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The effects of global and specific DNA-binding proteins on transcriptional regulation of the *bgl* operon

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Dennis Tran

Committee in charge:

Professor Milton Saier Chair
Professor Douglass Bartlett
Professor James Golden

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ABSTRACT OF THE THESIS

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Professor Milton Saier

The *bgl* operon is silent in wild-type strains of *Escherichia coli* and requires mutational activation, thereby allowing expression of functions necessary for the utilization of β-glucosides. Thus, it is normally cryptic under laboratory conditions, but nutrient scarcity in the presence of beta-glucosides can promote the occurrence of mutations that activates the *bgl* operon. In this work we characterized the transcriptional activities of different DNA-binding proteins, known to

regulate *bgl* expression, in the absence and presence of salicin, measuring β-galactosidase activities using transcriptional *lacZ* reporter gene fusions. We found that Crp is a substantial activator of the promoter region of *bgl* operon expression while Hns is a potent repressor. Our studies also determined the mechanism by which oligomerization of Hns allows formation of a loop between two binding sites, one in the promoter region and one internal to the downstream *bglG* gene, that is necessary and sufficient to silence *bgl* operon expression. IS5-mediated activation of *bgl* operon expression is therefore due to loss of the Hns binding site in the promoter region, blocking loop formation. To accomplish this, a mutant *hns* that abolishes its associative properties to loop but does not prevent binding to its two binding sites in the DNA, was used. We showed that although this mutant protein still binds to its binding sites in the DNA, it gives rise to increased *bgl* operon expression, comparable to that of a strain that is Bgl+ due to an IS5 insertional mutation, indicating that eliminating looping is sufficient to relieve repression.

Introduction:

Escherichia coli (*E. coli*) and the ancestral organisms from which it derived have adapted and evolved in response to environmental stresses since life first arose on Earth. In natural environments, *E. coli* has mostly survived under conditions of nutrient scarcity, leading to competition for limited resources. As a result, bacteria have evolved distinct mechanisms to derive utilizable substrates from their environment and obtain a fitness advantage over competitors. One of the ways bacteria were able to utilize this strategy is illustrated by the *bgl* operon of *E. coli* and the mechanisms of transposon insertion/excision which allow them to utilize nutritious plant-derived aromatic β-glucosides while avoiding toxic analogues of the same compounds.

As noted above, bacteria have evolved distinct mechanisms in order to obtain utilizable substrates from their environments and gain a fitness advantage over their competitors. One of the strategies bacteria such as *E. coli* use to accomplish this goal is a DNA (transposon) transposition mechanism that is associated with changes in cellular phenotypes. Sixty years ago, a geneticist, Barbara McClintock, discovered that transposable elements, known as "jumping genes", are DNA elements that can change position within the genome of an organism, thereby changing the organism's phenotype (Pray, 2008).

The ability of transposons to change from one genomic location to another has played a role in gene and genome evolution (Gao et al., 2015). There are three distinctly different known mechanisms of transposition: (1) a non-replicative "cut and paste" mechanism, where an integrase, acting with other proteins, excises the mobile element and integrates it at a distinct

target site, (2) a replicative “copy and paste” mechanism, where the transposon is first replicated, and then the replicated copy is integrated into the target site, and (3) a retro mechanism, in which an RNA copy of an *orf* containing the retro-transposon is converted into a cDNA at the insertion site, and the RNA in the DNA site is exchanged for the cDNA (Zhang and Saier, 2011). With the ability of DNA sequences to move from one location in the genome to another, insertional elements, such as IS1 and IS5, are among the most prevalent transposable elements in *E. coli* K12. These elements consist of one or more reading frames, one of which encodes the enzyme that catalyzes the transposition event, the transposase. Distinctive terminal sequences that set the IS elements apart from the flanking host DNA are present. IS elements can alter the functions of genes by activating or deactivating them (Zhang and Saier, 2011). Such types of mutations are necessary for activation of the *bgl* operon, allowing *E. coli* to utilize beta-glucosides and causing the bacterium to become *bgl* positive (Bgl+).

Following computational analysis, IS5 mutations have been shown to prefer regions of “superhelical stress-induced DNA duplex destabilization site” (SIDDs) in which regions in the chromosomal DNA more easily open into single-stranded bubbles (Humayun et al., 2017). Although IS5 insertion sites are not exclusive to SIDD sites, it is proposed that these SIDD regions have evolved to allow regulation of operons, like the *bgl* operon, so that a part of the bacterial population will have the *bgl* operon “off” (transcriptionally silent), allowing the organism to be resistant to toxic beta-glucosides, while another part of the population will be “on” (transcriptionally active), allowing it to utilize nutritious beta-glucosides. This mechanism has allowed the evolution of transposon-mediated adaptive and directed mutations in organisms including *E. coli*. Directed mutations are induced at a higher frequency under conditions that

allow the bacterium to avoid a stressful environment while taking advantage of a beneficial environment (Zhang and Saier, 2011). As a transposition event can be detrimental under some conditions but beneficial under others, living organisms have evolved mechanisms to control transposon movement in accordance with their specific needs.

As noted above, one example of this biological phenomenon involves the *bgl* operon in *E. coli* (Reynolds and Wright, 1981). This operon encodes all the necessary functions for the regulated uptake and utilization of aryl β-glucosides such as salicin and arbutin, as well as non-aromatic β-glucosides such as cellobiose, as carbon sources (Schnetz and Rak 1988). Normally, the *bgl* operon is cryptic or silent (not expressed) in wild-type *E. coli* strains. It can only utilize aryl β-glucosides when the operon is activated by a mutation, such as an IS insertional mutation upstream of the promoter (Choder and Wright, 1989).

The *bgl operon* includes three genes (*bglG*, *bglF* and *bglB),* all of which are essential for the transport and hydrolysis of β-glucosides (Schnetz et al., 1987). Early work on BglG has shown that it is a positive regulator of *bgl* operon expression (Mahadevan et al., 1987). Later, it was discovered that the *bglG* gene is flanked on both sides by two Rho-independent terminators; the product of this gene, BglG, functions to prevent termination at both sequences (Schnetz and Rak, 1988). Antitermination provides a regulatory mechanism for transcriptional control of *bgl* operon expression. Thus, BglG is an anti-terminator protein that binds to two RNA terminator structures to convert them into alternative antitermination structures.

The second gene in the *bgl* operon, *bglF*, is essential for β-glucoside uptake and phosphorylation, but it also plays a regulatory role, controlling the antitermination process (Schnetz and Rak 1988). Thus, BglF is both a phosphoenolpyruvate:sugar phosphotransferase system (PTS) enzyme and a regulator for the operon that prevents antitermination from occurring in the absence of a substrate β-glucoside or in the presence of a more easily metabolizable sugar such as glucose (Mahadevan et al., 1987). Consequently, expression of the *bgl* operon occurs only when a β-glucoside is present and other preferable carbon sources are absent. Antitermination is prevented by the product of the *bglF* gene as it is a negative regulator for the operon, a component of the PTS, that prevents antitermination in the absence of a β-glucoside (Schnetz and Rak 1990). This antitermination mechanism functions to positively regulate extension of the *bgl* operon mRNA. Moreover, the third gene in the operon, *bglB*, codes for phospho-β-glucosidase B that is responsible for hydrolyzing phosphorylated β-glucosides, such as salicin-P and arbutin-P, to release glucose-6-P and the aglycone (Harwani et al ., 2012). In summary, a cell is β-glucoside positive when it has a mutation within the *bgl* operon (most classically an IS insertional mutation upstream of the promoter) and a β-glucoside as a sole carbon source in the growth medium. This is possible because *bglG* is flanked on both sides by two rho-independent terminators, and its product, BglG, functions in anti-termination at both sites (Schnetz and Rak, 1988). A cell becomes *bgl* positive in response to a certain type of mutation in the *bgl* operon promoter region in the presence of a glucoside. The regulatory characteristics of the *bgl* operon are still not completely understood as the control mechanism by which IS insertion is regulated is not clear (Raveh et al., 2009).

Besides the three structural genes in the *bgl* operon responsible for the utilization of β-glucosides, several transcription factors are known to play roles in the regulation of *bgl* operon expression. For example, BglJ is a transcriptional regulator whose high-level constitutive expression causes activation of the Hns- and StpA-repressed *bgl* operon (Venkatesh et al., 2010). DNA-binding proteins may preferentially bind to single-stranded or double-stranded DNA, and these proteins play central roles in controlling transcriptional activities. Interestingly, DNA-binding proteins have been shown to regulate gene expression at multiple levels, causing high-level expression or silencing of operons (Hudson and Ortlund., 2014). BglJ expression relieves Hns repression of the *bgl* operon (Venkatesh et al., 2010). LeuO is a global transcription factor that is a regulator of the leucine biosynthesis operon of *E. coli*, and it is involved in the regulation of stress responses. It interferes with the silencing of *bgl* by Hns and thereby activates *bgl* operon transcription. Fis is a small basic DNA-binding protein that participates in essential cell processes such as rRNA and tRNA gene transcription (Ball et al., 1992), and Fis is a repressor of the *bgl* promoter (Caramel and Schentz, 2000). In our studies, the effects that deletion of each of these genes has on the *bgl* promoter and operon expression have been characterized. The results provide further insight into the possible mechanism that each gene plays in regulating expression of the *bgl* operon.

Crp, the cAMP receptor protein, is a global regulator in *E. coli* that binds cyclic AMP to regulate (usually to activate) expression of genes involved in carbon utilization. Crp regulates more than 180 genes by responding to the changing amounts of intracellular cAMP (Tsai et al., 2018). Cyclic AMP and Crp together form the cAMP-Crp complex that regulates gene expression. Crp-cAMP complex binding sites are within the promoter region of the *bgl* operon,

and this complex is known to activate *bgl* operon transcription (Madan et al., 2005). Crp can act either as an activator, a repressor, a corepressor, or a coactivator for the transcription of hundreds of genes in *E. coli* (Tsai et al., 2018).

E. coli histone-like heat-stable nucleoid protein (H-ns or Hns) is one of the major nucleoid proteins in *E. coli* that is involved in chromosomal stability and transcriptional regulation (Oshima et al., 2006). Hns binds to DNA and self-oligomerizes when bound to the DNA, and this creates a nucleoprotein complex that often represses transcription, either by blocking RNA polymerase binding or by trapping RNA polymerase (Dame et al., 2002). Therefore, Hns is an important negative regulator of transcription, and it decreases transcriptional read through of the *bgl* operon. Hns binds to the upstream promoter region of the *bgl* operon as well as a site within the *bglG* gene, causing the formation of a proposed loop between the two binding sites. It thus forms a bridge between adjacent DNA helices that are involved in transcriptional regulation and DNA compaction (Chib and Mahadevan, 2012). Hns plays an important role in regulating physiological adaptation to the environment, and as discussed above, it can play a role in regulating transposon-mediated directed mutation, determining how mutations can occur at higher frequencies when beneficial to the organism under stressful environmental conditions.

Microorganisms, such as *E. coli*, can survive extended periods of starvation as they can undergo rapid changes in gene expression during prolonged incubation in the stationary phase (Zambrano et al., 1993). RpoS is a stress/stationary phase-response sigma factor. RpoS improves biomass recycling and decreases the death rate, particularly after vegetative growth when cells

enter the stationary growth phase (Schink et al., 2019). It thus plays an important role in survival under environmental stress conditions in many γ-proteobacteria (Dong and Schellhorn, 2009). Environmental stresses, such as are observed when *E. coli* cells enter the stationary growth phase, regulate, and enhance the expression of many genes including the *bgl* operon, conferring a growth advantage in stationary phase (GASP) to Bgl+ cells (Harwani, 2014). Even when *rpoS* is expressed at low levels, mutation of *rpoS* affects the expression of a variety of genes (Dong and Schellhorn, 2009). Survivors of these stressful environments carry mutations that confer advantages to cells in the stationary phase. Therefore, not surprisingly, the first mutations that provide a survival advantage in the stationary growth phase are in the *rpoS* locus (Madan et al., 2008).

To examine the effects that RpoS has on the transcriptional activity of the *bgl* operon, *rpoS* deletion strains were used in comparison to the wild-type and Hns deletion strains to measure and analyze the transcriptional activities of the *bgl* operon during the stationary phase of *E. coli* growth.

As noted above, Hns is a global regulator that is known to silence the *bgl* operon. However, the repression mechanism is not fully understood as there is some evidence that Hns alone is not involved in silencing the *bgl* operon (Ohta et al., 1999). In this thesis, I explore in-depth the effects of each proposed transcriptional regulator. Deletion mutants were constructed for each of these genes, and these were analyzed by measuring *bgl* transcriptional activities (using a *lacZ* reporter gene) of both the promoter and intact P*bgl-bglG* operon fragment with and without salicin as the inducer of *bgl* operon expression. In these experiments, I used IS5

insertional mutations as a basis for generating strains to test the effect of Hns looping on transcription as these IS-mediated insertional mutations disrupt the ability of Hns to bind in the promoter region, and therefore to oligomerize and form its "repressive loop". Furthermore, I confirm an earlier suggestion that Hns binding to both the promoter and downstream *bglG* regions is insufficient to silence the operon, contrary to what others had claimed. Instead, I provide evidence that the ability of Hns to oligomerize and form a dsDNA loop is necessary and sufficient to provide the strong repressive mechanism that silences *bgl* operon expression. The impact of Hns on the transcriptional activity of the *bgl* operon was further investigated in the stationary growth phase in *E. coli*, and the effect of an *rpoS* deletion mutation was determined.

Materials and Methods:

Media

E. coli strains were cultured at 37 °C in LB broth, and LacZ assay experiments were conducted following growth in M63 media with either 0.5% glycerol, 0.5% salicin, or both 0.5 % glycerol and 0.5% salicin as carbon sources (Pardee et al., 1959). 10x M63 salt contains 15 mM of $(NH_4)_2SO_4$, 100 mM of KH_2PO_4, and $2x10^{-2}$ mM of $FeSO_4 \cdot 7H_2O$. After diluting to 1x M63 medium, it was supplemented with 10^{-4} % thiamine, 0.05% casamino acids and 1.7 mM MgSO4. This minimal medium was used for the growth of all cells in preparation for cell harvesting, washing, and disruption before toluene treatment for β-galactosidase assay.

Preparation of bacterial strains

Table 1. Primers and templates used in strain construction.

Name	Purpose	Sequence (5’ to 3’)
Pbgl-Xho-F	**Cloning Pbgl or Pbgl-bglG into pKDT**	**atactcgagtggcgatgagctggataaactgctg**
Pbgl-Bam-R	**Cloning Pbgl into pKDT**	**ttaggatcctgcagtaacaatccagtcatttattaatg**
bglG-Bam-R	**Cloning Pbgl-bglG into pKDT**	**taggatccttagactatttttctggctaactccgtc**
tPbgl-Xho-F	**Cloning truncated Pbgl or Pbgl-bglG**	**ctaaaattacacaaagttaataactg**
intS-P1	**Integration of PtetM2-bglG into the intS site**	**agatttacagttcgtcatggttcgcttcagatcgttgacagccgcactccatgtgtaggctggagctgcttc**
Ptet-R	**Integration of PtetM2-bglG into the intS site**	**gaattttggtgatttgcatgttcatggtacctttctcctctttaatgaattc**
bglG-F	**Integration of PtetM2-bglG into the intS site**	**gaaaggtaccatgaacatgcaaatcaccaaaattctc**

Table 1 continued.

IntS-bglG-P2	**Integration of PtetM2-bglG into the intS site**	**gatagttgttaaggtcgctcactccaccttctcatcaagccagtccgcccttcagtgttctttgcgcacgcgctc**
IntS-ver-R2	**Verification of integration**	**aaaggaatgaagtctatcatccaagtc**
bglG-R	**Amplification of km:T:Ptet-bglG for fusion PCR**	**gatcacaggttgccatcacctcaagaggaatatgac**
Hns-AB-F	**Amplification and chromosomal integration of tetA:sacB**	**ctcttcgtgcgcaggcaagagaatgtacacttgaaacgctggaagaaatgctcctaatttttgttgacactctatc**
Hns-AB-R	**Amplification and chromosomal integration of tetA:sacB**	**agccgcgctttcttcttcgcgacgttcgttaacaacaacttctaatttttcatcaaagggaaaactgtccatatgc**
Hns-100	**Template for Hns L30P amplification**	**cttcgtgcgcaggcaagagaatgtacacttgaaacgctggaagaaatgcctgaaaaattagaagttgttgttaacgaacgtcgcgaagaagaaagcgcgg**
Hns-F	**Amplification of Hns-100**	**cacttaaaattctgaacaacatccgtactcttcgtgcgcaggcaagagaatg**
Hns-R	**Amplification of Hns-100**	**gagtgcgctcttcaacttcagcagcagccgcgctttcttcttcgcgacgttc**

Table 1 continued.

Hns-ver-R	**Verification of Hns L30P on chromosome**	**agattattgcttgatcaggaaatcgtc**
Ptet-yjjQ-P1	**Chromosomal Ptet driving yjjQ/bglJ**	**tgatatgaaagtgaatgctaaggataatttattcgctaatctattaatttgtgtaggctggagctgcttc**
Ptet-yjjQ-P2	**Chromosomal Ptet driving yjjQ/bglJ**	**cataacaggtattttactgataacaattccatttttgcagcatcctggcaacatggtacctttctcctctttaatgaattc**
yjjQ-ver-R	**Verification of Ptet driving yjjQ/bglJ**	**aacagaacgtggatcttcactttcac**
Ptet-rcsB-P1	**Chromosomal Ptet driving rcsB**	**gaaaaatacatcagcgacattgacagttatgtcaagagcttgctgtagcaagtgtgtaggctggagctgcttc**
Ptet-rcsB-P2	**Chromosomal Ptet driving rcsB**	**gaacaagactatcggatggtcatcggcaataattacgttcatattgttcatggtacctttctcctctttaatgaattc**
RcsB-ver-R	**Verification of Ptet driving rcsB**	**tacttatcgccaggcatggagagatcg**
Ptet-leuO-P1	**Chromosomal Ptet driving leuO**	**ttatggattattatgctgtggtaaatgactcattccacggcaatggattctgtgtaggctggagctgcttc**
Ptet-leuO-P2	**Chromosomal Ptet driving leuO**	**ggtttgcttaactccgccgtctctggatgatctgtttgtacctctggcatggtacctttctcctctttaatgaattc**

Table 1 continued.

LeuO-ver-R	**Verification of Ptet driving leuO**	**tgttttgctcctgcatcacggcatcg**
pKDT Ptet	**Plasmid for cloning km:T**	

Construction of P*tet* and lacIq promoters driving *bglG* expression on the chromosome

Since overexpression of *bglG* is toxic to cells, P*tet* driven *bglG* cannot be cloned into a regular integration plasmid such as pKD13 before being moved to the chromosome. To make a chromosomal copy of P*tet* driven *bglG*, the region containing the km resistance gene (*km*^{r}), the *rrn*B terminator (*rrnB*T) and P*tet* (e.g., *km*^{r}:*rrnB*T:P*tet*) was amplified from pKDT (PMID: 20064380) using primers intS-P1 and intS-bglG-P2 (Table 1). The *bglG* gene was amplified from BW25113 chromosomal DNA using primers BglG-Kpn-F and BglG-Bam-R (Table 1). Please note the 35 bp region in the 3' end of the "*km*^{r}:*rrnB*T:P*tet*" fragment is the same as the first 35 bp region of the *bglG* fragment since the reverse primer IntS-bglG-P2 and the forward primer intS-P1 shared carried this 35 bp overlapping region in their 5' ends. After gel purification, the *km*^{r}:*rrnB*T:P*tet* fragment and the *bglG* gene were fused together by standard fusion PCR using their PCR products as templates and intS-P1and IntS-bglG-P2 as primers. The resultant fused product, "*km*^{r}:*rrnB*T:P*tet-bglG*", was gel purified and electroporated into the chromosome of BW25113 cells (expressing the λ-Red recombinase) to replace the *intS* gene using the Lamada-Red methods as described by Datsenko and Wanner (2000). After being confirmed by colony PCR and DNA sequencing, a correct clone was cultured in LB at 40 ^{o}C

overnight to remove the Lamada-red plasmid. The final strain carried the "*km*^{r}:*rrnB*T:P*tet-bglG*" construct at the *intS* locus and was named BW25113 P*tet-bglG* (Table 1).

To make lacIq driving *bglG*, the lacIq promoter was cloned from pZSint4 (Lutz and Bujard, 1997) into pKDT, yielding pKDT-lacIq. The region carrying the *km*^{r} gene, the *rrn*B terminator (*rrnB*T) and lacIq (that is, *km*^{r}:*rrnB*T:Iq) was amplified from pKDT-lacIq. The "*km*^{r}:*rrnB*T:Iq" fragment was fused to *bglG*, and the fused *km*^{r}:*rrnB*T:Iq-*bglG* product was then integrated into the *intS* site, yielding strain BW25113 Iq-*bglG*.

Construction of P*bgl-lacZ* and P*bgl-bglG-lacZ* transcriptional fusions

To make the *bglGFB* promoter-*lacZ* transcriptional fusion used to measure the promoter activities, the promoter region (-205 to +54 relative to the transcriptional start site, +1) without the first terminator upstream of *bglG*, was cloned into the XhoI/BamHI site of the integration vector, pKDT, yielding pKDT-Pbgl. The region carrying the km, *rrnBT* and P*bgl* (*km*^{r}:*rrnB*T:P*bgl*) was PCR amplified using oligos Pu1n-P1n and Pbgl-Z-P2 (Table 1) and then integrated into the chromosomal default strain EQ42 (Klumpp et al., 2009) to replace the *lacI* gene and the *lacZ* promoter. After being confirmed by PCR and sequencing, the promoter reporter, P*bgl* driving *lacZ* expression (that is, *km*^{r}:*rrnB*T:P*bgl* drives *lacZ*) was transferred into BW25113 and the various mutants by P1 transduction.

Using primers P*bgl*-Xho-F/*bglG*-BamH-R (Table 1), the region carrying the *bglGFB* promoter and the first gene, *bglG*, including the 2nd terminator downstream of the *bglG* translational stop codon (the 205th nucleotide to the 1127th nucleotide relative to the

transcriptional start site) was amplified from BW25113 genomic DNA. The PCR products were digested with *Xho*I and *BamH*I and then inserted into the same sites of the plasmid pKDT [Klumpp et al., 2009), yielding plasmid pKDT P*bgl-bglG*. Present in this plasmid, the "*km*:*rrnB*T:P*bgl-bglG*" DNA fragment was amplified using primers P*bgl*-Z1/*bglG*-Z2 and then integrated into the chromosome of default strain EQ42 (Klumpp et al., 2009) to replace the region containing *lacI* and the entire *lacZ* promoter. After being confirmed by colony PCR and DNA sequencing, the P*bgl-bglG-lacZ* transcriptional fusion was transferred to BW25113 and BW25113 P*tet-bglG* by P1 transduction, yielding strains BW25113 P*bgl-bglG-lacZ* and BW25113 P*tet-bglG* P*bgl-bglG-lacZ*, respectively (Table 1).

To make a truncated P*bgl-bglG-lacZ* transcriptional fusion (tP*bgl-bglG-lacZ*), a smaller DNA region (-93 to +1127 relative to the transcriptional start site, +1) was cloned into pKDT, yielding pKDT-tP*bgl-bglG*. This DNA fragment, "*km*^{r}:*rrnB*T:tP*bgl-bglG*", was integrated into the EQ42 chromosome, yielding tP*bgl-bglG* that drives *lacZ* expression.

Construction of the HnsL30P strain using positive/negative selection

H-NS (Hns) is the primary silencer of the *bgl* operon. The H-NS protein usually exists in oligomeric forms that contribute to its biological activity (Smyth et al., 2000), promoting formation of structures such as DNA loops and bridges (Rimsky, 2004 and Dame et al., 2006). The N-terminal domain is responsible for H-NS oligomerization. The leucine residue at position 30 is essential for H-NS:H-NS binding (Smyth et al., 2000). To test the possible looping mechanism by which H-NS silences the *bgl* operon, we used a two-step recombineering protocol based on TetA-SacB positive-selection and counter-selection (Li et al., 2013) to change the

leucine codon CTG (88 to 90 relative to the first H-NS codon ATG) to a proline codon CCT in the *hns* gene. "TG" in the leucine codon was first replaced by the *tetA-sacB* cassette that was amplified from the chromosomal DNA of strain T-SACK (Li et al., 2013) using chimeric oligos Hns-AB-F and Hns-AB-R (Table1). These long oligos carry the appropriate homologous arms flanking the "TG" nucleotides on the *hns* gene. The replacement of "TG" by *tetA-sacB* in some tetracycline (Tc) resistant mutants was confirmed by colony PCR and sequencing. A 100-bp single strand DNA fragment, which covers the region of the *hns* gene (38 to 138 relative to ATG) with CT replacing "TG" in the middle, was synthesized. This fragment was amplified using oligos Hns-F and Hns-R (Table 1), and PCR products were electroporated into the cells of a Tc resistant mutant that expressed Lambada-Red proteins encoded by pKD46 (Datsenko and Wanner, 2000). After one-hour incubation, the electroporated cells were applied onto TetA/SacB counter-selection agar (plus 6% sucrose and 24 mg fusaric acid per liter). After incubation at 42C for about two days, about 10 colonies resistant to sucrose and fusaric acid were purified on LB agar plates and tested for both sensitivity to Tc and resistance to sucrose. Several Tc-sensitive/sucrose-resistant colonies were confirmed for the replacement of the *tecA-sacB* cassette by "CT" by PCR and sequencing. The resultant altered strain was named Hns_L30P, in which the 30th codon is changed from leucine to proline.

Constitutive expression of positive *bgl* operon regulators

BglJ, RcsB and LeuO have been reported to positively regulate *bgl* operon expression (Ohta et al., 1999). To examine if these proteins elevate *bglGFB* promoter activity and/or activate the operon under various conditions, the regulatory region of each of these genes was replaced by a strong, constitutive *tet* promoter (P*tet*). Using pKDT-P*tet* as template, the region carrying the kanamycin resistance gene (*km*^{r}), the *rrnB* transcriptional terminator (*rrnB*T) and

P*tet* (that is, *km*^{r}:*rrnB*T:P*tet*) was amplified using a pair of chimeric oligos (Table 1). The PCR products were gel purified and then electroporated into wild type BW25113 cells expressing the l-Red recombinase. The cells were incubated with shaking at 37 ^oC for 1 hour and then applied onto LB + Km agar plates. The Km^r colonies were verified for the replacement of the promoter region of the target gene by the "*km*^{r}:*rrnB*T:Ptet " cassette by PCR and sequencing. The resultant strains were named P*tet-bglJ*, P*tet-rcsB* and P*tet-leuO*, respectively. To make the *bglJ rcsB* double expression strain, the *kmr* gene from the P*tet-bglJ* strain was flipped out using pCP20. The P*tet-recB* cassette was transferred to the P*tet-bglJ* strain, yielding the P*tet-bglJ*/P*tet-rcsB* double expression strain.

To determine the effects of high-level constitutive expression of positive regulators on the *bglGFB* promoter and operon, these expression cassettes (P*tet-bglJ*, P*tet-rcsB* and P*tet-leuO*) were individually or doubly transferred to various *lacZ* reporter strains.

Strains List

Table 2. List of strains used in this study

BW25113	Wild-type
BW25113:km P*bgl*-Z	Resistant to km with the promoter reporter driving *lacZ* gene expression.
BW Bgl+ km: P*bgl*-Z	Insertion of *lacZ* at the end of the *bgl* operon, allowing utilization of salicin. The strain is resistant to km with the promoter driving *lacZ* gene expression.

Table 2 continued.

Δ*bglG*:km P*bgl*-Z	Insertion of the km gene into *bglG*, rendering the strain resistant to km with the promoter driving expression of the *lacZ* gene
Δ*bglJ*:km P*bgl*-Z	Elimination of *bglJ;* km resistant with the promoter driving *lacZ* gene expression
Δ*crp* km: P*bgl*-Z	Elimination of the *crp* gene; resistant to km with the promoter driving *lacZ* gene expression
Δ*fis*:km P*bgl*-Z	Elimination of *fis* ; resistant to km with the promoter driving *lacZ* gene expression
Δ*hns*:km P*bgl*-Z	Elimination of *hns* ; resistant to km with the promoter driving *lacZ* gene expression
Δ*leuO*: km P*bgl*-Z	Elimination of *leuO*; resistant to km with the promoter driving *lacZ* gene expression
Δ*rcsB*:km P*bgl*-Z	Elimination of *rcsB*; resistant to km with the promoter driving *lacZ* gene expression
Δ*cyaA*:km Glp+ P*bgl*-Z	Elimination of *cyaA*; resistant to km with the promoter driving *lacZ* gene expression The *glpFK* operon is constitutively expressed due to an IS5 insertion.
*hns*L30P km P*bgl*-Z	*hns* with an amino acyl change from leucine to proline at the 30th codon; resistant to km with the promoter driving *lacZ* gene expression
Bgl+ Δ*hns* km P*bgl*-Z	Elimination of *hns* with an insertion in the *bgl* operon; resistant to km with the promoter driving *lacZ* gene expression
BW25113 Δ*bglJ:*km	Elimination of *bglJ*; resistant to km with the promoter driving *lacZ* gene expression
BW25113 P*bgl-bglG*-Z	Wild-type operon reporter with the *bglG* gene intact between the promoter and the *lacZ* reporter gene
Δ*bglG* km P*bgl-bglG*-Z	Elimination of *bglG*; resistant to km with the operon driving *lacZ* gene expression.
Δ*bglJ* km P*bgl-bglG*-Z	Elimination of *bglJ*; resistant to km with the operon driving *lacZ* gene expression.

Table 2 continued.

Δ*crp* Glp+ km P*bgl-bglG*-Z	Elimination of *crp*; resistant to km with the operon driving *lacZ* gene expression.
Δ*fis* km P*bgl-bglG*-Z	Elimination of *fis* ; resistant to km with the operon driving *lacZ* gene expression.
*hns*L30P km P*bgl-bglG*-Z	A mutant *hns* with an amino acyl change from leucine to proline at the 30th codon; resistant to km with the operon driving *lacZ* gene expression
Δ*hns* km P*bgl-bglG*-Z	Elimination of *hns* ; resistant to km with the operon driving *lacZ* gene expression.
Δ*leuO* km P*bgl-bglG*-Z	Elimination of *leuO*; resistant to km with the operon driving *lacZ* gene expression.
Δ*rcsB* km P*bgl-bglG*-Z	Elimination of *rcsB*; resistant to km with the operon driving *lacZ* gene expression.
Δ*stpA* km P*bgl-bglG*-Z	Elimination of *stpA*; resistant to km with the operon driving *lacZ* gene expression.
Δ*cyaA* km Glp+ P*bgl-bglG*-Z	Elimination of *fis* and IS5 insertional mutation to Glp+ to allow the cells to utilize glycerol as a carbon source even in the absence of *crp*. Resistant to km with the operon driving *lacZ* gene expression.
Bgl+ IS5 km P*bgl-bglG*-Z	IS5 insertion in the native *bgl* operon; able to utilize salicin; resistant to km with the operon driving *lacZ* gene expression.
BW 25113 km IS5 P*bgl*-Z	Wild type with the IS5 insertional mutation within the promoter driving *lacZ* gene expression.
BW 25113 Bgl+ km IS5 P*bgl*-Z	IS5 insertion in the native *bgl* operon; able to utilize salicin; resistant to km with the insertional mutation within the promoter driving *lacZ* gene expression.
*hns*L30p km: IS5 P*bgl*-Z	A mutant *hns* with a change from leucine to proline at the 30th codon; resistant to km; insertional mutation within the promoter driving *lacZ* gene expression.

Table 2 continued.

Δ*crp* Glp+ IS5 P*bgl*-Z	Elimination of *crp*, and one IS5 insertional mutation to Glp+ to allow the cells to utilize glycerol as a carbon source; resistant to km with another IS5 insertional mutation within the promoter driving *lacZ* gene expression.
Δ*hns* km IS5 P*bgl*-Z	Elimination of *hns*; resistant to km with an insertional mutation within the promoter driving *lacZ* gene expression.
Bgl+ Δ*hns* km IS5 P*bgl*-Z	Elimination of *hns* with an insertion in the native *bgl* operon; able to utilize salicin and is resistant to km with an insertional mutation within the promoter driving *lacZ* gene expression.
BW25113 IS5 P*bgl-bglG*-Z	Wild type with an IS5 insertional mutation within the operon driving *lacZ* gene expression.
Bgl+ IS5-P*bgl-bglG*-Z	Insertion in the native *bgl* operon; able to utilize salicin and is resistant to km with an IS5 insertional mutation within the operon driving *lacZ* gene expression.
Δ*crp* Glp+ IS5 P*bgl-bglG*-Z	Elimination of *crp* and IS5 insertional mutation to Glp+ to allow the cells to utilize glycerol as a carbon source; resistant to km with an insertional mutation within the *bgl* operon driving *lacZ* gene expression.
Δ*hns* IS5 *Pbgl-bglG*-Z	Elimination of *hns*; resistant to km with an insertional mutation within the operon reporter driving *lacZ* gene expression.
Δ*hns*L30P IS5 P*bgl-bglG*-Z	A mutant *hns* with a change from leucine to proline at the 30th codon with resistance to km and insertional mutation within the operon driving *lacZ* gene expression.
BW 25113 *t*P*bgl-bglG*-Z	Wild type with the IS5 insertional mutation within the promoter driving *lacZ* gene expression.
Bgl+ BW 25113 *tPbgl-bglG*-Z	IS5 insertion in the native *bgl* operon; able to utilize salicin and is resistant to km with a truncation mutation within the operon driving *lacZ* gene expression.

Table 2 continued.

Δ*hns tP*b*gl-bglG-Z*	Elimination of *hns*; resistant to km with a truncation mutation within the operon driving *lacZ* gene expression.
*hns*L30P *tP*b*gl-bglG-Z*	A mutant *hns* with a change from leucine to proline at the 30th codon; resistant to km; truncation mutation within the operon driving *lacZ* gene expression.
lacIq-bglG P*bgl-bglG-Z*	*lacIq* promoter driving *bglG* expression; resistant to km with the operon driving *lacZ* gene expression.
P*tet-bglG* P*bgl-bglG-Z*	*ptet* promoter driving *bglG* expression; resistant to km with the operon driving *lacZ* gene expression.
Δ*rpoS* km P*bgl-Z*	Elimination of *rpoS*; resistance to km with the promoter driving *lacZ* gene expression
Δ*rpoS* km: IS5 P*bgl-Z*	Elimination of *rpoS* with an IS5 insertional mutation within the promoter driving *lacZ* gene expression; resistance to km with the operon driving *lacZ* gene expression.
Δ*rpoS* km: P*bgl-bglG-Z*	Elimination of *rpoS*; resistance to km with the operon driving *lacZ* gene expression.
Δ*rpoS* km IS5 P*bgl-bglG-Z*	Elimination of *rpoS* with an IS5 insertional mutation within the operon driving *lacZ* gene expression.

β-Galactosidase (LacZ) Assay:

o-Nitrophenyl-β-galactoside (ONPG), a synthetic substrate of LacZ, when cleaved, yields galactose and o-nitrophenol. The yellow color, produced by the release of o-nitrophenol and its dissociation to the anionic form, was used to measure β galactosidase activity and thereby to quantitatively estimate the activity of the promoter or operon to which the *lacZ* gene was fused. Samples were collected during the exponential or stationary growth phase. LacZ assays were all done in 37 °C.

Growth and cells:

1. The inoculum was grown in LB medium overnight.
2. Thirty µL of this cell culture in LB medium were used to inoculate 3 mL of M63 medium for overnight growth with shaking in the presence of 0.5% glycerol or 0.5% salicin or both as the carbon source(s).
3. The cultures were inoculated into 5 ml of the same medium, and the cells were grown at 37°C with shaking (600 rpm), starting at an OD_{600} of 0.03.
4. To measure the OD at 600 nm, four samples (300 µL for promoter reporter strains, and 650 µL for operon reporter strains) were collected during the exponential growth phase before freezing.
5. Frozen samples were stored at -20 °C for subsequent measurement when necessary.

LacZ Assay

1. The collected samples were diluted with the LacZ assay buffer and brought to a total volume of 1000 µL.
2. 25 µL chloroform was added to each sample which was vortexed twice for ten seconds.
3. Samples were then put into a 37°C water bath, and 200 µl of 4mg/mL β-ONPG was added to each sample (at intervals of 10 seconds for the different samples).
4. After yellow color development, the reaction was terminated by adding 500 µL of 1 M sodium carbonate to each sample, and the reaction duration as well as the optical densities at 420 nm and 550 nm were recorded.
5. The following equation was used to calculate β-galactosidase activity :

$$\frac{1000\ x\ (OD_{420nm} - 1.75\ x\ OD_{550nm})\ x\ Dilution\ Factor}{Min.\ of\ Reaction\ x\ Volume\ of\ sample\ (mL)}$$

Equation 1. The equation was used to calculate the β-galactosidase activity in Miller units (Miller, 1972).

β-Galactosidase activity was measured as described using equation 1. The slope of the graph was determined using four time points. The slope was calculated using the optical density at 600 nm of each sample plotted on the X-axis and the β-galactosidase activity units on the Y-axis.

Electroporation:

To construct the strains used for these experiments, electroporation was performed to introduce DNA into the bacterial cells using a pulse of electricity to open pores in the cell membrane. This was done mainly to remove the Km resistance marker in the plasmid, as the empty plasmid of pcp20 will remove the gene responsible for Km resistance and replace it with a gene conferring resistance to ampicillin. However, pcp20 is heat-sensitive; after heating the cells at 40°C, the cells are sensitive to both ampicillin and kanamycin.

P1 Transduction:

P1-transduction allows the transfer of DNA from one bacterium to another (Thomason et al., 2007). P1 bacteriophages were grown in cells having the element to be moved, and the phage was used to infect recipient strains as discussed by Thomason et al., 2007.

***E. coli* Strains**

The wild-type strain used in these experiments was *E. coli* BW25113. We used two different types of promoter reporters and three different types of operon reporters. The first promoter-reporter is labeled P*bgl*-Z and has a *lacZ* fusion to the promoter region of the *bgl*

operon. The second promoter-reporter is labeled IS5 P*bgl*-Z and is the same as the previous reporter except that it has an IS5 insertional mutation in front of the *bgl* operon. Different reporter strains were constructed to measure the promoter and operon levels of expression using β-galactosidase activity as a measure of the transcriptional level under different environmental conditions, different gene variants, and in the presence of various transcription factor gene deletions and overexpression vectors.

Strains were created with the intact *bgl* operon in one place of the chromosome with the *lacZ* fusion reporters at two different positions in the native *bgl* operon, one immediately following the *bgl* promoter, and a second just after the complete *bglG* gene.

Results

Significance of Crp to *bgl* operon promoter activity

To determine the activity of the promoter region of the *bgl* operon, a promoter-*lacZ* reporter was constructed (see Methods). The wild-type strain was compared to other isogenic strains in which various regulatory genes had been deleted. These experiments were performed in M63 media with 0.05% CAA and 0.5% glycerol as the primary carbon source.

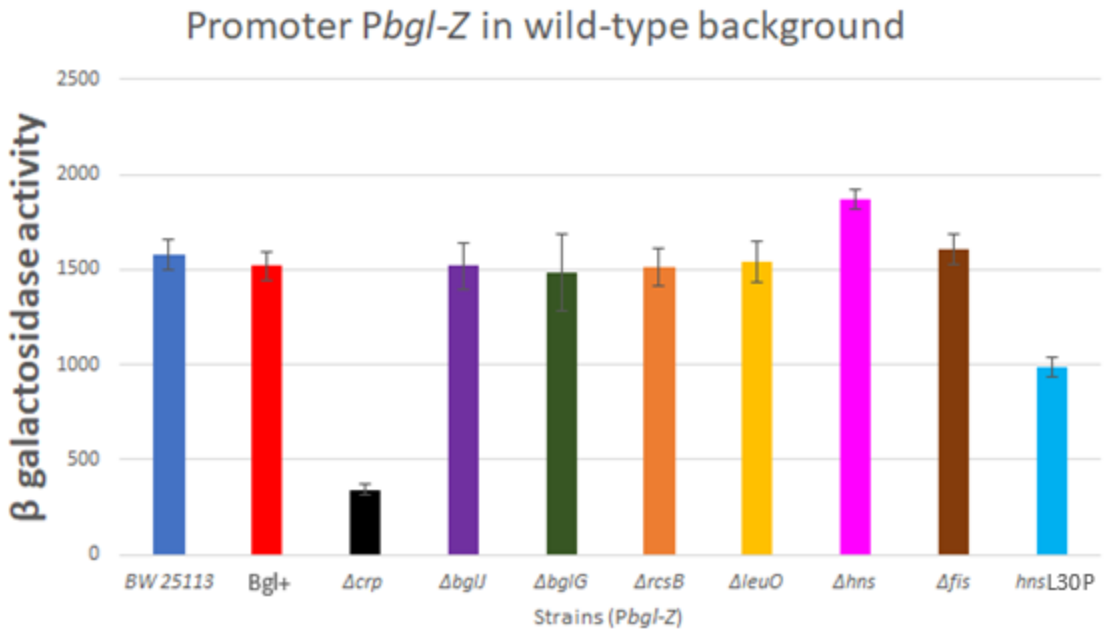

Figure 1. β-galactosidase activities measuring the *bgl* promoter activities in the wild-type genetic background with different deleted or mutated genes, as described under Materials and Methods and Table 2. Cells were grown in M63 medium with glycerol as the primary carbon source. Error bars represent standard deviations for the various samples. Samples were collected at OD_{600} values of 0.2, 0.4, 0.6 and 0.8, and β-galactosidase activities were determined using the slopes of the OD values; β-galactosidase activities were calculated using Equation 1.

As shown in Figure 1, deletion of the genes encoding most of the transcription factors shown previously to influence *bgl* operon expression when overexpressed, except the *crp* gene, had little or no effect on promoter activity compared to the wild-type strain. However, the loss of the cAMP receptor protein (Crp) resulted in ~5-fold lower activity than the wild-type strain and other deletion mutants. Thus, Crp plays an important role in promoter activation. Deletion of *hns* gave rise to a 20% increase in activity, while the L30P mutation in the *hns* gene that prevents oligomerization of this nucleoid protein had substantially lower promoter activity, presumably because this mutant protein binds to its binding sites with higher affinity than the wild type protein.

M63 minimal media with salicin as the carbon source and inducer were used to explore promoter activity under inducing conditions. All deletion strains used in Fig. 1 were included in this experiment to compare the effects of these deletion and point mutations on promoter activity under both non-inducing and inducing conditions.

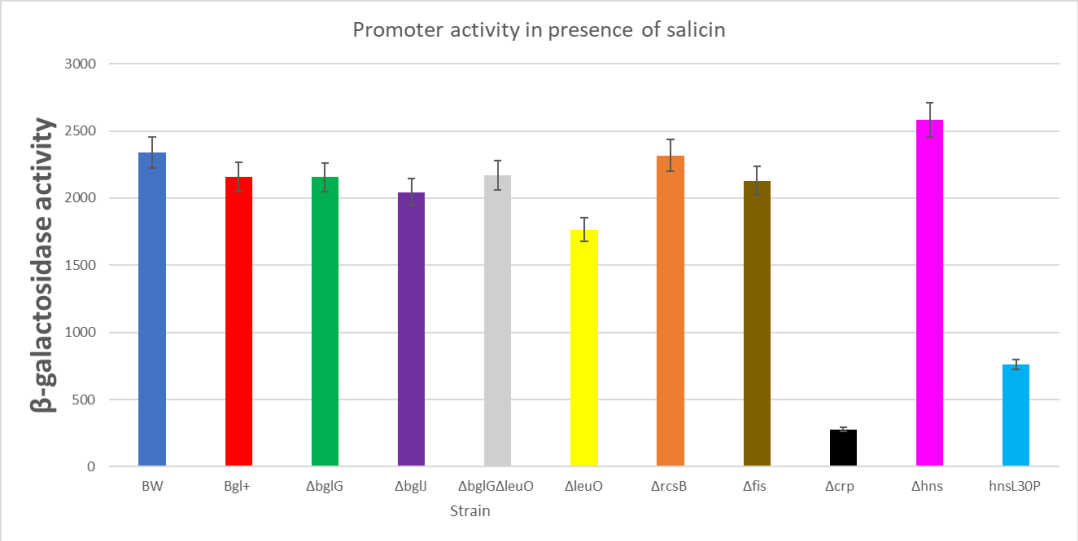

Figure 2. β-Galactosidase reporter gene activities used to measure the effects of various deletion and point mutations on *bgl* operon promoter activity under the same conditions as for Figure 1, except that 0.5% salicin was included in the media. Error bars represent the standard deviation for each set of samples.

In the presence of salicin, the deletion of *crp* reduced the promoter activity by a factor of 7. Although deletion of Hns had only a slight positive impact on the β-galactosidase activity compared to the wild-type and other deletion strains, HnsL30P had 2.5x decreased activity. Thus, both effects (loss of Crp and this mutation of *hns*) showed larger reduction in promoter activity with salicin present than under non-inducing conditions (compare Figures 1 and 2). Of particular note is the fact that in both Figures, HnsL30P had lower β-galactosidase activity compared to the wild-type and the other deletion strains, except for the *crp* deletion. This may be due to the increase in affinity that HnsL30P appears to have relative to the wild type protein (see

Discussion). It is also worth noting that deletion of the leuO gene reduced promoter activity by about 20%.

DNA looping by Hns is essential for full *bgl* operon silencing

The *bgl* operon is known to be silenced in a wild-type *E. coli* strain by Hns, but the mechanism by which Hns causes this repression is not fully understood (Sankar et al., 2009). To test the hypothesis that Hns-mediated DNA looping is involved, the transcriptional activity of the *bgl* operon was tested using different deletion strains, including an *hns* deletion strain and the strain in which leucine was changed to a proline in the 30th codon of the *hns* gene. By changing this amino acid, the Hns protein can still bind to the promoter (and presumably to the *bglG* site within the *bgl* operon, but it cannot oligomerize to form the repression loop. As noted above, the results shown in Figures 1 and 2 clearly suggest that this mutation causes the protein to bind more tightly to its binding site in the *bgl* promoter region, and that binding has a negative effect on promoter activity.

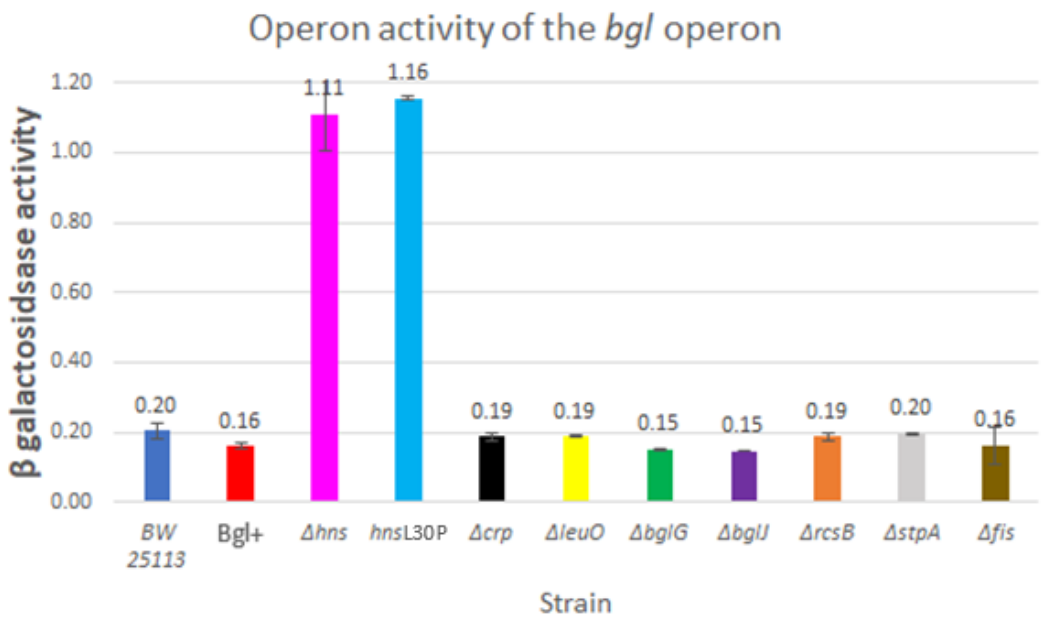

Figure 3. β-galactosidase activities of the *bgl* operon measured using the *lacZ* reporter gene. Different deleted genes were tested as in Figures 1 and 2 with cells grown in M63 medium with glycerol as the carbon source. LacZ assays were performed for each strain as described under Methods. Error bars indicate the standard deviation values for each set of strains and conditions.

Figure 3 shows that the wild-type and most of the deletion strains, (except for that of *hns* and the *hns*L30P mutant), have similar β-galactosidase activities, showing that only these two mutations had substantial effects on expression. Moreover, Figure 3 demonstrates that the *hns*L30P strain had activity similar to that of the *hns* deletion strain; both have about 6x increased expression compared to the wild-type strain and the other deletion mutants. As the experiments shown in Figure 3 were performed in media without salicin, the low β-galactosidase activity suggests that the *bgl* operon is largely silenced by Hns.

To determine the β-galactosidase activity of the *bgl* operon when the operon is induced by inclusion of salicin in the growth media, strains with *bglG-lacZ* operon reporter fusions were

tested using M63 media with salicin as the carbon source. Into the *bglG-lacZ* fusion reporter strain, we introduced the different deletion mutations.

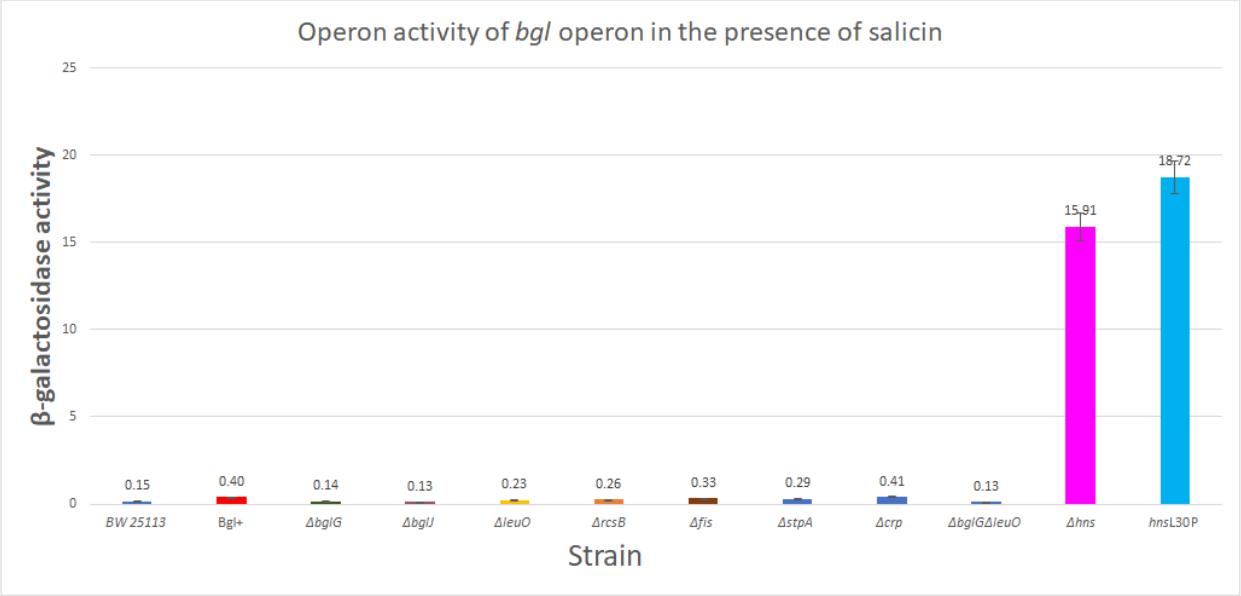

Figure 4. Expression of the *bgl* operon as measured with a LacZ reporter gene fusion with different deleted genes in strains having the *bgl* promoter and the *bglG* gene in front of the *lacZ* gene. Cells were grown in M63 medium with salicin as the carbon source. Error bars for the LacZ assays are shown with the standard deviation values for the different strains indicated by the vertical bars.

The data shown in Figure 4 suggests that both deletion of *hns* and the *hns*L30P mutation prevented Hns from oligomerizing to form the repression loop. In the presence of salicin, both strains had over 100x increased activity compared to the other strains. The wild-type and the other deletion mutants had low β-galactosidase activities, similar to those shown in Figure 3. Therefore, the increased activity of the Δ*hns* and *hnsL30P* mutants suggests that most of the growth advantage for the utilization of salicin is due to prevention of Hns-mediated DNA looping. It is interesting to note that with both Hns binding sites intact, the repressive effects of HnsL30P, both in the presence and absence of salicin, are not observed. Presumably the effect of the mutation on looping overwhelms the effect on promoter repression caused by direct binding in the promoter region.

High level expression of *bglG* is prevented by Hns looping

To determine the effects of BglG on this activity, Figure 5 shows the effects of two levels of *bglG* overexpression. A *lac-Iq* promoter was used to produce a moderate amount of BglG while a *ptet* promoter was used to produce a higher amount of BglG.

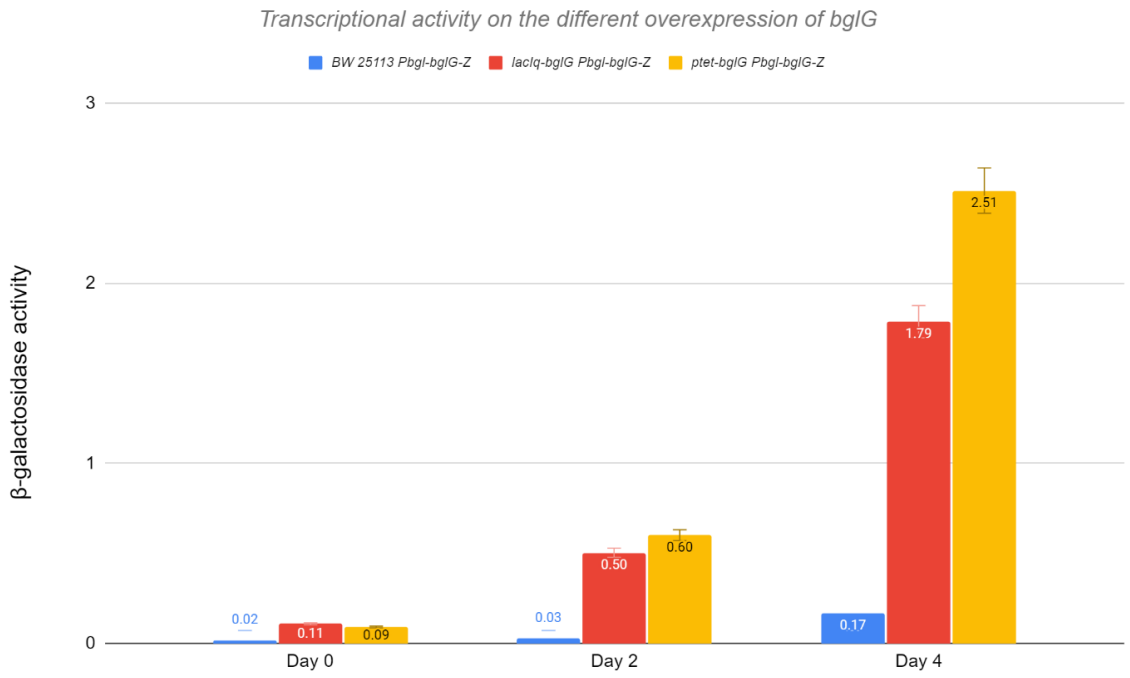

Figure 5. β-Galactosidase activities of the *bgl* operon (promoter + *bglG* in front of *lacZ*) with salicin present. *lacIq-bglG* produces more BglG protein than the wild-type strain while *ptet-bglG* produces still more. Error bars indicate the standard deviations of these samples. Strains were grown in M9 medium instead of M63 with 0.5% salicin, and cells were grown at 30°. Plates were then washed with 1.5 mL of 1x M9 salts on their respective day and collected. LacZ assays were then performed for each strain.

The data in Figure 5 show the differences between the wild-type, *lacIq* promoter fused to *bglG*, and the *ptet* promoter fused to *bglG*. Plates were grown in 30°C to allow proper incubation of each strain and were then washed with 1x M9 salt for LacZ assay measurement. With wild type Hns, very low β-galactosidase activity was observed, indicating that the *bgl* operon is largely silenced. Day 0 shows little activity for all three strains as expected as *lacZ* assays were

performed shortly after inoculation. On day 2 of incubation, the wild type had an activity of about 0.02 while use of the *lacIq* promoter and the *ptet* promoter to overexpress *bglG* had β-galactosidase activities of about 0.5 and 0.6 Miller units, respectively. However, on day 4m the *ptet* promoter had about 40% more activity than the *lacIq* promoter.

Elimination of the upstream Hns binding site by IS insertion or elimination of the site in *bglG* prevents Hns loop-dependent *bgl* operon silencing

As indicated above, for a wild type *E. coli* cell to utilize β-glucosides, a mutation (such as IS insertion) within the *bgl* operon promoter region is required. However, eliminating the downstream *hns* binding site in *bglG* also interrupts the ability of Hns to oligomerize and form its repression loop (Figure 6).

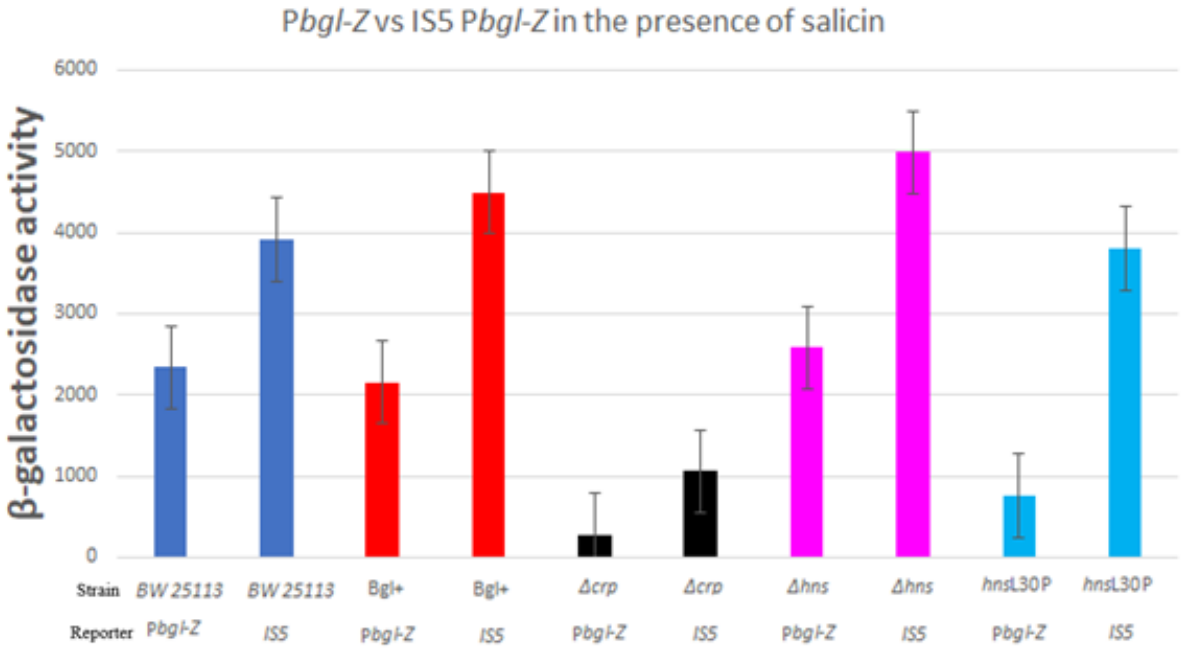

Figure 6. Promoter activities of strains lacking or having an insertional mutation (IS5 P*bgl-z*) or lacking the *hns* binding site in the *bglG* gene. This experiment was conducted in M63 media with glycerol and salicin as carbon sources. Error bars indicate standard deviations.

Using a *lacZ* reporter with an IS insertional mutation in the promoter region, the β-galactosidase activity increased about 2-3x compared to the strains without the insertion. The wild-type, BW25113, increased from 2000 to 4000 Miller units when the insertional mutation was present in the promoter region. The other strains examined similarly had about a 2-3x increase in LacZ activity due to the insertional mutation (IS5 P*bgl-Z*). Apparently, the insertional mutation not only disrupts the ability of Hns to oligomerize and form a loop but may also influence the action of other transcription factors.

As Figure 5 explored the effects of the insertional mutation on the promoter-reporter in the presence of salicin, Figure 6 explores the impact of the insertional mutation on the P*bgl-bglG-lacZ* fusion reporter in the presence of salicin.

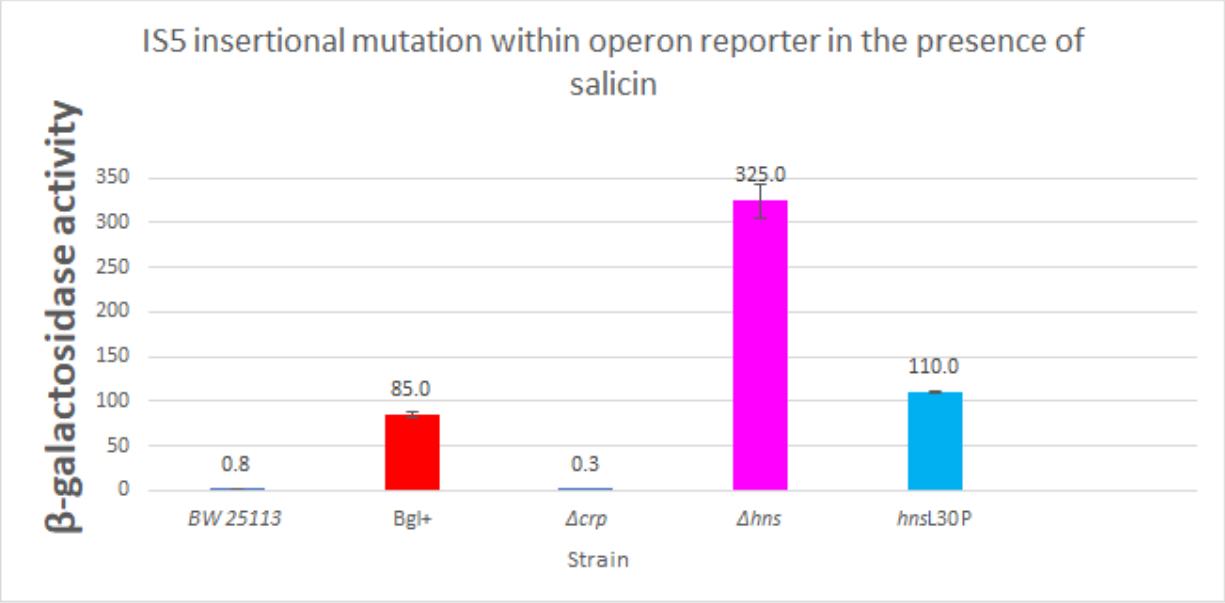

Figure 7. *bgl* operon reporter activity with the IS5 insertional mutation within the P*bgl* region of strains expressing the *lacZ* reporter gene (P*bgl-bglG-Z*). The experiment was carried out in M63 media with salicin as the carbon source. Error bars indicate the standard deviation values.

The wild-type, BW 25113, and the *crp* deletion strain showed β-galactosidase activities of about 0.3, a number much lower than observed for the other strains tested. The Bgl+ IS5 P*bgl-bglG-Z* strain had an activity of about 85 units, which indicates that the cells are Bgl-positive and can utilize salicin. Moreover, the HnsL30P-bearing strain had an activity of about 113 units. As this strain can still produce HnsL30P that can bind to its site in the *bglG* site but cannot oligomerize to form the repression loop because its binding site in the promoter region may have been destroyed by IS5 insertion, the β-galactosidase activity is seen to be slightly higher than the Bgl+ strain. In the presence of salicin, deletion of Hns gives rise to β-galactosidase activity of about 325 units. These results suggest that the absence of the repressive loop allows the cells to utilize salicin.

Seeing the impact of the IS insertion mutation on LacZ activity in the *bgl*-P*bglG-lacZ* reporter strain, we next examined the effect of the mutation that eliminated the *hns* binding site in the promoter region (Figure 8).

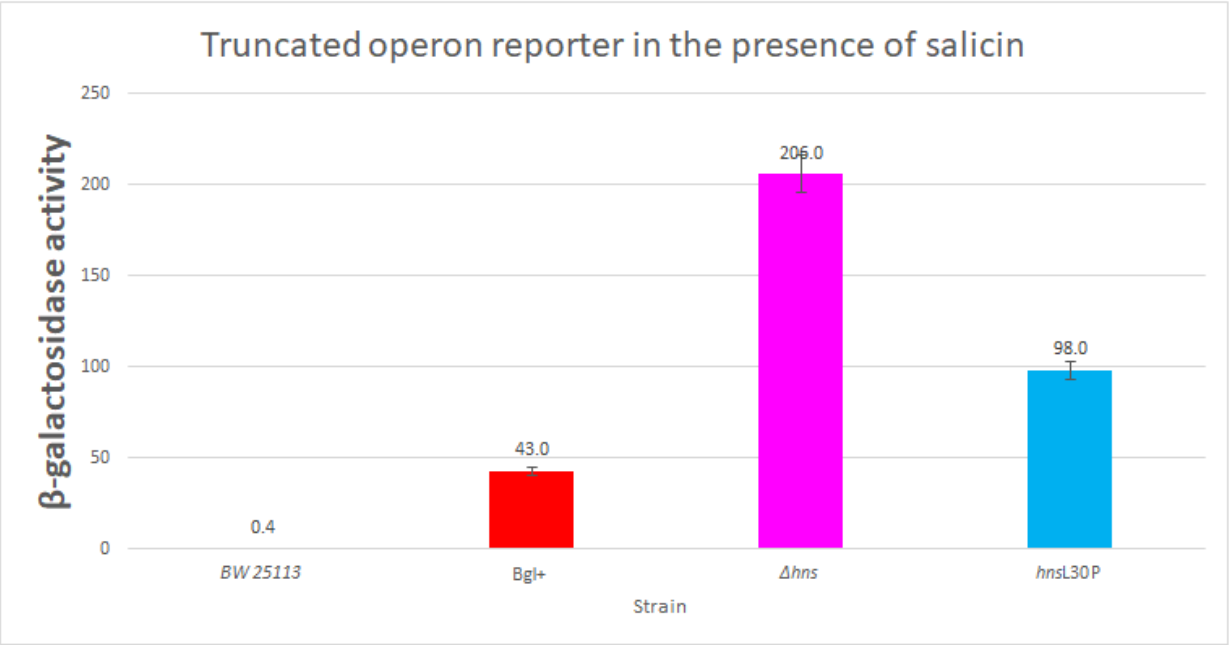

Figure 8. *bgl* operon reporter activities using a reporter where the upstream promoter region of the *crp* binding site is truncated. The experiment was conducted in M63 with salicin as the sole carbon source. Error bars represent standard deviations.

The wild-type strain (BW25113) serves as a control with very low β-galactosidase activity, indicating that the *bgl* operon is silenced. The IS5 insertion (Bgl+), deletion of *hns*, and the *hns*L30P mutation gave rise to strains that had a 43, 207, and 97 Miller units, large increases in β-galactosidase activity compared to the wild-type, respectively. These activities are similar to those seen in Figure 6, which demonstrates that the same pattern is observed when *hns* is either deleted or Hns cannot oligomerize to form the repressive loop. The inducer, salicin, increases the β-galactosidase activity expected. The results also suggest (1) that Hns still has a repressive effect on operon expression following IS5 insertion into its activating site, and (2) that the binding a HnsL30P still has a mild repressive effect possibly because it can bind in the *bglG* gene.

Hns regulates *bgl* operon expression in the stationary phase

Previous studies have examined the role of RpoS on *bgl* operon expression during the stationary phase of growth (Madan et al., 2008)). RpoS is a sigma factor that regulates the general stress response. The effects of an *rpoS* deletion mutation in comparison to an *hns* deletion under stressful conditions (stationary phase) is shown in Figure 9.

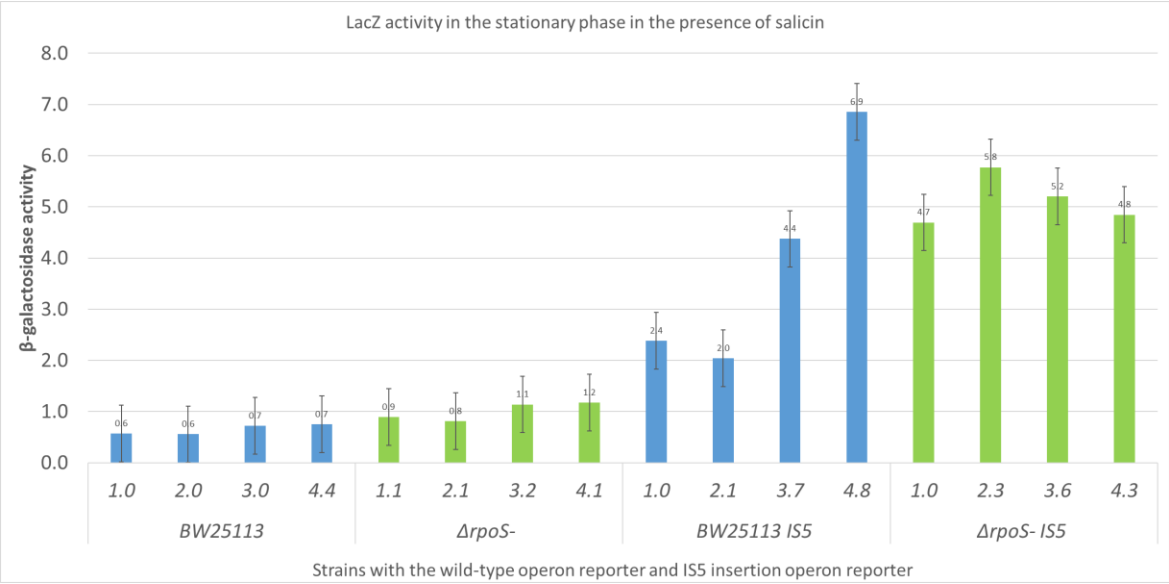

Figure 9. Induction of *bgl* operon (P*bgl-bglG-Z*) expression during entry into the stationary growth phase (OD units of about 1 represent log phase; OD units of about 2 represent entering stationary phase; OD of about 3 and 4 represent cells in the stationary phase. Cells were grown in M63 media with 0.5% glycerol and 0.5% salicin present as carbon sources. The X-axis indicates the strain and the optical density (OD, 600nm) at which each sample was collected. The Y-axis presents the β-galactosidase activity expressed in Miller units. The P*bgl-bglG-lacZ* operon reporter with or without the IS5 insertional mutation in the operon promoter region was used for all measurements.

Each strain was collected at four different optical densities first in the log phage and last in the stationary phase. Deletion of *rpoS* in the wild type genetic background with the IS insertional mutant in the *bgl* operon and the *lacZ* reporter gene showed low β-galactosidase activity, about 0.2 and 1 units, respectively. These results are consistent with the hypothesis that IS5 insertion exerts its effect on operon expression, at least in part, by destroying the Hns binding site in the promoter region, thereby preventing formation of the repression loop.

Discussion:

Figures 1 and 2 reveal the effects of various mutations in transcription factor-encoding genes on the promoter activities of the *bgl* operon without and with salicin, respectively, using a LacZ reporter to measure transcriptional activities. Except for the *crp* deletion strain, other mutants examined had similar β-galactosidase activities compared to the wild-type, *BW25113*. In this regard, it is important to note that the effects of most of these transcription factors were shown by others to exert their effects only when expressed at greater than wild type levels. As there was little increase in β-galactosidase activity when salicin was present, it appears that the promoter activity is not appreciably affected by this inducer. Deletion of genes other than *crp* had little impact on *bgl* promoter activity, but loss of Crp substantially lowered the β-galactosidase activity in comparison to the other strains including the wild-type *BW25113* strain with the p*bgl-lacZ* fusion promoter reporter. Thus, Crp is the primary activator of the *bgl* promoter. The *hns* deletion strain had only a 20% increase in β-galactosidase activity compared to the wild type, *BW 25113*, showing that Hns has little repressive effect on the promoter under these conditions. Also, in both figures, the HnsL30P protein was seen to have a significantly lower β-galactosidase activity (about 3x) compared to the wild-type strain. This supports the hypothesis that when there is an amino acid change of leucine to proline in the 30th codon position of the *hns* gene, there is an increase in its binding affinity for its binding site in the *bgl* operon promoter region. This results in lower promoter activity, confirming the fact that the L30P mutation does not prevent Hns from binding to its binding sites in the DNA, and may enhance its affinity for this binding site. This amino acid substitution does, however, abolish oligomerization as reported previously (Ref), and therefore the formation of the repressive loop

is prevented. All other regulators examined do not appreciably influence promoter activity when their encoding genes are deleted. It is evident that under normal conditions, the *bgl* operon promoter has high activity, even in the absence of salicin.

Figures 3 and 4 explore the same conditions as in Figures 1 and 2, except that the P*bgl-bglG-lacZ* fusion was used to measure the activity of the operon. Even under non-inducing conditions (no salicin present), the *hns* deletion and the *hns*L30P mutant strains displayed a 5x increase in β-galactosidase activities. Figure 4 demonstrates that using salicin as the inducer causes the *hns*L30P strains compared to the wild-type and other deletion strains to have a greater effect. The fact that cells expressing *hns*L30P have similar operon activity, but lower promoter activity compared to the *hns* deletion strain suggests that HnsL30P has binding affinities in the promoter region and possibly also in the *bglG* region, than the wild type protein. We suggest that HnsL30P binds more tightly to the promoter region of the *bgl* operon although it may bind more loosely to the *bglG* region.

The data reported here confirms that the *bgl* operon is silenced by Hns, but additionally suggests DNA looping is required for strong repression, and that the wild-type cells cannot utilize salicin as a carbon source because of this repression. HnsL30P has similar operon activity compared to the deletion of *hns*, but this is not true when only the promoter activity is measured.

When *hns* was deleted, or when its ability to oligomerize to form a loop between the two *bgl* operon binding sites was lost, β-galactosidase reporter activity significantly increased compared to the wild-type strain. This fact emphasizes the effect of the looping mechanism by

Hns and indicates why, when its gene is deleted, *bgl* operon activation results. This provides strong evidence that the binding of Hns to its two sites in the promoter and the *bglG* gene is not sufficient to silence the *bgl* operon. Instead, Figures 3 and 4 suggest that the ability of Hns to oligomerize and form a repression loop is of prime importance to the repression mechanism. The LacZ activity in the presence of the *hns*L30P mutant was significantly higher compared to the wild-type.

Figure 5 investigates the consequences of *bglG* overexpression and its effects on operon expression in the presence of salicin. The data show that overexpression of *bglG* increases the β-galactosidase activity compared to the wild-type reporter strain. More specifically, the higher increase in expression of *bglG* the higher increases of operon expression. However, Hns is still functional and can bind both sites and oligomerize to form its repression loop in all three strains. The effect of Hns can be seen in Figure 5 as all three strains in both types of media still have relatively low β-galactosidase activity compared to Figure 4 where the *hns* deletion and *hns*L30P strains both have activities of around 16 Miller units. This demonstrated that the *bgl* operon is still partially silenced, even with the overexpression of *bglG* in the presence of salicin. It further leads to the conclusion that the Hns repression loop is necessary for silencing the *bgl* operon as the overexpression of *bglG* is not sufficient to fully activate *bgl* operon expression.

In Figures 6 and 7, the effects of an IS5 insertional mutation, which prevents Hns from binding in the promoter region and therefore forming a repression loop, are shown. In Figure 6, the data reveal a 2 to 3x increase in β-galactosidase activity due to the IS5 insertional mutation, which is believed to destroy the upstream Hns binding site (Zhang and Saier, 2009). As a

consequence, the insertional mutation prevents formation of the Hns repression loop, and consequently, IS5 insertion eliminates Hns-mediated repression. *crp* mutants express the *bgl* operon promoter at very low levels (Figure 6), and the IS5 insertion causes a three-fold increase in promoter activity. This reveals the importance of Crp for controlling the *bgl* promoter (Figures 1 and 2) as when Crp is eliminated, the promoter and operon activities are low, despite having an IS5 insertion in the promoter region, driving *lacZ* gene expression. As a result, Crp is shown to have an important role in controlling the promoter region of the *bgl* operon.

To obtain high rates of *bgl* operon transcription, a mutation in the *bgl* operon of wild type *E. coli* must arise, and an inducer such as salicin must be present. Figure 7 explores the consequences of the IS5 insertional mutation on *bgl* operon transcription with an insertion mutation in the promoter region and *lacZ* fused to the end of *bglG* in the presence of salicin. The wild-type and *crp* deletion strains both have very low activity, less than 1 Miller unit, because Hns is still produced and can bind and oligomerize to form the repression loop. The Bgl+ strain is under optimal conditions when IS5 and the inducer, salicin, are present. It is evident that the Hns looping mechanism can repress *bgl* operon expression because the *hns*L30P strain had 30% increased activity compared to the IS5 Bgl+ strain, which is able to utilize salicin. Hns is apparently the dominant repressor for the operon, and IS5 insertion prevents Hns from binding to the upstream binding site, or at least inhibits binding, blocking its ability to oligomerize to form the repression loop. Although HnsL30P cannot oligomerize and form its repression loop, Hns is still being produced and can bind to both of its binding sites in the promoter and the *bglG* region of the *bgl* operon, which is why the *bgl* operon expression level is lower than when *hns* is

deleted. This conclusion was further confirmed by deleting the *hns* binding site in the promoter region.

Figure 8 is similar to Figure 7 as the same environmental conditions apply but the upstream promoter region of the Crp binding site is truncated instead of using the insertional mutation as for Figure 7, which is believed to remove the binding site of Hns in the promoter region. The β-galactosidase activity observed for the *hns* deletion strain showed a 2x increase compared to the HnsL30P mutant strain, similar to the result shown in Figure 7. The L30P mutation rendered the cells Bgl+ although its expression was lower than when *hns* was deleted. This presumably resulted because although the HnsL30P strain can't oligomerize to form its repression loop, it can still bind to both of its sites, in the promoter region and within the *bglG* gene. Losing the ability to form the Hns repression loop for any reason allows the transcriptional activity of the *bgl* operon to increase. Thus, Figure 8 shows that Hns binding is not sufficient to silence the expression of the *bgl* operon.

Figure 9 focuses on the effect that *rpoS* has on *bgl* operon expression as cells enter and are in the stationary growth phase. In this experiment, the *bglG-lacZ* fusion reporter was used with and without the IS5 insertion. The wild-type and *rpoS* deletion strains displayed low β-galactosidase activity (about 0.4 units) compared to the *hns* deletion strain. Deletion of *rpoS* showed a 2x increase in the log growth phase. However, in the stationary phase there is no observed difference between the wild-type and the *rpoS* deletion strain. Hns is still present and can oligomerize to form its repression loop in both the wild-type and *rpoS* mutant strains. Thus,

Hns remains the major repressor and *rpoS* plays essentially no role in regulating *bgl* operon expression in the stationary growth phase.

In this thesis, I have altered the Hns repression loop by using a strain where Hns can bind to both of its binding sites (in the promoter region and in *bglG)* but cannot oligomerize to form a repressive DNA loop. In the future, we shall examine the strength of the loop between the two Hns binding sites. This experiment should provide more insight into how the Hns repression loop fully operates, possibly revealing the minimal strength needed to keep the *bgl* operon silent. The precise Hns binding sites need to be defined, and manipulation of these binding sites could then be revealing.

In summary, I have explored the transcriptional activities of the promoter and *bgl* operon using mutated versions of *lacZ* fusion reporter strains with and without the inducer, salicin, and the *crp*, *rpoS* and *hns* genes, in the log phase of growth. The data demonstrates that Crp is the primary activator for the *bgl* promoter while Hns is the primary repressor. Moreover, the binding of Hns to the promoter and *bglG* regions is not sufficient to silence *bgl* operon expression. Possibly the IS5 insertion was able to eliminate the Hns binding site in the promoter region, and consequently the ability to loop the DNA. This looping mechanism appears to be largely responsible for *bgl* operon silencing.

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