exponential phase culture. This represented an approximately 20% decrease in baseline expression from the HS143 batch experiment. Activation of the gene again originated in the late exponential phase of growth and increased to a value of approximately 980 MU. This again represented about a 50% decrease compared to the HS143 experiment. Gene expression increased by a factor of 2.7 fold between the exponential to middle stationary phase of growth for the HS143m strain. Shellhorn and Stone (1992) observed a similar decrease in gene expression for the merodiploid strain and attributed this trend to the competitive inhibitory effect of the merodiploid’s active RpoS sigma factor. Since RpoS is apparently not responsible for the

![Graph](image)

**Figure 8.** Summary for batch culture of HS143m / rpoS⁺ (rpoS::lacZ⁺). Extracellular acetate and glucose along with intracellular activity of β-galactosidase are plotted against cell density.
transcription of the rpoS gene, the RpoS sigma factor thus competes for a limited amount of RNA polymerase in the cell and effectively reduced the number of transcriptional events for the rpoS sequence.

Cell density profiles for this experiment are shown in figure 8. The β-galactosidase profile indicated that gene activation occurred at OD₆₀₀ of 1.5 and increased along with cell densities into the stationary phase. Gene expression again paralleled an initial increase in acetate concentration and a depletion of glucose in the media. These trends were similar to those obtained from HS143. As expected, extracellular glucose concentration declined with increasing cell density until exhaustion at cell densities above an OD₆₀₀ of 17. Acetate accumulation in the extracellular media was concurrent with glucose consumption throughout the early exponential phase. However, unlike the HS143 strain, the HS143m strain appeared to reutilize the acetate as an additional carbon source. The final cell density for the HS143m strain was also higher. An OD₆₀₀ of approximately 16 was obtained after 20 hours compared to an OD₆₀₀ of 8 for the HS143 strain. The two experiments suggest that the HS143m strain with the active rpoS regulon appeared to be a hardier strain. Moreover, qualitatively it was observed that the meridiploid strain survived prolonged storage periods better than the haploid strain.

Three possible factors involved in rpoS regulation can be speculated from the above set of batch data. First, high cell density may play a role in gene activation. This idea stems from the experiments conducted by Huisman and Kolter (1994) which determined the
connection between \textit{rpoS} activation and a homoserine lactone dependent signaling pathway in \textit{E. coli}. The cell density dependent response involves the production and excretion of metabolic byproducts and possibly signaling molecules (such as homoserine lactone or its derivatives) by exponentially growing cells. These compounds then, through some yet unknown mechanism, provide the cells with information on local cell density. Above a critical signal level, the cells then induce the expression of \textit{rpoS} and a number of other genes crucial for stationary phase viability. Indeed, density sensing may have evolved to prepare microbial cultures for the stressful environmental conditions and nutrient limitations that are likely due to excess biomass accumulation in the system.

Both sets of batch data indicated that gene activity was maintained at a basal level until the cell density of the system reached a \textit{OD}_{600} of roughly 1.2-1.5. After an \textit{OD}_{600} of about 1.2-1.5, both cultures activated gene expression and increased the intracellular concentration of the gene product while accumulating fermentor biomass. The activation of the gene could possibly be explained by the accumulation of a density dependent substance. This substance would accumulate along with the biomass and activate the gene once a critical concentration was reached.

\textbf{Glucose} limitation or carbon starvation in general may also be a regulator of gene activity. Lange and Hengge-Aronis (1991) established that the addition of cAMP, a well-known intracellular sensor of glucose availability, negatively regulated gene expression. Glucose was also found to suppress catalase synthesis (Moustafa and Fridovich, 1978), thus suggesting yet another possible link between glucose and the \textit{rpoS} regulon. Here, as
in the cell density case, the activation of rpoS and the rpoS regulon by incipient carbon limitations would create a hardier and more resistant cellular state or may possibly induce metabolic changes that would be needed to maintain viability once carbon becomes limiting or environmental conditions become unfavorable for growth. Again, both sets of batch data indicated that gene expression increased while glucose levels dropped in the system. This observation supports the hypothesis that glucose concentration has an inverse relationship with gene activity.

Finally, extracellular acetate may influence rpoS expression. It is evident from the batch data that acetate accumulated with biomass throughout the exponential phase of growth. This data corroborates studies performed by other laboratories (Heather et al., 1990; Jensen and Carlsen, 1990; Shimizu et al., 1988). The accumulation of acetate in extracellular media has often been linked with reductions in cell growth (Smulders et al., 1986; Adams and Hall, 1988; Luli and Stohl, 1990; Koh et al., 1992) and recombinant productivity (Bauer et al., 1990; Jensen and Carlsen, 1990; Konstantinov et al., 1990; George et al. 1992; Sun et al., 1993). Acetate’s inhibitory effect has often attributed to the destruction of the cell’s proton motive force and thus the overall reduction in the energy charge of the cell (Slonzewski et al., 1981; Diaz-Ricci et al., 1990; Aristidou, 1994). However, activation of the rpoS regulon by acetate may provide a genetic mechanism for the reduced cellular productivity. In this context, acetate may be acting as an intercellular signal molecule for high cell density or harsh environmental conditions. As mentioned previously, this hypothesis is suggested by the observation that
accumulation of acetate in both the HS143 and HS143m runs had striking parallels to the increase in specific gene activity.

3.2 Chemostat Experiments

Chemostat studies were performed using HS143 to investigate the three speculative factors influencing rpoS activation. These factors included externally added glucose, externally modulated acetate, and changes in cell density. Chemostats were chosen for this study since they provided a convenient means to regulate bacterial growth rate. The notion of balanced growth suggests that cells grown at a constant dilution rate in a chemostat should have fixed transcriptional and translational rates. Thus for a given dilution rate, the effect of various parameters can then be examined without interference from growth rate. Dilution rates were also varied to simulate a range of growth conditions from slow to fast growth.

3.2.1 The Effect of Glucose on HS143

Chemostat experiments were performed with LB media to determine the effect of externally supplemented glucose on rpoS expression in HS143. Glucose was added to the feed medium with a concentration of 28 mM (5 g/l). Continuous runs scanning dilution rates from 0.2 h\(^{-1}\) to 1.0 h\(^{-1}\) were conducted using LB alone as a base set (table 4) and LB+28 mM Glucose (table 5) as the experimental set.
Table 4. Chemostat results for HS143 using LB media.

<table>
<thead>
<tr>
<th>Dilution Rate ($h^{-1}$)</th>
<th>OD$_{600}$</th>
<th>Residual Acetate (mM)</th>
<th>Residual Glucose (mM)</th>
<th>β-Galactosidase Activity (MU)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.2</td>
<td>4.1</td>
<td>5.0</td>
<td>0.4</td>
<td>1309</td>
</tr>
<tr>
<td>0.4</td>
<td>2.9</td>
<td>2.3</td>
<td>0.1</td>
<td>1320</td>
</tr>
<tr>
<td>0.6</td>
<td>2.1</td>
<td>2.8</td>
<td>0.1</td>
<td>840</td>
</tr>
<tr>
<td>1.0</td>
<td>0.6</td>
<td>2.3</td>
<td>0.2</td>
<td>449</td>
</tr>
</tbody>
</table>

Table 5. Results for HS143 using LB media supplemented with 28 mM of glucose.

<table>
<thead>
<tr>
<th>Dilution Rate ($h^{-1}$)</th>
<th>OD$_{600}$</th>
<th>Residual Acetate (mM)</th>
<th>Residual Glucose (mM)</th>
<th>β-Galactosidase Activity (MU)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.2</td>
<td>7.1</td>
<td>17.3</td>
<td>7.8</td>
<td>958</td>
</tr>
<tr>
<td>0.4</td>
<td>5.0</td>
<td>14.2</td>
<td>15.0</td>
<td>1111</td>
</tr>
<tr>
<td>0.6</td>
<td>2.0</td>
<td>7.7</td>
<td>17.7</td>
<td>587</td>
</tr>
<tr>
<td>1.0</td>
<td>1.2</td>
<td>6.0</td>
<td>22.9</td>
<td>464</td>
</tr>
</tbody>
</table>

The results for this set of experiments are also shown in Figure 9. The graph suggests that under experimental conditions glucose supenmentation decreased the specific gene expression by approximately 25% for dilution rates of 0.6 $h^{-1}$ and lower (99% confidence level based on a two sample Student t-test). This is consistent with batch results from this study and other studies linking glucose availability to $rpoS$ expression (Moustafa and Fridovich, 1978; Lange and Hengge-Aronis, 1991). The decrease in gene expression due to glucose addition is most likely associated with carbon limitations in the fermentor. This would explain the observation that gene activity was equivalent within experimental error for the dilution rate of 1.0 $h^{-1}$. It is evident from table 5 that as the dilution rate was
lowered, cell density increased simultaneously with a similarly dramatic decline in glucose levels. This strongly suggests that carbon sources in the reactor were being depleted as the dilution rate was lowered. At a dilution rate of 1.0 h⁻¹, the glucose concentration for the supplemented case was 22.9 mM, an 18% decrease from feed. Carbon availability was probably high enough to suppress gene expression in the LB base case to a level equivalent to that in the LB+28 mM Glucose case.

![Graph](image)

**Figure 9.** The effect of externally supplemented glucose on rpoS expression in the strain HS143 (rpoS::lacZ'). Chemostat experiments were performed using LB and LB supplemented with 28 mM of glucose as reactor feed. Gene expression in terms of β-galactosidase activity (MU) is plotted versus dilution rate for the two different media. Error bars indicate the standard deviation due to sampling error for one experimental run.

### 3.2.2 The Effect of Externally Supplemented Acetate

In recent years acetate has been clearly established as a potent inhibitor of cell growth and productivity (Smulders *et al.*, 1986; Adams and Hall, 1988; Bauer *et al.*, 1990; Luli and
Stohr, 1990; Jensen and Carlsen, 1990; Konstantinov, et al., 1990; George et al. 1992; Koh et al., 1992; Sun et al., 1993). Acetate inhibition is most often attributed to the reduction in the cell’s energy charge due to proton motive force decoupling (Slonzewski et al., 1981; Diaz-Ricci et al., 1990; Aristidou, 1994). However, a second proposed mechanism involved in inhibition due to acetate suggests that acetate may activate the rpos gene and regulon which then induces a reduction in cell activity and protein production through genetically controlled means (Melvey et al., 1990; Schellhorn and Stones, 1992). The current study investigated the proposed link between extracellular acetate and rpos expression through chemostat experiments in complex media. Three dilution rates were chosen to mimic growth rate corresponding to the stationary (0.2 h⁻¹), transitional (0.6 h⁻¹), and exponential (1.0 h⁻¹) cultures in a batch reactor (μ_max = 1.2 h⁻¹). The strain HS143 (rpos::lacZ⁺) was employed to monitor gene expression through β-galactosidase activity. A wide range of reactor acetate concentrations were obtained by adding appropriate quantities of sodium acetate to the feed media.

Acetate probably acted as a growth inhibitor in these chemostat experiments. The presence of high residual acetate concentrations in the reactor significantly reduced the optical density of HS143 (rpos::lacZ⁺) at all dilution rates as figure 10 suggests. At a dilution rate of 0.6 h⁻¹, a remarkable 88% reduction in cell density was observed for an acetate concentration of 47 mM (corresponding to an acetic acid concentration of approximately 2.9 g/l).
Figure 10. The inhibition of growth by acetate. Chemostat experiments were conducted to observe the effect of residual acetate on cell density in the strain HS143 (rpoS::lacZ'). Cell density was monitored spectrophotometrically and is reported in terms of Optical Density at 600nm (OD_{600}).

Figure 11 implies that the reactor acetate concentrations under study significantly modulated gene activity. The graph suggests that residual acetate levels of greater than 17 mM increased the transcriptional activity of the rpoS gene by 60% to as much as 170% under these experimental conditions. This trend strongly suggests that acetate acts as an activator of rpoS activity. This set of experiments implies that mutants deficient in rpoS expression may be less sensitive to the inhibitory effects of acetate and therefore may be more productive when acetate levels are comparatively high.
**Figure 11.** The effect of residual acetate on *rpoS* expression in HS143 (*rpoS::lacZ*'). Chemostats were used to scan the effect of residual acetate concentrations on gene activity at three fixed dilution rates. Studies were conducted using LB media supplemented with 28 mM (5 g/l) of glucose and various initial concentrations of sodium acetate. Residual acetate concentrations were measured by gas chromatography. Gene activities are reported in terms of MU of β-galactosidase.

### 3.2.3 The Effect of Medium Quality on HS143

The third factor speculated to induce *rpoS* expression is cell density. Chemostat experiments were conducted using the strain HS143 (*rpoS::lacZ*') to examine the effect of this parameter on gene expression. Manipulation of cell density was achieved through changes in media composition. The three compositions of media that were employed are listed in table 6.
Table 6. Media used to investigate the cell density dependence of rpoS.

<table>
<thead>
<tr>
<th>Medium</th>
<th>NaCl (g/l)</th>
<th>Tryptone (g/l)</th>
<th>Yeast Extract (g/l)</th>
<th>Glucose (mM)</th>
<th>Compositions relative to LB+28 mM Glucose series</th>
</tr>
</thead>
<tbody>
<tr>
<td>LB+28 mM Glucose</td>
<td>5</td>
<td>10</td>
<td>5</td>
<td>28</td>
<td>NaCl 1</td>
</tr>
<tr>
<td>Enriched</td>
<td>5</td>
<td>32</td>
<td>20</td>
<td>55</td>
<td>1</td>
</tr>
<tr>
<td>Reduced</td>
<td>5</td>
<td>2</td>
<td>1</td>
<td>28</td>
<td>1</td>
</tr>
</tbody>
</table>

The results from the cell density versus dilution rate plot (figure 12) demonstrates the effect of varying media composition on chemostat cell density.

Figure 12. Changes in cell density due to media quality. Initial concentrations of glucose, tryptone, and yeast extract were manipulated to generate a wide range of cell densities under chemostat conditions. Cell density was monitored spectrophotometrically and is reported in terms of Optical Density measured at 600 nm (OD$_{600}$).
As expected from the relative ratios of various components, the enriched media had the highest cell density with a maximum of approximately 19.7 optical density units, the reduced media produced the lowest cell densities with a minimum of approximately 0.2, while LB+28 mM Glucose produced cell densities between the two (Figure 12).

A summary of the cell density data and relative ratios to the LB+28 mM Glucose set is shown in Table 7. Closer comparison between Table 6 and Table 7 hints that the constituents of the tryptone in culture probably limited chemostat growth. This conjecture follows from the comparison between the ratios of compositions to the ratios of the cell densities. The ratios of the resulting cell densities in the enriched media case more closely mimics the ratio of the tryptone concentrations than the yeast extract or the glucose.

Table 7. Cell density results from chemostat experiments using three different media compositions. Entries are in units of OD_{600} (left block) and ratios of sample OD_{600} to OD_{600} of the LB+28 mM Glucose series (right block).

<table>
<thead>
<tr>
<th>Medium</th>
<th>D = 0.2 h^{-1}</th>
<th>D = 0.4 h^{-1}</th>
<th>D = 0.6 h^{-1}</th>
<th>D = 1.0 h^{-1}</th>
<th>OD_{600}</th>
<th>OD_{600}/OD_{600}</th>
</tr>
</thead>
<tbody>
<tr>
<td>LB+28 mM Glucose</td>
<td>7.1</td>
<td>5.0</td>
<td>2.0</td>
<td>1.2</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Enriched</td>
<td>19.7</td>
<td>13.9</td>
<td>7.6</td>
<td>4.7</td>
<td>2.8</td>
<td>2.8</td>
</tr>
<tr>
<td>Reduced</td>
<td>1.3</td>
<td>0.7</td>
<td>0.4</td>
<td>0.2</td>
<td>0.2</td>
<td>0.1</td>
</tr>
</tbody>
</table>
The effect of media quality induced cell density changes on rpoS induction is shown in figure 13. The graph suggests that dilution rates of 0.4 h\(^{-1}\) and higher produced no significant change in gene expression. Stated differently, no effect was observed between optical densities of 0.4 to 19.7 under these particular experimental conditions. Variation in gene expression among the three media did occur for a dilution rate of 0.2 h\(^{-1}\). This variation is probably not directly related to cell density. In the case of the enriched broth, the relative increase in gene activity with respect to the LB+28 mM Glucose case was most likely due to the relative increase in residual acetate concentration and possibly other spent media components. Chemostat acetate levels rose to 47.0 mM in the Enriched broth, compared to 17.3 mM in the LB+28 mM Glucose media and 5.7 mM in the Reduced broth. In the Reduced media case, the increase in rpoS expression could be attributed to a downward shift in osmotic pressure caused by the conversion of scarcely concentrated species in the media into biomass. In the Reduced broth experiments, it was observed that the media itself had comparatively little buffering capacity and pH had to be controlled with 1 N instead of 3 N concentrated acid and base. This indicated low buffering and most probably low osmotically involved components. This effect would be further amplified at the lower dilution rate where larger amounts of solution material would be incorporated into biomass accumulation. It is reasonable to speculate that shifts in osmotic pressure should have some type of effect on rpoS expression since a significant number of genes in the rpoS regulon genes (cfa, treA, otsA, otsB, osmB, osmE, and osmY for instance) are involved in osmotic protection. However, this speculation should be further investigated.
In the Reduced media case, it is also possible that the media may have been nitrogen source limiting. This media contained abundant amounts of carbon in the form of glucose, however, the yeast extract and the tryptone concentrations were reduced by a factor of five. At the higher cell density observed at a dilution rate of 0.2 h⁻¹, nitrogen source concentrations may have been low enough to trigger stress responses in the cell that would ultimately activate the rpoS gene.

Figure 13. The effect of media quality on rpoS activity. Feed concentrations of glucose, tryptone, and yeast extract were manipulated to observe the effect of cell density on gene expression. Gene expression in terms of β-galactosidase activity (MU) is plotted versus dilution rate for the three different media. Error bars indicate the standard deviation due to sampling error for one experimental run.
3.3 Metabolic Fluxes Using HS143

Other metabolic data was also collected from the chemostat experiments with HS143 (rpoS::lacZ'). Specifically, concentrations of specific metabolites were monitored to determine the effect of dilution rate and feed composition on cellular fluxes. Glucose and acetate were examined in particular due to their relative importance with respect to cell growth and productivity.

The specific glucose uptake rate in complex media is shown in figure 14. The concept of balanced growth in a chemostat implies that the uptake of glucose should be proportional to cellular growth (dilution) rate. However, figure 14 implies that glucose flux increased non-linearly with dilution rate. This trend can be attributed to the loose regulation of glucose uptake by growing cells as observed by Han et al., (1992) and Chou et al. (1994). Under conditions of excess environmental glucose, E. coli consumes glucose at a rate exceeding that required for cellular growth and energy requirements.

Cells cultivated in Reduced media had strikingly higher rates of glucose uptake compared to cells grown in all other media investigated in this study. This is possibly due to the fact that the Reduced media had the lowest concentration of preassembled biosynthetic building blocks found in the complex components of the media (Yeast Extract and Tryptone). The Enriched media along with the LB+28mM Glucose+30mM Acetate
media had lower glucose consumption rates than the other media under study. This could be due to the overabundance of complex material available to the cells in the Enriched media runs. The relatively high concentration of Yeast Extract and Tryptone available to the cell would relieve some of the biosynthetic and energetic burden required for growth and thus lower glucose demand. Glucose uptake by the cells grown in LB+28mM Glucose+30mM Acetate may have been lower due to the addition of Acetate as a secondary carbon source.

![Graph showing glucose flux vs dilution rate](image)

**Figure 14.** Glucose fluxes for HS143(rpoS::lacZ') grown in complex media under aerobic conditions. Chemostat studies were undertaken to observe the effects of dilution rate and feed composition on the uptake of glucose. Fluxes are reported in terms of mM of glucose consumed per unit cell density (OD600) per unit time. A value of 45 mM/OD600 h was obtained for the Reduced media at a dilution rate of 1.0 h⁻¹.

Closely tied to the phenomenon of glucose over-consumption in *E. coli* is the production and excretion of acetate under aerobic conditions (Han *et al.*, 1992; Ko *et al.*, 1993).

**Figure 15** implies that acetate production by the cells dramatically increased with dilution
rate for all media under study. This data is consistent with the carbon overflow hypothesis which predicts that the loosely regulated uptake of glucose leads to carbon overflow of the central metabolic pathway (Demain, 1972; Meyer et al., 1984; Holms, 1986; Han et al., 1992). As a consequence of this overflow, the cell is forced to balance its carbon flux through the acetate production pathway. The acetate synthesized by the cell is then excreted into the extracellular environment.

![Graph showing acetate fluxes](image)

**Figure 15.** Acetate fluxes for HS143(rpoS::lacZ') grown in complex media under aerobic conditions. Chemostat studies were undertaken to observe the effects of dilution rate and feed composition on the production of acetate. Fluxes are reported in terms of mM of acetate excreted per unit cell density (OD_{600}) per unit time.

In this study, the LB media and the Enriched media had the lowest specific rate of acetate production. It is reasonable to expect that the LB media alone would have a low level of acetate production since carbon overflow in this case would be relatively lower due to the absence of glucose in the media. The reduction of acetate flux by cells grown in the Enriched media is consistent with the decreased rate of glucose uptake by cells grown in
this media. Conversely, cells grown in Reduced media had the highest levels of glucose uptake and relatively high levels of acetate production. However in this case the ratio of acetate to glucose flux would support the conjecture that the increased acetate flux alone does not fully account for the dramatic increase in glucose flux through the cells.

The metabolic data collected in this study emphasizes the importance of media composition with respect to cellular fluxes. Similar observations concerning metabolic fluxes and acetate excretion have been made by Han et al. (1992). The data from this study suggests that acetate excretion can be attenuated by manipulation of the balance between carbon and nitrogen sources. Therefore, it should be possible to reduce the impact of acetate on cellular productivity by media optimization.
Recommendations for Further Study

Chapter 4

The current work opens up numerous doorways for investigation into the behavior of the \textit{rpoS} and other stationary phase induced genes. The next logical step in this research would be to study the difference in recombinant protein production between \textit{rpoS}\textsuperscript{-} and \textit{rpoS}\textsuperscript{+} strains when acetate is added to the feed media.

A possibly significant outcome of \textit{rpoS} deletion besides increased protein productivity (Chou \textit{et al.}, 1994) is a change in cell membrane and wall composition (Huisman and Kolter, 1994). A practical study that could be conducted on the \textit{rpoS}\textsuperscript{-} strains is the determination of cell disruption efficiencies of these strains compared to \textit{rpoS}\textsuperscript{+} strains. The modifications in cellular membrane composition could possibly increase cracking efficiencies and protein recovery downstream.

One possible direction for further study on acetate's effect on the cell would be to determine the relationship, if any, between acetate and the ppGpp synthesis pathway. This study has confirmed the relationship between \textit{rpoS} activation and extracellular acetate levels, however the study did not establish if acetate activates the gene specifically or if gene activation is a consequence of a more sophisticated activation scheme. Investigators such as Gentry \textit{et al.} (1993) have linked the expression of RpoS to the intracellular signal molecule ppGpp. Since ppGpp is synthesized by the product of the
relA and spoT genes in E. coli, elimination of these genes in a rpoS::lacZ' strain would demonstrate if acetate activated the rpoS gene through a ppGpp dependent pathway.

Another promising direction for the study of stationary phase genes and gene products is the integration host factor (IHF). Preliminary studies conducted by Avis et al. (1994) on himA and himD/hip, the structural genes for IHF, indicate that these genes have a role in protein synthesis and degradation. Mutation of these genes may provide a conducive environment for recombinant protein production.
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