THE PENNSYLVANIA STATE UNIVERSITY
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DEPARTMENT OF BIOCHEMISTRY AND MOLECULAR BIOLOGY

REGULATION OF *sacX* EXPRESSION BY SacY AND NusG in *Bacillus subtilis*

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A thesis
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of the requirements
for a baccalaureate degrees
in Biochemistry and Molecular Biology and Finance
with honors in Biochemistry & Molecular Biology

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ABSTRACT

Transcription in *Bacillus subtilis* is regulated at the levels of initiation, elongation, and termination. During elongation, the activity of RNA polymerase is modified by pausing events stimulated by signals in the template or nascent transcript, protein factors such as NusG, or a combination of both. In antitermination regulatory mechanisms, formation of secondary structures in the 5' untranslated region of the nascent RNA transcript promotes continued transcription elongation and readthrough; these structures are often stabilized by factors that promote gene expression.

The *sacX* gene is involved in sucrose metabolism. The 5' untranslated region of *sacX* contains a ribonucleic antiterminator (RAT) that is stabilized by SacY or SacT in the presence of sucrose growth conditions. In the absence of sucrose, SacY or SacT do not bind to the RNA, the RAT is not stabilized, and an intrinsic termination hairpin forms such that transcription terminates in the 5' untranslated region.

The *sacX* leader also contains a TTNTTT motif characteristic of NusG-mediated pausing. In sucrose growth conditions, pausing is thought to allow more time for binding of SacY and SacT that promote transcript readthrough and gene expression. Without NusG or the sequence elements needed to promote pausing, there is insufficient time for regulatory factor binding even in sucrose growth conditions, preventing transcript readthrough and gene expression. This expression pattern was confirmed *in vivo* using *sacX-lacZ* transcriptional fusions. Additionally, *in vitro* transcription assays confirmed the presence of a strong NusG-dependent pause site that depends on the TTNTTT sequence motif in the *sacX* leader.

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Chapter 1

Introduction

The ubiquity of bacteria is contingent on their ability to survive and adapt in a variety of environmental conditions. A key component of environmental adaptation in bacteria is the ability to import and process a variety of external nutrients into useful compounds for growth, necessitating a complex set of metabolic pathways and corresponding enzymes. A further layer of complexity is added when considering the expression of genes involved in metabolism. A myriad of regulatory networks coordinates and optimizes metabolic gene expression to promote bacterial survival and efficiency in processing nutrients. In *Bacillus subtilis,* glucose is the preferred carbon source; however, other sugars, such as sucrose, can be used when glucose is not present. Through differential gene expression, bacteria can promote the uptake and metabolism of sucrose or other alternative sugars in the absence of glucose.[1]

Bacterial transcription is carried out by RNA Polymerase (RNAP), which uses a DNA template to produce mRNA, which is then translated into protein. The conversion of DNA into mRNA through transcription is a key level of regulating gene expression and can occur at the levels of initiation, elongation, and termination. Processivity of RNAP is defined by continuous movement along the DNA template and production of mRNA during transcript elongation; however, this processivity is marked by pausing events which allow time for the nascent transcript to form secondary structures or interact with regulatory factors.[2] These factors or structures may interact with RNAP, altering its activity. In antitermination regulatory systems, secondary structures formed by the nascent mRNA transcript, which can be stabilized by external factors,

promote continued elongation or termination of transcription, increasing or decreasing gene expression, respectively. Pausing of RNAP is often critical for these regulatory events to occur, such as in the *trp* operon of *B. subtilis and E. coli*.[3]

RNAP pausing is often signalled by sequence motifs present in the template DNA or nascent transcript,[4] and is further modulated by the activity of NusG. Conserved in all domains of life, NusG is a general transcription elongation factor that exerts different effects on pausing in various organisms. In the *B. subtilis trp* operon, NusG-dependent pausing allows for binding of protein factor TRAP and secondary structure formation in the nascent transcript such that expression of the operon is reduced in the presence of tryptophan. Additionally, in the *ribD* riboswitch, binding of FMN to the mRNA aptamer is aided by NusG-dependent pausing.[3,5] In contrast, NusG functions as an anti-pausing factor in *E. coli.*[10]

Analysis of NusG-dependent pausing in other gene regulatory systems has revealed two factors necessary for pausing, namely: a conserved TTNTTT sequence in the nontemplate DNA strand within the paused transcription bubble, as well as a pause hairpin 11 to 13 nucleotides from the 3' end of the nascent RNA transcript. Transcriptome-wide studies of RNAP pausing in *B. subtilis* using an RNET-seq technique revealed NusG-mediated pausing at 1,600 sites containing the consensus TTNTTT motif, as well as pause hairpins in a majority of the strongest pausing sites.[6] One pause site was identified in the leader region of the *sacX* gene, which is controlled by an unusual mechanism utilizing a set of regulatory mRNA hairpins not commonly found in canonical antitermination mechanisms found in other organisms and elsewhere in the *B. subtilis* genome.

The Bgl/Sac family of genes in *E. coli* and *B. subtilis* encode for various proteins involved in the metabolism of β-glucosides and includes *bglG* in *E. coli* as well as *sacX* in *B. subtilis*. In the

canonical *bglG* model of anitermination, phosphorylation of BglG by the phosphotransferase system leads to the formation of an active BglG RNA-binding homodimer which stabilizes a ribonucleic antiterminator (RAT) located in the 5' untranslated region (5' UTR) of target genes, promoting transcription readthrough. A similar system exists in the 5' UTR of *sacX*, with binding of SacY or SacT stabilizing a RAT formed in the nascent transcript and promoting transcription readthrough. In the absence of active SacY or SacT an intrinsic terminator hairpin forms, aborting transcription in the 5' UTR and limiting *sacX* expression.

The structure and RNA-binding activities of SacY and SacT have been well characterized. Like BglG, both proteins contain a regulatory domain and RNA-binding domain. In the presence of sucrose, phosphorylation of residues at the regulatory domains of SacY or SacT drive activation, dimerization, and binding to RNA. Critically, in the absence of the regulatory domain altogether, SacY and SacT are artificially locked into the active RNA-binding state.[7] Studies of the interaction between SacY and the RAT hairpin show that the RNA-binding domain of SacY is 55 amino acids in length; NMR footprint analyses have found that key binding residues include Asn8, His9, Asn10, Gly25, Gly27, and Phe30. All residues protrude in lobes from the RNA interaction surface of the SacY dimer and many are conserved in homologous proteins, including SacT, LicT, and BglG. Notably, the aromatic residue Phe30 within each monomer is crucial to stacking interaction with the minor groove of the RNA hairpin and has the greatest contribution to stabilization of the hairpin. Additionally, the aromatic residue Phe47 is buried within the dimer interface and contributes significantly to both monomer folding and dimer integrity.[8]

Despite numerous data characterizing the interaction between the RAT of *sacX* and regulatory proteins SacY and SacT, as well as data indicating similarity between other regulatory mechanisms such as in the *bgl* operon of *E. coli,* no cohesive model exists for explaining

antitermination of *sacX.* The model proposed herein asserts that, unlike other systems, the RAT hairpin is extruded from an anti-antitermination hairpin. Upon stabilization of the RAT by SacY or SacT, a separate antitermination hairpin prevents formation of the terminator. The key difference is that for *sacX* the antiterminator and RAT are distinct entities. This model includes a pause hairpin, consistent with previous data indicating a strong NusG-dependent pause site and TTNTTT motif in the *sacX* leader. Pausing of transcription just downstream of the RAT allows time for regulatory factor binding and stabilizing the RAT before further elongation (Fig. 1).

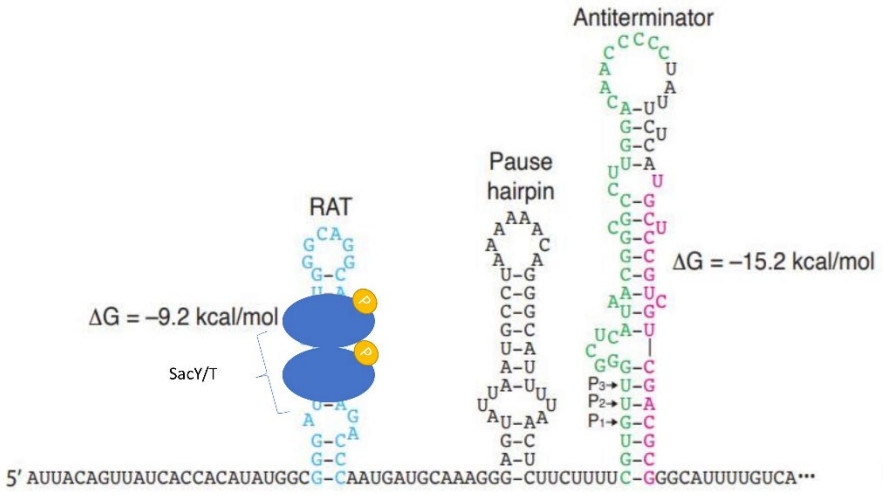

Figure 1. Antitermination complex. Pausing provides more time for SacY or SacT binding to the RAT, stabilizing it and allowing formation of the antitermination hairpin. The antitermination hairpin sequesters the magenta sequence of the termination hairpin, promoting readthrough.

In the absence of SacY or SacT binding, the RAT and pause hairpins still form; however, as transcription continues following pausing both structures form a much larger and more stable anti-antiterminator hairpin. The first hairpin of the termination complex sequesters the entire RAT sequence, contains the pause hairpin at its apex, and sequesters part of the antitermination hairpin. Downstream of this hairpin the terminator hairpin itself forms; this sequence is sequestered in the antitermination hairpin in the presence of bound SacY or SacT. Transcription is then attenuated by the established mechanism of intrinsic termination through shearing of RNA polymerase from the transcription bubble in the U-rich tract downstream of the terminator hairpin (Fig. 2).[9]

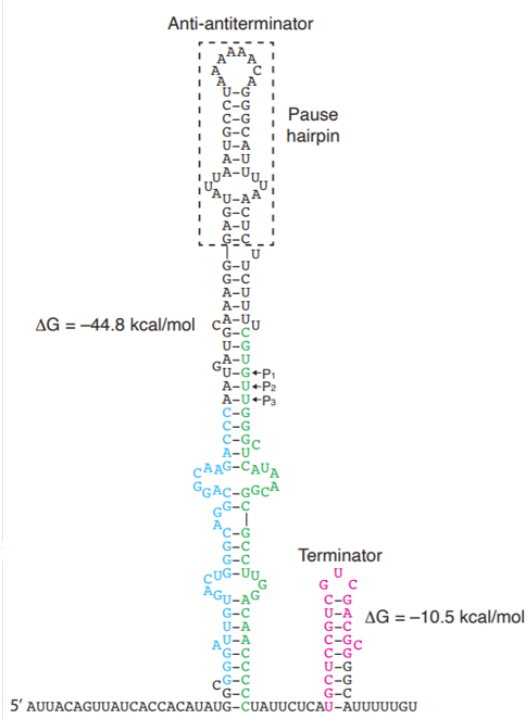

Figure 2. Termination complex formed in the absence of RAT stabilization. The RAT sequence and a portion of the antiterminator are sequestered by the anti-antiterminator, which contains the pause hairpin at its apex. Downstream, the terminator hairpin forms and aborts transcription.

Chapter 2

Results

Initial investigation of the effect of sucrose on *sacX* expression began with the construction of transcriptional and translational *sacX-lacZ* fusions integrated into the *B. subtilis* chromosome. Expression assays were conducted with cells grown in minimal media containing either 0.5% glucose, 0.5% sucrose, 1% glucose, or 1% sucrose. Under 0.5% growth conditions, expression in sucrose media increased twofold compared to glucose for the translational fusion strain; this figure increased to threefold for the transcriptional fusion strain. Furthermore, at a 1% concentration of sugar, a greater increase in expression was observed when comparing sucrose to glucose. As a result of these preliminary studies, all further assays were carried out using the transcriptional fusion and in 1% glucose or sucrose growth media.

To examine the effects of NusG-mediated pausing, the *sacX-lacZ* transcriptional fusion was chromosomally integrated into WT and Δ*nusG* strains. Furthermore, a *sacX-lacZ* transcriptional fusion was created with a TTNTTT pause motif mutated to TTNAAA and integrated into the WT and Δ*nusG* strains (Fig. 3). In the WT strain, sucrose produced a threefold increase in expression compared to glucose. In strains with altered pausing capability, this increase was reduced to less than twofold (Fig. 4). These data indicate that NusG-mediated pausing is critical for normal expression of *sacX* in response to extracellular sucrose *in vivo.* The global lack of NusG, in addition to targeted elimination of the critical pausing motif, reduced expression of *sacX* to the same level as the single Δ*nusG* mutant strain as expected.

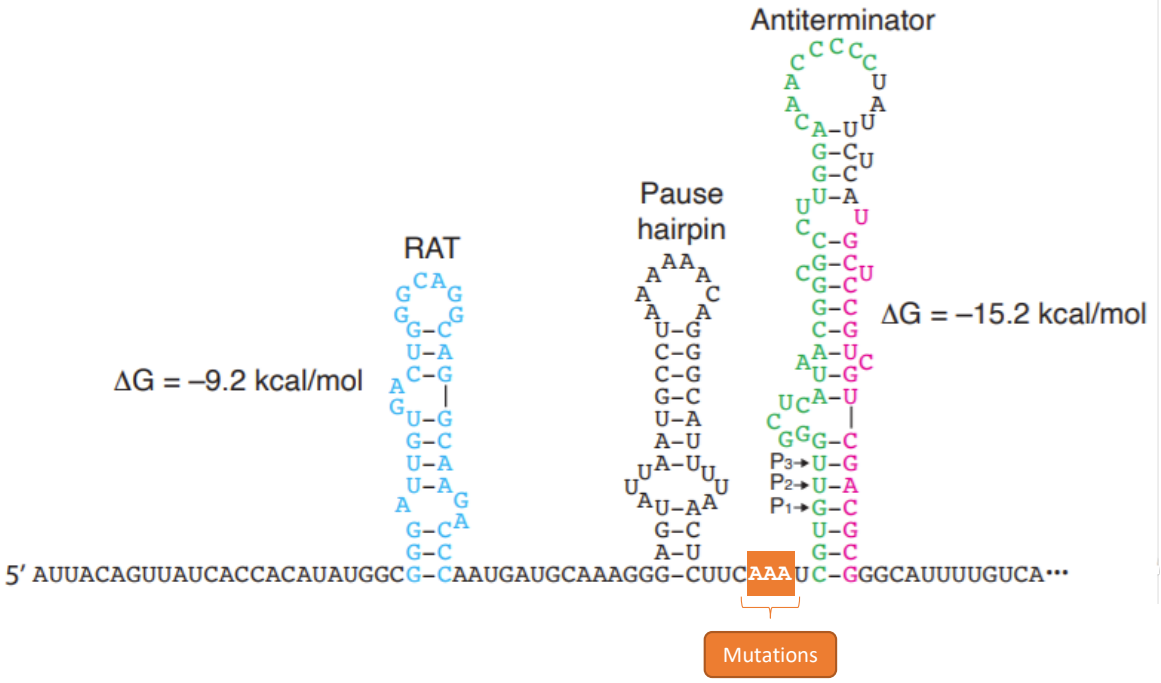

Figure 3. Mutant pause motif. TTNTTT motif is mutated to TTNAAA, reducing NusG-mediated pausing activity at the specific pause site in the *sacX* leader region.

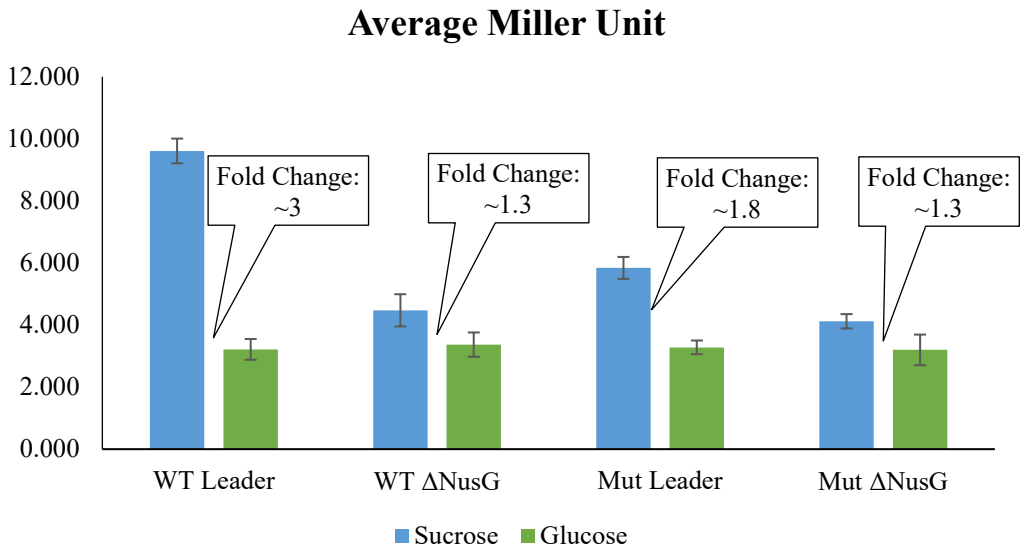

Figure 4. Expression of *sacX-lacZ* transcriptional fusions as measured by β-galactosidase activity. Increased expression of *sacX* was observed in sucrose media for the WT strain, but this effect was reduced when NusG-mediated pausing was eliminated by deleting *nusG,* mutating the pause motif, or both, thereby demonstrating the role of pausing in regulating *sacX* expression.

To elucidate the exact roles of SacY and NusG in *sacX* transcription, a series of *in vitro* transcriptional studies were performed using a set of four templates: wild-type (WT), pause-deficient (P), RAT deletion (R), and a combination of pause-deficient and RAT deletion (PR). The first of a series of pausing assays showed a distinct pause site *in vitro* that depended on both the presence of NusG and the TTNTTT motif. Strong pausing activity was only observed in the WT and R templates, which contained an active pausing motif, and only with added NusG.

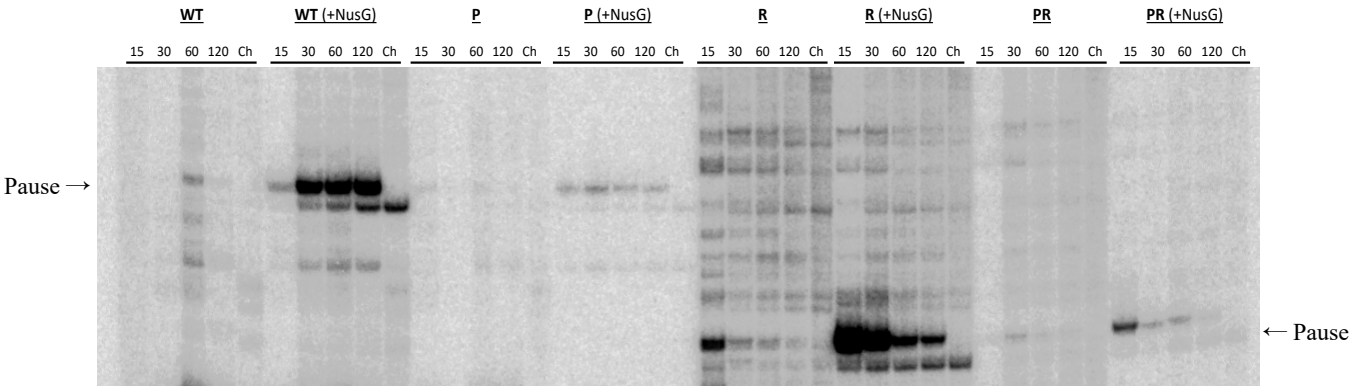

Figure 5. *In vitro* transcriptional pausing visualised in a polyacrylamide gel. Pause bands indicating pause sites were observed in the wildtype (WT) and RAT-deletion (R) templates in the presence of NusG. No strong pauses were observed in the pause-deficient (P) and RAT-deletion/pause-deficient (PR) templates containing a mutated TTNAAA motif, regardless of the presence of NusG. Pausing is dependent on both protein factors and sequence elements.

Chapter 3

Discussion

NusG-mediated pausing is a well-studied event in the *B. subtilis* transcription cycle and occurs throughout the transcriptome for a variety of purposes. In the *sacX* leader, pausing stimulated by NusG provides time for regulatory factor binding. Binding of regulatory factors SacY and SacT stabilizes the RAT sequence, promoting transcript readthrough and expression of *sacX.* Because regulatory factors are known to activate expression of sucrose metabolism genes, *B. subtilis* grown in media containing sucrose as the primary carbon source express the *sacX* leader fused to *lacZ* at a higher level than those grown in media containing glucose as the primary carbon source.

The critical NusG-dependent pausing event for this process was demonstrated by the reduced expression of *sacX* when NusG-dependent pausing was eliminated. While growth in sucrose media increased expression of the *sacX* leader approximately 3-fold compared to expression in glucose media, this increase was reduced significantly in the pause-defective strains. Pausing was reduced specifically during transcription of *sacX* by mutating the consensus TTNTTT motif located in the *sacX* leader to TTNAAA, decreased the increase in expression from 3-fold to 1.8-fold. Integration of the *sacX-lacZ* fusion into a strain lacking NusG reduced expression even further to just 1.3-fold above the glucose-grown control. When integrating the mutant leader into the Δ*nusG* strain, expression was comparable to the Δ*nusG* strain. These data suggest that the complete absence of NusG has modest indirect effects on *sacX* expression.

The *in vitro* data confirms the presence of a strong NusG-dependent pause site in the *sacX* leader. The presence of the pause band indicates paused RNA polymerase at the 15, 30, 60, and 120 sec timepoints; this band was only observed in templates containing the TTNTTT motif and only with NusG present. These data confirm the presence of a strong NusG-dependent pause site in the *sacX* leader, originally identified in sequencing data through the presence of the TTNTTT sequence motif, and provide continued evidence for the canonical NusG-dependent pausing model. Furthermore, the combination of *in vivo* and pausing assay data demonstrate a clear role of NusG-mediated pausing in antitermination systems by providing time for regulatory factor interaction with the nascent transcript. Investigation of pause sites found in other 5' UTR regions of the *B. subtilis* genome may further characterize the role of NusG in antitermination systems as a critical factor for proper functioning and gene expression. Finally, investigation of other pause sites outside the 5' UTR may provide a more complete picture of the capacity of NusG-mediated pausing to exert changes in expression throughout the *B. subtilis* transcriptome.

Despite the relative ease of pause site identification *in vitro*, it was more difficult to identify the corresponding patterns of termination predicted based on *in vivo* expression data. All four templates prepared for the *in vitro* transcription studies were assayed for termination in the presence of purified SacY and SacT in concentrations ranging from 1 μM – 3 μM; however, bands indicating transcription termination did not appear as expected considering the proposed model of *sacX* antitermination. Further investigation will aim to control for confounding factors and determine the necessary conditions for *sacX* antitermination *in vitro.*

Chapter 4

Materials and Methods

Strain and Plasmid Construction

Bacterial strains used in this study are included in Table X. The sacX leader region (nt –169 to +387) was amplified from the *B. subtilis* chromosome and, using pTZ19R as a vector, cloned into E. coli strain NEB5α; generating strain PLB2023 containing plasmid pTW1. The transcriptional fusion was generated by subcloning the *sacX* fragment from pTW1 and into pDH32 to generate pTW3, while the translational fusion was cloned into ptrpBGI-PLK to generate pTW2. The transcriptional and translational fusions generated PLBS999 and PLBS990, respectively, following integration into the genome of PLBS338. After initial β-galactosidase assay experiments, pTW3 was cloned into ΔnusG strain PLBS538 to generate PLBS990(2). Strain PLBS998 was generated by mutating the *sacX* leader pause site using the QwikChange protocol from Agilent, then integrating the new *sacX-lacZ* fusion into the genome of PLBS338.

β-galactosidase Assay

Transcriptional and translational fusions to *lacZ* were grown overnight at 37°C in minimal media supplemented with acid casein hydrolysate (ACH) and experimental sugar source. After inoculation into fresh minimal media, cells were grown at 37°C and collected during mid-exponential growth. Pelleted cells were resuspended with Tris-HCl (pH 7.5) to wash cells, then re-pelleted and resuspended in 1 mL Z-buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 50 mM β-mercaptoethanol), then diluted to 4 mL with Z-buffer. Cell density was determined by absorbance at 600 nm, then 0.1 mL was removed and diluted to 1 mL with more Z-buffer for assaying. Cells were incubated with 10 µL of lysozyme (10 mg/mL) at 37°C for 5 min, then with 10 µL Triton X-100 (10%) at 28°C for 5 min. Reactions were started by adding 0.2 mL ortho-nitrophenyl-β-galactoside (ONPG) (4 mg/mL) in 0.1 M phosphate buffer (pH 7.0) and incubation was continued at 28°C, then stopped with 0.5 mL of 1 M

Na2CO3. Absorbance was measured at 420 nm and 550 nm, and readings were used to calculate Miller units of β-galactosidase activity.

In Vitro Transcription

The *sacX* leader region was fused with a strong promoter followed by a C-less cassette and tested for pausing by single-round in vitro transcription. Four templates were used with modifications to the *sacX* leader: WT, deletion of the RAT sequence, mutation of the TTNTTT motif to TTNAAA, and a template containing both the RAT deletion and mutant pause motif. Templates were purchased as gBlocks gene fragments from Integrated DNA Technologies and amplified by PCR.

In vitro transcriptions were started with the formation of a halted elongation complex for 5 min at 37 °C in a 20-µL reaction containing 100 nM DNA template, ATP and GTP (40 µM each), 1 µM UTP, 50 µg/mL acetylated bovine serum albumin (BSA), 75 µg/mL (0.19 µM) *B. subtilis* RNAP holoenzyme, 0.38 µM housekeeping sigma factor SigA, and 1 µCi of [α-^{32}P]UTP at 37 °C with no CTP. Halted complexes were diluted with 1x transcription buffer, 100 µg/mL acetylated BSA, and KCl to a final KCl concentration of 17 mM. Elongation at 25 °C was resumed by addition of all four NTPs in addition to 100 µg/mL heparin ± 1 µM NusG. Final concentrations of NTP and KCl were 150 µM and 10 mM, respectively. Aliquots of the elongation mixture were removed at 15, 30, 60, and 120 seconds to determine pausing; the last aliquot was continued for 20 min at 25 °C.

Table 1: Strains Used in this Study

Strain	Description	Source
NEB5α	*fhuA2 Δ(argF-lacZ)U169 phoA glnV44 Φ80 Δ(lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17*	New England BioLabs
PLBS338	Prototroph	11
PLBS538	PLBS338/*nusG::kan* Kan^r	12
PLB2023	NEB5α/pTW1 Ap^r	This Study
PLBS990	PLBS338/*amyE::P$_{sacX}$-sacX-lacZ* TLN fusion (-169 to +387) Cm^r	This Study
PLBS990(2)	PLBS338/*amyE::P$_{sacX}$-sacX(mutant leader)-lacZ* TXN fusion (-169 to +387) Cm^r	This Study
PLBS998	PLBS538/*amyE::P$_{sacX}$-sacX-lacZ* TXN fusion (-169 to +272) Kan^r	This Study
PLBS999	PLBS338/*amyE::P$_{sacX}$-sacX-lacZ* TXN fusion (-169 to +272) Cm^r	This Study

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EDUCATION

The Pennsylvania State University | Schreyer Honors College — **University Park, PA**
Smeal College of Business | Bachelor of Science in Finance — *Class of 2022*
Eberly College of Science | Bachelor of Science in Biochemistry

RELEVANT EXPERIENCE

Sumitomo Mitsui Banking Corporation — **New York, NY**
Summer Analyst | Strategic Credit — *Jun 2021 – Aug 2021*

- Supported the Leveraged Finance team on private equity sponsored LBOs, dividend recapitalizations, and acquisitions
- Participated in all aspects of the investment and due diligence process, including credit analysis, industry research, and financial modeling; culminating in presentations on prospective investments to credit committee members
- Conducted industry research on managed care companies, specialty practice groups, and medical supply companies to provide more detailed insight and analysis on current portfolio companies and support pitches on future transactions
- Attended meetings with company executives to monitor investments within the $2.00 bn Large Corporate Group and develop financial projections and credit metrics; summarized findings into credit memos for senior management
- Completed a 10-week internship program among various working groups within the Strategic Credit Division, concluding with a capstone M&A project involving capital markets activity, LBO valuation, and qualitative credit analysis

Nittany Lion Fund, LLC — **University Park, PA**
Lead Associate | Healthcare Sector — *May 2019 – Present*

- Collaborated with fellow sector analysts to oversee a portfolio of stocks within the biopharma, medtech, and managed care industries as a manager of the Nittany Lion Fund, a student-run equity hedge fund with over $14.00 million in AUM
- Maintained knowledge of current issues affecting the healthcare sector and individual subsectors, including FDA regulatory activity, political rhetoric surrounding healthcare reform, and industry competiton within therapeutic areas
- Analyzed market news and research reports to develop comprehensive sector, subsector, and company theses; implemented portfolio decisions by presenting investment ideas to other managers for critique and voting before each transaction
- Outperformed the S&P 500 Healthcare benchmark by ~2.00% in FY2020 due to carefully selected investments

CFA Institute Research Challenge — **Pittsburgh, PA**
Americas Regional Finalist — *Jan 2020 – Apr 2020*

- Created a thirty-page written equity research report and corresponding slide presentation simulating an initiation of coverage on Evoqua Water Technologies, a global supplier of water treatment products located in Pittsburgh, PA
- Formulated a comprehensive and forward-looking investment thesis among team members and built a valuation based on comparables and a DCF reflecting key qualitative and quantitative drivers; presented findings to a panel of judges
- Won local challenge in Pittsburgh, PA, advancing the team to the Americas Regional Competition in New York, NY

Babitzke Laboratory — **University Park, PA**
Thesis Research — *Jan 2019 – Present*

- Developed and conducted experiments to collect meaningful data within the research field of RNA transcriptomics
- Utilized classical and next-generation microbiology techniques including restriction digest analysis, PCR amplification, Sanger sequencing, termination-sequencing, and *in vitro* transcription to produce reliable and high-quality data for analysis
- Presented research findings at weekly meetings and poster symposia in order to educate fellow scientists regarding new discoveries and to discuss potential connections between the research work of laboratory and department members
- Validated the existence of a new form of RNA antitermination mechanism related to RNA polymerase pausing in *Bacillus subtilis;* synthesized research data into a comprehensive written articles to be published in various journals for peer review

WORK EXPERIENCE

LionTutors — **State College, PA**
Statistics and Chemistry Tutor — *Jan 2019 – Present*

- Increased student understanding of classroom material through both one-on-one and group tutoring formats
- Studied written and video training materials and practiced teaching techniques with more experienced tutors
- Conducted live reviews in a classroom setting ahead of major class exams; demonstrated effective problem-solving techniques and provided detailed examples with the goal of increasing students' test scores and overall class grade

SKILLS AND INTERESTS

Language: German (Conversational Level)

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