Exploring the Regulation of a *Bacillus pumilus* Bioplastic Degrading Protein

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DEDICATION

I dedicate my work to all of the microbes that have helped me along the way. Sorry for the bleach.

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Thank you to Dr. Reynolds and the Reynolds lab for teaching me about the breadth of microbiology. Without the lab, I wouldn't know how to make my data any CRISPR and I wouldn't have gotten to work on the cutting edge of science.

I am very grateful to the many members of the microbiology department that have helped me along the way and made the department a fun place to work. I would especially like to thank the labs on the 6th floor (Fozo, Burcham and Goodrich-Blair) for letting us borrow supplies and bounce ideas. I would like to thank my dissertation and preliminary exam committee for helping me identify gaps in my knowledge and improve my thinking as a scientist. Thank you to Dr. Fozo and Sarah Kauffman for being additional science mentors, especially for benchwork.

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ABSTRACT

Biodegradable plastics are being adopted to reduce the environmental impacts associated with petroleum-based plastics, including long environmental residence times, and reliance on fossil fuels as a feedstock. Microbial degradation of bioplastics is still poorly understood, which leads to inconsistent degradation outcomes and challenges for implementation. *Bacillus pumilus* B12 degrades the biodegradable bioplastic polylactic acid (PLA) via the protease AprE, but the regulatory mechanisms controlling *aprE* expression in *B. pumilus* B12 are largely unknown. To explore this question, we needed to establish protocols to introduce DNA into this organism, which had no devoted genetic tools. To bridge this gap, we developed a conjugation- based method to introduce DNA in diverse *Bacillus* species that is applicable to environmental and industrially relevant species.

We are leveraging this conjugation technique to begin exploring *aprE* regulation in *B. pumilus* B12. Despite belonging to the same genus there is a considerable difference in the *aprE* upstream regulatory region sequence of *B. pumilus* B12 and the extensively studied *B. subtilis*. Thus, we wanted to begin defining subregions of the upstream regulatory region that influence expression, especially transcription factor binding sites. To do this, we employed the technique known as 'promoter bashing' and generated a series of transcriptional reporters to GFP with varying lengths of the upstream regulatory region. These reporters provide indications of repressor and activator binding sites, including for Spo0A. Additionally, we used site directed mutagenesis (SDM) to mutate the bases corresponding to conserved DNA binding motifs for putative transcription factors (ScoC, SinR, CodY). We observe that SinR acts as a partial repressor of AprE in our minimal media conditions but ScoC has no measured impact

In this dissertation we show that expression of *aprE* is repressed by the addition of inosine, adenine and adenosine, which supports previous work from our lab which showed that adenine repressed PLA degradation. In other *Bacillus* species, AprE is a highly regulated secreted protein expressed during stationary phase, but the mechanism for this nucleotide- mediated repression remains to be elucidated, as no one transcription factor abrogated the repressive effect of inosine addition. We hypothesize that purine addition disrupts the activity of a pleiotropic regulator, which is reflected in the repression of additional genes. Overall, we show that *aprE* expression is controlled over 500 bases upstream of the predicted open reading frame and that there may be more regulatory elements than described in *B. subtilis.* Further, we describe *aprE* repression by inosine for the first time.

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CHAPTER 1 THE ROLE AND REGULATION OF SUBTILISINS IN BIOPLASTIC DEGRADATION

Portions of this chapter are intended for publication.

The document was written by Elise Phillips. Amanda Swets helped find and enter sources for Table One. Dr. Reynolds helped edit the document and refine its scope.

Abstract

The biobased biodegradable plastic polylactic acid (PLA) provides some advantages over many petroleum- based plastics, including improved biodegradation and reduced global warming potential. However, it is poorly degraded in mesophilic environmental conditions, thus it is essential to expand our knowledge of how mesophilic PLA degraders control PLA degrading enzymes. Through building an understanding of regulatory mechanisms controlling PLA degrading enzymes we can more adequately generate hypotheses to improve degradation efficiency. The genus *Bacillus* is a promising group of species on which to perform these kinds of studies because of their ubiquity, potential for genetic modification and known enzymatic activity against biodegradable plastics. The primary enzyme that PLA degrading *Bacilli* use is subtilisin (*aprE*). In this review we describe the role subtilisins and other serine proteases play in bioplastic degradation and how the regulation of subtilisin may impact degradation outcomes.

Introduction

Plastics are highly versatile materials because they can be manufactured to have practically any physical property and shape. This versatility, in addition to low cost, has led plastics to become a nearly ubiquitous material, with uses in medical, consumer and agricultural industries (1–4). However, most plastics are derived from crude oil and natural gas, which means they are nonrenewable and reintroduce stored carbon to the

environment. Additionally, their production generates greenhouse gases(5). These materials will be referred to as petroplastics throughout this dissertation. Many commonly used petroplastics are considered non-biodegradable because they have long residence times in the environment ranging from a couple of years to centuries(6), so they accumulate in and pollute the environment. A substantial, yet preliminary mechanism for degradation of non-biodegradable petroplastics is through abiotic oxidation and fracturing, termed 'weathering', that occurs when plastics are exposed to UV radiation and contact with wind, waves and rough surfaces (7–10). However, this mostly leads to generation of micro- and nanoplastics which have additional problems associated with them(11–16). Biomolecule- derived biodegradable plastics (bioplastics) are a promising alternative because they are renewable, use actively cycling carbon, are typically less greenhouse gas intensive, are more susceptible to enzymatic degradation, and thus have significantly shorter lifespans in the environment, typically under a few years (6, 17–19).

Biodegradable Plastics Offer Some Solutions to Petroplastics

The chemical structure of biodegradable plastics makes them more amenable to degradation by enzymes. Compared to nondegradable plastics, the chemical structure of biodegradable plastics tends to be more functionalized with ester bonds compared to the hydrocarbon polymers (typically, alkanes) that constitute nondegradable plastics (Figure 1A)(20–22). For example, polycaprolactone is more readily degraded than polyethylene (PE) and polyethylene terephthalate (PET) is more susceptible to degradation than polystyrene (PS). These structures are represented for comparison in Figure 1A.

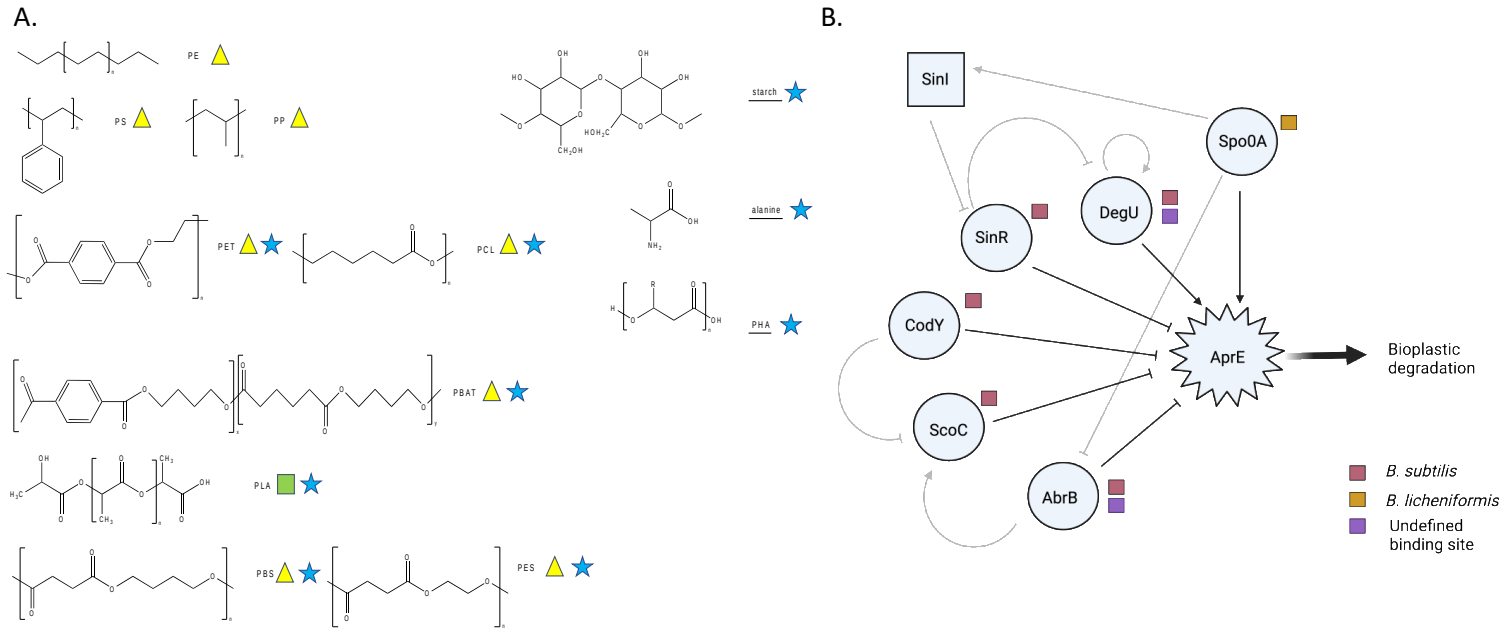

Figure 1-1: Regulation of enzyme genes that can degrade specific biopolymers. A. Chemical structures of plastics and biomacromolecules. Plastic monomers shown include polyethylene (PE), polystyrene (PS), polypropylene (PP), polyethylene terephthalate (PET), polycaprolactone (PCL), Polybutylene adipate terephthalate (PBAT), polylactic acid (PLA), polybutylene succinate (PBS), polyethylene succinate (PES), polyhydroxyalkanoate (PHA). Petroleum based polymers are marked with a yellow triangle. Biobased polymers are marked with a green square. Biomacromolecules are underlined. Biodegradable polymers, regardless of source, are marked with a blue star. B. Regulation of AprE. Transcription factors are shown in circles. Direct regulation of aprE is shown with black arrows, while regulation of transcription factors is shown in grey. Small squares indicate the organism(s) in which the transcription factor has been described. Figure 1B was generated in BioRender.

Compared to esters, alkanes are very stable. Ester bonds make polymers more likely to be sensitive to enzymatic degradation because they can be hydrolyzed. Further, alkanes require structural changes such as oxidation (enzymatic or abiotic), carboxylation or addition to fumarate to be microbially degraded through hydrolysis(23, 24).

Biodegradable plastics are defined based on their ability to be mineralized to carbon dioxide within a set timeframe. The European standards for biodegradability in industrial composting and anaerobic digestion is 90% CO_2 transformation within 6 months, while in soils the standard is 90% within two years at ambient temperature (25). Biodegradable plastics can be derived from modern biological (termed biobased) or petroleum- derived starting materials, however, not all biobased plastics are biodegradable. Fully degradable bioplastics offer opportunities to integrate them into a circular economy, where the starting materials can be polymerized into plastics and then degraded back to the starting monomer to be made into a different object. This circularity reduces the need to continually generate starting materials or find ways to dispose of the waste (26, 27).

In 2022, the three most widely used biodegradable plastics were PLA, starch blends and polybutylene adipate terephthalate (PBAT), with PLA being the most widely used (457 thousand tons produced globally)(28). Degradation time varies significantly depending on the type of plastic polymer due to different chemical and physical properties. Poly(hydroxyalkanoate) (PHA), poly(ε -caprolactone) (PCL), poly(hexamethylene carbonate) PHC, poly(tetramethylene succinate) PTS, and poly(butylenesuccinate) PBS are degraded relatively easily by environmental isolates, but

fewer organisms degrade PLA, which is largely attributed to PLA's relatively high glass transition temperature(29).

Serine Proteases Including Subtilisins Degrade Bioplastics

Plastic polymers are too large to cross cell barriers, thus initial degradation occurs extracellularly. Secreted enzymes are therefore required for organisms to degrade plastics, because they break the polymers into smaller oligomers and monomers extracellularly. Most plastic degrading enzymes are natively active against biomacromolecules, such as lipids, proteins and complex carbohydrates, like cutin in plant cell walls(30–32). Plastic polymers have modest structural similarities to biomacromolecules, and as a result some enzymes can act promiscuously on plastic polymers to break them down. One such group of promiscuous enzymes are serine proteases, which are a type of α/β hydrolase. The α/β hydrolase superfamily includes proteases, lipases and peroxidases among others(33). α/β hydrolases are characterized by an eight-stranded β- sheet and α- helices on both sides with a common active site catalytic triad. The catalytic triad of these enzymes includes three residues, a nucleophilic residue, an acidic residue and a histidine. Enzymes within this family that have larger active site volumes tend to be able to bind and act on a wider array of substrates(34).

Many families of serine hydrolases are implicated in plastic degradation, including cutinases, lipases, and subtilisins. In a survey of commercial serine proteases, including α-chymotrypsin, trypsin, elastase, proteinase K and subtilisin, the enzyme activity of each was tested against an array of biodegradable plastics (PLA, PHB, PES, PEA, PBS, PBS/A, PBS/C and PCL). Proteinase K and subtilisin were effective against

all of the tested plastics except for PHB(35). This suggests that proteinase K and subtilisin are widely applicable and the investigation of similar proteins may uncover even more efficient, broad range enzymes capable of bioplastic degradation. In fact, mining environmental metagenomes for hydrolases with a α/β hydrolase fold has even been an effective tool for identifying new bioplastic degrading enzymes(36).

Cutinases from diverse organisms have been observed to have specificity for various bioplastics. Cutinases from *Thermobifida alba*, *Ideonella sakaiensis and Bacillus sp*. KY0701 have activity against PLA, PET, and PCL, respectively, and *Pseudozyma antarctica* cutinases degraded PBS, PBSA, PCL and PLA (37–41).A cutinase from *Amyclotopsis mediterannei* had activity against both PBS and PCL(42). Additionally, cutinases from *Thermobifida fusca* were isolated and observed to fully break down PBAT films more quickly when two key residues below the TPA-binding tryptophan (His to Ser and Phe to Ile) were mutated(43). Further, it was posited that cutinases are a promising enzyme family for degrading plastics with bulky aromatic groups like PBAT and PET, as their wider active sites (compared to lipases) are more capable of accommodating aromatic rings(44).

Lipases are similar to cutinases, but have a ‘cap’ covering the active site. Despite this hinderance to binding, lipases have also been observed to degrade a range of biodegradable plastics. Lipases from *Candida antarctica* (45), and *Pseudomonas cepacian* (46) were observed to degrade PBS, but the efficiencies of the lipases are difficult to compare because of differences in the methodologies utilized for measuring PBS degradation. Lipases from *Aspergillus niger* have been observed to have activity

against PLA, PCL and PES (47) and a lipase from *Sphingomonas* Sp2 was active against PLA(48).

Alkaline serine proteases are also regularly identified as the enzymes responsible for plastic degradation. Subtilisins, alkaline serine proteases produced by multiple *Bacillus* spp., show activity against PLA, however not all so to the same extent (49, 50). Alkaline serine proteases are also very important industrially, with uses ranging from laundry detergent and leather processing to silver recovery from X-ray films(51). This importance is highlighted by a 2019 market value of $2.24 billion (52) and the production of 18,500 tons of *B. licheniformis* and *B. subtilis* subtilisin in 1998 (53). *Bacillus* spp. are ubiquitous organisms in the environment, including soil and marine systems where PLA degradation occurs slowly(54–56). A substantial portion of industrial alkaline serine protease produced is made by Bacilli(57). Additionally, many genetic tools are available for studying *Bacillus* species, thus making them a particularly promising genera to focus on because their protease production can be made more efficient though genetic manipulation(58–60). There are also genetic tools being developed for emerging/ environmental strains, which can be challenging to manipulate(61–63). Many of subtilisins are natively produced under specific conditions, so it is useful to characterize these conditions. While characterization of subtilisin expression has been done in some species (particularly *B. subtilis* and *B.* licheniformis) and under some conditions, those that are relevant to plastic degradation are understudied. Holistically investigating this specific group of proteins will enable us to manufacture efficient biodegradation proteins and better predict how native organisms will behave in an environmental setting.

Polylactic Acid

Of all of the bioplastics that can be degraded by enzymes, polylactic acid (PLA) may be the most important to focus on in the near future. This is because it is the most commonly used bioplastic on the market, as described above, but also is not as easily degraded, as described below. Polylactic acid (PLA) is composed of repeating monomers of lactate. These monomers are then polymerized through ring opening of dilactide dimers or direct polymerization of monomers (64). Lactate can be derived from crops including maize and sugarcane, and there is potential to produce it from agricultural waste materials (65, 66). PLA is considered capable of being processed into more products than other biodegradable plastics, yet is brittle compared to many other polymers (64). Thus, PLA is often used as part of a blend in consumer products. PLA has a variety of uses including packaging, biomedical products and agricultural products. PLA has physical properties most similar to the plastics polyethylene terephthalate (PET) and polystyrene (PS). In 'cradle to grave' life cycle assessments, PLA offers substantial reductions in global warming potential (measured as a combination of CO_2, renewable and nonrenewable energy, acidification and eutrophication) compared to PET when the PLA is recycled rather than landfilled because there are high costs associated with intensive agriculture used for many, but not all, PLA feedstocks (17, 67). Compared to petroleum- based plastics, PLA is thought to produce about half the mass of greenhouse gases during production(17). Even further reductions in greenhouse gas emissions can be obtained by using waste products to produce lactate (68).

For many years, PLA was believed to be degraded exclusively abiotically. However, the first PLA degrading organism was described in 1996 (69). Since then,

dozens of PLA degrading organisms have been described, but few studies extend the work past identifying the organism and sometimes the enzyme. To date, enzymes capable of biodegradation have only been identified from bacteria and fungi, yet this does not preclude the possibility of degradation by archaea or non-fungal eukaryotic phyla, as these organisms also produce related enzymes(31, 70–74). Table 1 lists PLA degrading organisms described since 2018. These recently described PLA degrading organisms are highly diverse and represent Gram positive, and Gram negative bacteria as well as fungi. The isolation locations were primarily soil, but also included waste water sludge.

PLA degrades more readily in temperatures above its glass transition temperature (T_g), ~55 °C, because at this temperature crystalline portions of the plastic become amorphous. Crystalline plastics are harder to break down because their polymer chains are tightly packed, which prevents access by the degrading enzyme and water, while amorphous regions are more readily accessible (64, 75, 76). PLA is successfully degraded at the elevated temperatures found in industrial composting facilities and anaerobic digesters(18). In contrast, PLA is poorly degraded under many environmental conditions including soil, marine, freshwater, and home composting(18, 77) because the temperature is typically below the T_g. Thus, it is important to explore biological processes to make PLA degradation more efficient under mesophilic conditions.

Subtilisins, described above, including some derived from *Bacillus subtilis*, *Bacillus pumilus* and *Bacillus licheniformis* have been shown to degrade PLA and PLA coated cellulose paper (49, 78–80). PLA is slow to degrade in the environment, yet

Bacillus spp. are well distributed in the environment, so optimizing conditions where Bacilli produce PLA degrading enzymes offers a solution for environmental PLA waste.

Regulation of Subtilisin (AprE) in *Bacillus* species

Understanding the way subtilisins or other plastic degrading proteins are regulated can inform applications through optimizing subtilisin gene expression conditions or modifying regulators of the plastic degrading protein. Subtilisin and other plastic degrading enzymes are secreted by the microorganism to break polymers into subunits small enough to be imported into the cell. Secreting enzymes is energy intensive, and thus is highly regulated. The primary secreted protease produced by many *Bacillus spp.* is subtilisin E, which is known as AprE(81, 82). It is directly regulated by 6 or more transcription factors depending on the species (Figure 1B) (83–88). Some of the transcription factors are within the regulons of one another, which amplifies their effects and underscores the interconnectedness of this process (Figure 1B). This multitier regulation ensures that AprE secretion is appropriately timed and highlights its importance to the cell.

AprE is produced during early stationary phase and is involved in scavenging proteins for carbon and nitrogen. In *Bacillus* species, early stationary phase genes are regulated by transcription factors known as transition state regulators. These regulators can either act as repressors or activators depending on the cellular conditions, so proper tuning is a necessary consideration. This tuning occurs intracellularly through the degree of phosphorylation for transcription factors like DegU and Spo0A and through transcription factor feedback loops (86, 89–91). As a result, subtilisin production happens

heterogeneously at the population level (92). Transition state regulators can also influence genes involved in later stationary phase phenotypes including sporulation.

Disruption of these regulatory networks can improve protease production. Because *aprE* is repressed by AbrB, ScoC, CodY and SinR in *B. subtilis*(85, 88, 93–97), these are potential targets to improve protease production. To enable *aprE* expression during exponential phase, which is likely to be the condition found in a chemostat, a *codY scoC abrB* triple knockout mutant can be created(86). Individual knockouts of these transcription factor genes however, do not highly express *aprE* during exponential growth. It takes construction of the triple mutant to uncouple *aprE* expression from growth phase and causes more subtilisin to be produced. Sporulation genes including *spoIIG* (SigE) and *spoIIA* (SigF) have also been deleted to improve AprE secretion in lab strains of *Bacillus* by avoiding and thereby prolonging the amount of time the enzyme is produced (98, 99). Deletion of *spo0A* however did not improve protease production and this may be due to its role activating *aprE*(83). Genetic modifications of a plastic degrading organism are limited by the availability of genetic tools, and environmental application is limited by regulatory guidelines for the use of modified organisms in environmental settings. Thus, investigating the role of nutrients and other molecules to improve plastic degrading protein expression from natural soil isolates is a valuable undertaking.

AprE is known to be regulated by a handful of nutrients and environmental stimuli. Salt stress upregulates AprE production causing the degradation of proteins to small peptides. These peptides are precursors to low molecular weight molecules that

may act as osmoprotectants (100). Additionally, isocitrate (101) and glutamine (102) availability alter the expression of *aprE*, likely through disruption of central carbon or nitrogen metabolism, which regulates many of *aprE's* transcription factors. Further defining the stimuli that subtilisin activators (*e.g.* DegU) respond to would provide an avenue to improve protease production.

Nucleotide derivatives are also involved in controlling the regulators of *aprE*, which should impact the protease gene as a result. Nucleotide second messengers are used by cells to relay messages about nutrient conditions, and a range of stressors including osmotic stress. One second messenger that impacts processes related to stationary phase is cyclic-di-AMP (C-di-AMP). C-di-AMP is thought to deactivate SinR, and therefore biofilm formation in *B. subtilis* (103). C-di-AMP also a ligand of a large class of riboswitches that exhibit a *ydaO* motif(104). This motif is often correlated with regulating cell wall metabolism, osmotic stress, and sporulation, so may also have a role in AprE regulation. The nucleoside inosine has been implicated as a sporulation inducer, while other closely related nucleotides had 65% or less efficacy for inducing sporulation(105). This suggests that while many of these processes are regulated by nucleotides species, they are also fairly specific. Additionally, nucleotide triphosphate concentration changes can be used as a signal of cell status. CodY becomes deactivated under conditions where GTP concentration drops (106), and the transcription factor binds DNA more effectively when it is bound to GTP(107). Significant drops in ATP concentration are associated with the end of exponential growth and the initiation of

sporulation(108). Additionally, (p)ppGpp regulates GTP homeostasis, which influences CodY activity(109, 110).

Conclusions and Future Considerations

Understanding the conditions that effect plastic degradation efficiency in the lab may provide clues to improve plastic degradation in the field. Compared to field studies, culture- based studies offer opportunities to understand how specific variables impact biodegradation. *In vitro* studies of *Bacillus pumilus* B12 indicated that PLA degradation was impacted by the addition of adenine, potassium phosphate, maltose and mannitol(111), while *Tritirachium album* and *Lentzea waywayandensis* were stimulated by proteins, peptides and amino acids(112). It seems that biostimulation with amino acids is a good initial strategy to explore, as this is effective for stimulating multiple organisms to degrade plastics.

Gaining a holistic understanding of the processes governing microbial plastic degradation will provide multiple avenues to ameliorate global plastic waste. *Bacillus spp.* produce subtilisins that are capable of degrading a range of bioplastics and offer a suitable organism for studying the protein, its regulation, and environmental applications. As production of bioplastics continues to grow, strategies for disposal are essential to prevent a problem similar to the current state of petroplastic waste.

This dissertation seeks to connect previously observed PLA- degradation phenotypes in *B. pumilus* B12 with the regulatory processes controlling protease production. First, we describe a conjugation method for introducing plasmid DNA into *B. pumilus* B12 and other *Bacillus* species. This methodology enables time resolved tracking

of *aprE* expression and is simpler than RNA extraction and RT-qPCR. We then use promoter bashing to begin identifying activator binding sites and operators in the upstream regulatory region of *B. pumilus* B12 *aprE.* We show that there are likely additional important regulatory features than those predicted based on known transcription factor binding motifs in *B. subtilis* and *B. licheniformis.* Finally, we observe that adenine, adenosine and inosine repress *aprE,* which supports previous work showing that adenine reduces PLA degradation. Inosine was the strongest repressor and we observe that it's repression of *aprE* likely involves multiple of *aprE's* transcription factors.

Appendix

Table 1-1 PLA degrading organisms described since 2018

Organism(s)	Plastic	Isolation Location	Citation
Actinomadura sp. TF1	PLA	Soil	(113) Sriyapai *et. al* 2018
Streptomyces sp. APL3	PLA	Soil	(113)Sriyapai *et. al* 2018
Pseudomonas geniculata WS3	PLA	Soil/ wastewater sludge	(114)Bubpachat *et. al* 2018
Stenotrophomonas pavanii CH1	PLA	Soil/ wastewater sludge	(114)Bubpachat *et. al* 2018
B. pumilus B12	PLA	Soil	(111) Bonifer *et. al* 2019 (115) Bordel *et. al* 2022
Co-culture: *Pseudomonas mendocina* and *Actinomucor elegans*	PLA, PBAT and blended	Soil	(116) Jia *et. al* 2021
Aspergillus flavus	PLA and PLA/ Jute	Culture collection University of Gothenburg	(117) Karimi-Avargani *et. al* 2020
Geobacillus thermoleovorans	PLA, PLA-OMMT5 and PLA-QAC0.4	Enriched from compost	(118) Castro-Aguirre *et. al* 2018
Co-culture: *Nocardioides zeae EA12, Stenotrophomonas pavanii EA33, Gordonia desulfuricans EA63, and Chitinophaga jiang-ningensis EA02*	PLA	Plastic waste from landfill	(119) Mistry *et. al* 2022
Streptomyces sp. KKU215	PLA	Botanical garden soil	(120)Yottakot and Leelavatcharamas 2019
Amycolatopsis sp. SNC	PLA	soil	(121)Decorosi *et. al* 2019
Amycolatopsis sp. SST	PLA	soil	(121)Decorosi *et. al* 2019
Amycolatopsis sp. SO1.1	PLA	soil	(121)Decorosi *et. al* 2019
Amycolatopsis sp. SO1.2	PLA	soil	(121)Decorosi *et. al* 2019
Paenibacillus sp. T8 LB10-50	PLA, PHB, PLA/PHB	PLA/PHB transparent foil, soil mesocosm	(122) Jeszeová *et. al* 2018
Streptomyces cyaneogriseus T8 FSS40	PLA, PLA/PHB	PLA/PHB transparent foil, soil mesocosm	(122) Jeszeová *et. al* 2018
Trichoderma harzianum T8 SAB1-f	PLA, PLA/PHB	PLA/PHB transparent foil, soil mesocosm	(122) Jeszeová *et. al* 2018

Table 1-1 continued

Bacillus megaterium T12 LB10-60	PLA	carbon black PLA/PHB filled foil, soil mesocosm	(122) Jeszeová *et. al* 2018
Bacillus licheniformis T12 LB10-65	PLA	carbon black PLA/PHB filled foil, soil mesocosm	(122) Jeszeová *et. al* 2018
Rhodococcus sp. T21 AIA10; T21 AIA11	PLA, PHB, PLA/PHB	carbon black PLA/PHB foil exposed for 90 days to sun light, soil mesocosm	(122) Jeszeová *et. al* 2018
Rhodococcus sp. T21 AIA12; T21 LB10-54	PLA, PHB	carbon black PLA/PHB foil exposed for 90 days to sun light, soil mesocosm	(122) Jeszeová *et. al* 2018
Actinomucor elegans T21 PDA1-f	PLA	carbon black PLA/PHB foil exposed for 90 days to sun light, soil mesocosm	(122) Jeszeová *et. al* 2018

CHAPTER 2 CONJUGATION-MEDIATATED PLASMID TRANSFER ENABLES GENETIC MODIFICATION OF DIVERSE BACILLUS SPECIES

A version of this chapter was originally published by Elise Phillips, Jordan Cannon, Yue Zhou, Kyle Bonifer and Todd Reynolds

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This article has been revised since publication to update the strain table, and renumber the figures and references. Jordan Cannon cloned GFP into the backbone plasmid. Aeric (Yue) Zhou conjugated the plasmid into *P. megaterium.* Dr. Reynolds provided guidance and feedback. I cloned the *oriT*, performed conjugations, growth curves, sporulation assays, and wrote the manuscript.

Abstract

Performing genetic manipulations in *Bacillus* strains is often hindered by identifying conditions appropriate for DNA uptake. This shortcoming limits our understanding of the functional diversity within this genus and the practical application of new strains. We have developed a simple method for increasing the genetic tractability of *Bacillus spp.* through conjugation- mediated plasmid transfer via a diaminopimelic acid (DAP) auxotrophic *E. coli* donor strain. We observe transfer into representatives of the *Bacillus* clades *Subtilis, Cereus, Galactosidilyticus* and *Preistia megaterium* and successfully applied this protocol to 9 out of 12 strains attempted. We utilized the BioBrick 2.0 plasmids pECE743 and pECE750 as well as the CRISPR plasmid pJOE9734.1 to generate a xylose-inducible GFP- expressing conjugal vector, pEP011. The use of xylose- inducible GFP ensures ease of confirming transconjugants, which enables users to quickly rule out false positives. Additionally, our plasmid backbone offers the flexibility to be used in other contexts, including transcriptional fusions and overexpression, with only a few modifications.

Introduction

Bacillus subtilis has become a model Gram- positive organism in part due to lab strains' natural competence, which enables rapid genetic manipulation (123). However, as the conditions that induce competence are highly variable, identifying appropriate conditions for genetic manipulation of other isolates presents a substantial challenge. *Bacillus* spp. are widely utilized for commercial enzyme production, and novel isolates have the potential to contribute to plastic degradation (124), agricultural efficiency(125), and antibiotic production(126). Thus, expanding the repertoire of DNA introduction techniques for a broad range of Bacilli will encourage further work on novel isolates.

Implementation of previously- described strategies to improve transformation efficiency of *Bacillus spp.* is impeded by initially identifying reliable transformation conditions, which can be further impacted by restriction modification systems and/ or a reliance on recombination into the host genome(127). Working to improve transformation efficiency by inducing natural competence through overexpression of *comK*(128) and *comKS*(129) is effective when competence occurs, but competence conditions are highly strain specific, which limits the study of new strains.

Alternative techniques for genetic manipulation include electroporation, protoplast transformation, particle bombardment and conjugation. Conjugation has been utilized to introduce DNA into difficult-to-transform *Bacillus spp.* and is less strain specific than some of the aforementioned methods. Triparental mating successfully generated transconjugants in two *Bacillus* clades, as well as *Paenibacillu*s, but did not produce *Preistia megaterium* (formerly *Bacillus megaterium)* transconjugants(62). An elegant conjugation- based knockout system in *Preistia megaterium* utilizes

pasteurization as a counter selection(63), which limits the user's study of sporulation because sporulation mutants would be unable survive pasteurization. We sought to identify a protocol that enables conjugation into diverse Bacilli and uses a simple counterselection strategy. We have developed a biparental mating system that introduces plasmid- encoded xylose- inducible GFP into *Bacillus spp.* and employs a diaminopimelic acid (DAP) auxotrophic *E. coli* for counterselection. We observe efficient transformation in *Bacillus* clades *Subtilis, Cereus, Galactosidilyticus* and *Preistia megaterium*. This combination of *E. coli* donor and oriT expands *Bacillus subtilis* toolkits, such as the BioBrick Box plasmids(130), to additional species. The plasmid backbone, pEP011, can be easily modified to create transcriptional fusions and protein overexpression constructs for a wide range of *Bacillus* spp., further broadening its utility.

Methods

Bacterial Growth

Cells were grown in Luria-Bertani (LB) broth or plates with appropriate antibiotics: ampicillin (100 ug/mL) for *E. coli* and erythromycin plus lincomycin (2 and 25 ug/mL, respectively) for *Bacillus* spp. *E. coli* EZ180 cells (used as the donor strain) were supplemented with 0.3 mM diaminopimelic acid (DAP). For fluorescence measurements with the xylose inducible promoter, cells were grown in minimal salts medium (MSM) [per liter 1 g $(NH_4)_2SO_4$, 1.5 g KH_2PO_4, 0.5 g K_2HPO_4, 0.2 g $MgSO_4*7H_2O$, 1 g NaCl] supplemented with 1% (wt/vol) fructose and 5% (vol/vol) LB. For fluorescence measurements with native promoters, MSM was supplemented with 1% wt/vol mannitol.

All strains are listed in Table 1. All tables are in the appendix at the end of the chapter. Representatives of *Bacillus* clades were acquired from the *Bacillus* Genetic Stock Center (BGSC). *Bacillus* strains NB5, NB6 and NB9 were isolated from a contaminated bottle of the detergent Tween-40 and identified using 16S sequencing.

Difco Sporulation Media(131) [per liter 8g Nutrient Broth No 4 (Difco), 1 g KCl, 1mM $MgSO_4$, 1 mM $Ca(NO_3)_2$, 10 μM $MnCl_2$, 1 μM $FeSO_4$] was used to induce sporulation.

Vector Construction

Plasmid construction and maintenance was performed using NEB DH5 α *E. coli*. pECE743 was used as the backbone plasmid because of its xylose- inducible promoter and the ability to utilize red-white screening to determine the success of GFP insertion into the plasmid(130). The origin of transfer (*oriT*) was amplified from pJOE9734.1 using primers EPO61 and EPO62 (Table 3), both of which introduced a *Pci*I site. This fragment was cloned into the *Pci*I site in the pECE743 plasmid (132) resulting in plasmid pEP004. The GFP open reading frame was digested from pECE750 and ligated into the *Xba*I and *Spe*I cut sites in pEP004 to create plasmid pEP011. Plasmids were transformed into calcium chloride induced chemically competent EZ180 cells. For native promoter experiments, PCR amplified promoter fragments were digested and inserted into pEP011 with the *XbaI* and *AatII* cut sites. Plasmids described in this study are listed in Table 2. The sequences of pEP011, pEP024 and pEP036 are found in supplemental data.

Conjugation

Conjugation was performed by inoculating *E. coli* EKP23 (EZ180 + pEP011) at 0.2 OD in 25 mL and growing to early stationary phase (between six and seven hours) in LB medium supplemented with the non-canonical amino acid diaminopimelic acid (DAP) and ampicillin (LB*DAP*Amp) at 30 °C. Simultaneously, the *Bacillus* strain to be conjugated was inoculated into 25 mL LB at 0.2 OD and grown to mid logarithmic phase (~1.3 OD) at 30 °C. The cultures were spun down and resuspended in 10 mL of PBS and kept on ice until both strains reached the appropriate growth phase. One OD_{600} of cells from each culture was combined in a microfuge tube, mixed by gentle pipetting and incubated on the bench for five to ten minutes. Three 5 µL spots of the mix were plated on LB DAP and incubated overnight (18 hours) at 37 °C. The three spots were scraped into 5 mL of LB erythromycin/lincomycin and recovered for 24 hours at 30 °C. Cells were then serially diluted and plated on LB erythromycin/ lincomycin and incubated overnight at 30 °C. Single colonies were streaked and screened by colony PCR with primers EPO92 and EPO93.

Fluorescence Measurements Over Time

Overnight cultures of transconjugants were diluted to 0.1 OD_{600}, and then grown in minimal medium for 2.5 hours at 30 °C. Cultures were aliquoted into wells and then 0.5% (w/v) xylose was added to appropriate wells. Fluorescence was measured using a BioTec Cytation 5 plate reader using λ= 483nm excitation, 513nm emission followed by an absorbance reading at λ=600 nm, while shaking at 30 °C for 24 hours, with reads every 15 or 30 minutes for 3 biological replicates.

For native promoter GFP expression, cells were inoculated into LB erythromycin/ lincomycin at 0.01 OD and grown 16 hours overnight. Cells were washed in PBS and then inoculated into MSM mannitol at 0.2 OD or sporulation medium, LB or 50% LB at 0.1 OD. All media were supplemented with erythromycin/ lincomycin as described in "Bacterial Growth". Fluorescence measurements were then taken for 36 hours as described above.

Fluorescence Microscopy

Cells were grown as described for fluorescent measurements, but they were induced with xylose for 6 hours prior to visualization via fluorescence microscopy. Cells were then imaged with a Keyence BZ-X700 fluorescence microscope using λ= 470/40nm excitation, 525/50 emission for GFP.

Sporulation Assay

Cells were inoculated into DIFCO sporulation media erythromycin/ lincomycin or LB erythromycin/ lincomycin at 0.01 OD and grown for 24 hours at 30 °C. Cells were then heat killed by incubating for 20 minutes at 85°C(133). Heat killed cells were serially diluted and then plated onto LB and LB erythromycin/ lincomycin. Phase bright and phase dark cells were quantified with phase contrast microscopy [Olympus BX50] and FIJI image processing software.

Plasmid Copy Number Quantification

To assess plasmid copy number, qPCR was used to determine the abundance of plasmid relative to a single chromosomally- encoded gene, *ftsZ.* Total DNA was extracted with the Winston Hoffman phenol/chloroform protocol (134) after a 6-hour induction with

xylose. qPCR was performed with PowerUp SYBR Green Master Mix (Applied Biosystems/ ThermoFisher) using the manufacture's protocol and the primers qEPO61, 62, 117 and 118. Data was acquired and analyzed with QuantStudio3 software (Applied Biosystems). GFP expression was normalized to *ftsZ* using Livak's method ($2^{-\Delta\Delta C_T}$).

Results and Discussion

We are interested in developing a strategy for genetic manipulations in the soil isolate *B. pumilus* B12 (111) to explore the mechanism behind its ability to degrade the bioplastic poly-L-lactic acid and understand how this process is genetically regulated to improve its biotechnical applicability. Initially, many unsuccessful attempts were made to introduce DNA, including electroporation at multiple voltages (plasmids, linear single stranded DNA and linear double stranded DNA), protoplasted cells, and media-induced natural competence(135–141). We then decided to switch to conjugation, a technique known for the diverse organisms that can receive DNA from a single host. We used *E. coli* EZ180, which has conjugation transfer genes from the plasmid RK4 integrated into its genome (142) and has been observed to act as a donor for diverse bacterial clades(143–145). The genomic integration of the RK4 transfer genes makes it possible to utilize vectors that contain a functional origin of transfer (*oriT*) for successful conjugation, ensuring ease of cloning and allowing the use of many potential backbones. Additionally, *E. coli* EZ180 has a DAP auxotrophy, which enables easy counterselection and removal of the donor bacteria from the mixed culture. To generate our conjugal vector, we cloned the *oriT* from pJoe9734.1, which another group used to conjugate CRISPR machinery into *Bacillus subtilis* JABs33 (132). To probe the efficiency of

conjugation, a xylose- inducible GFP from pECE750 was inserted into the *oriT* containing pECE743. This plasmid, pEP011 (Figure 2-1a), was transformed into the donor *E. coli* EZ180 strain for conjugation.

Using this approach, we observed that conjugation between log phase *Bacillus pumilus* B12 and early stationary phase *E. coli* EZ180 produces numerous transconjugants. For *B. pumilus* B12 expressing pEP011, fluorescence above background is statistically significantly different from control groups after 75 minutes of xylose induction ($p=0.182$) and steadily increases over time when measured in a plate reader (Figure 2-2). This difference is also observable via microscopy after 6 hours of induction (Figure 2-3). After 6 hours incubation with xylose, the majority of plasmid harboring cells are robustly fluorescent (Figure 2-3a), however, without induction, only a few cells exhibit low fluorescence (Figure 2-3b). This suggests that xylose induction is not very leaky. Without the plasmid, no fluorescence is observed (Figure 2-3c and d), indicating that conjugation was successful with pEP011. After conjugation, we observe that the plasmid is maintained after sporulation because we obtain a similar (not statistically significantly different) number of CFU's when grown with and without erythromycin selection (Figure 2.4).

Next, in order to measure whether this conjugation method can be used in other *Bacillus* spp., we chose representatives of 3 major *Bacillus* clades (*Subtilis, Cereus, and Galactosidilyticus*), the closely related *Prestia megaterium* (146) in addition to

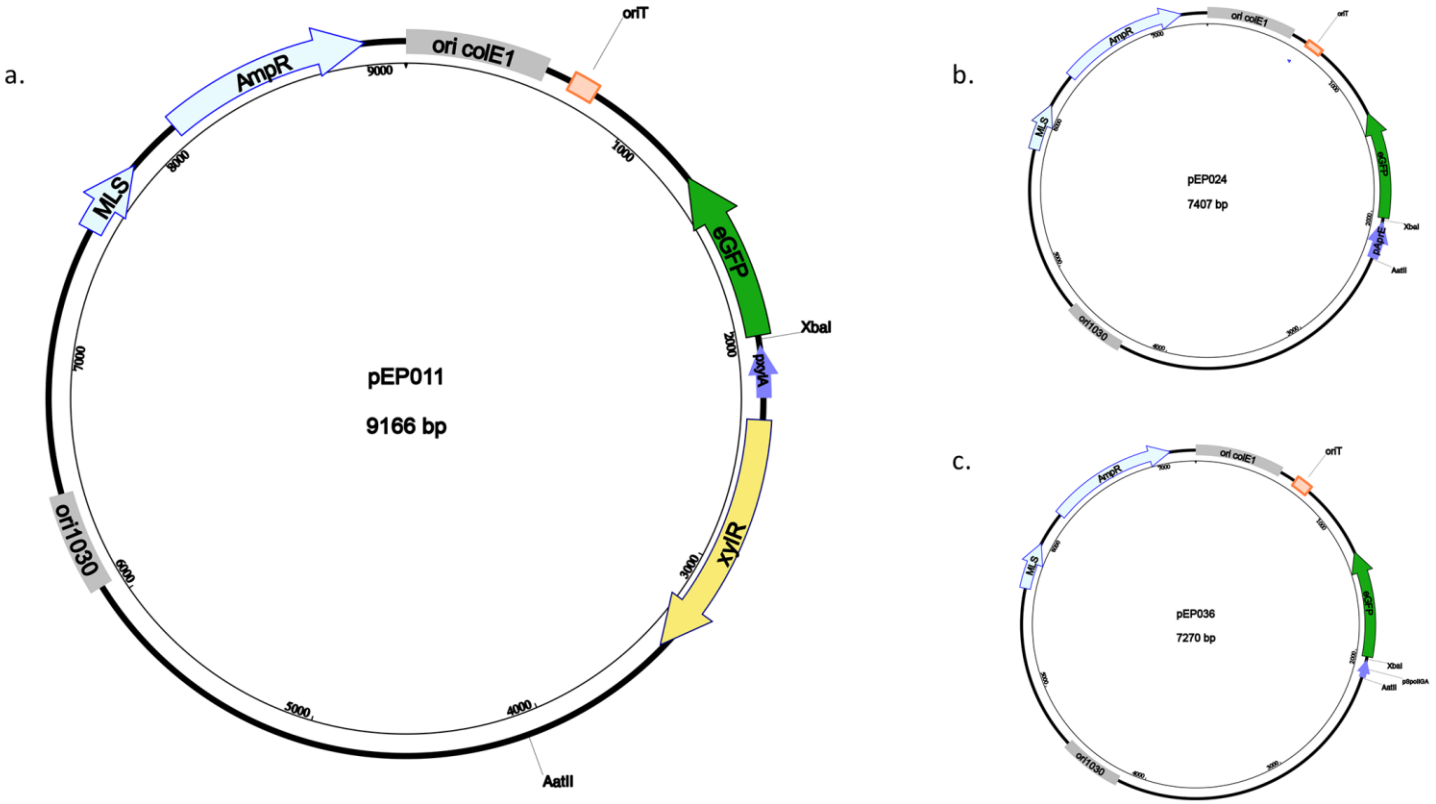

Figure 2-1: Plasmid diagram of pEP011, pEP024, & pEP036. Light blue represents antibiotic resistance markers. MLS confers resistance to erythromycin/ lincomycin for selection in *Bacillus* strains, while AmpR confers resistance for selection in *E. coli*. Ori colE1 is the *E. coli* origin derived from *colE1* plasmid. Ori1030 is the replication origin for *Bacillus.* The origin of transfer (oriT) is denoted in orange. A) pEP011. Xylose de-represses XylR (yellow), which activates *pXylA* (dark blue) thereby inducing *gfp* (green) expression. B) pEP024. pAprE (dark blue) induces *gfp* (green) expression. C) pEP036. pSpoIIGA (dark blue) induces *gfp* (green) expression

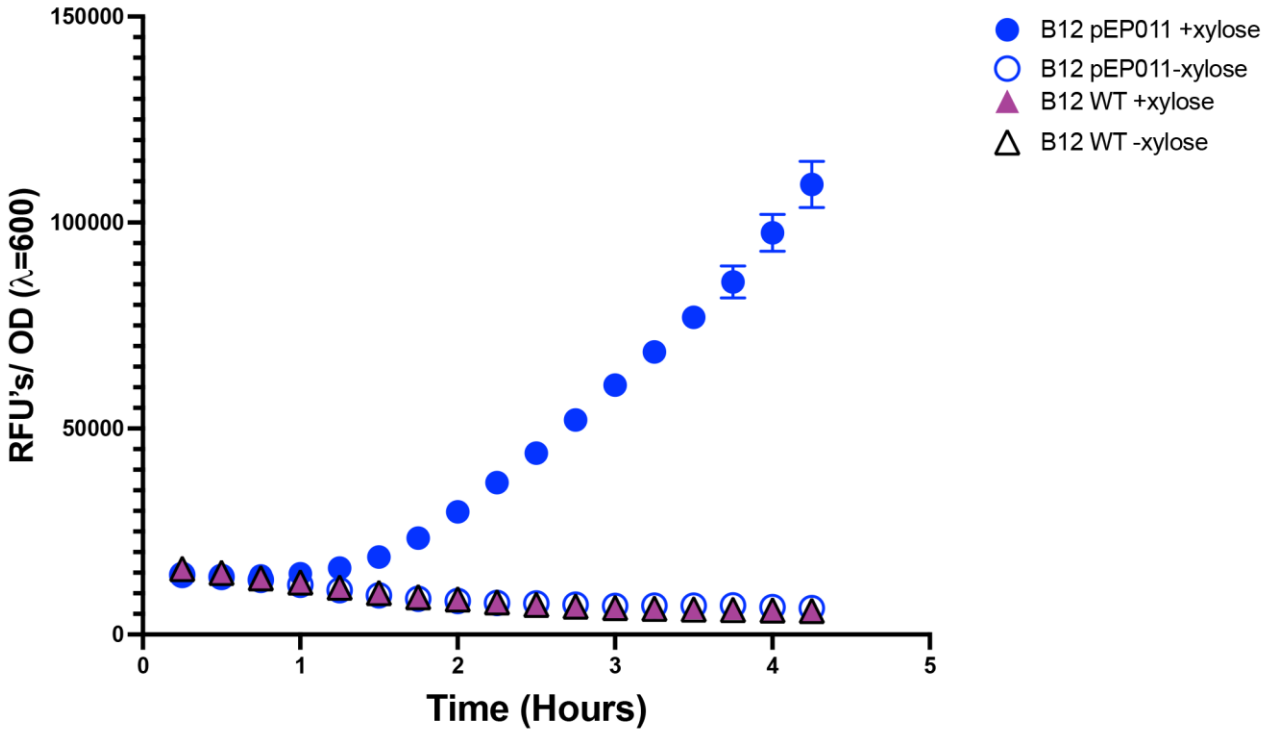

Figure 2-2: The pEP011 plasmid was successfully conjugated into *Bacillus pumilus* B12, and GFP was induced over time following xylose addition. Fluorescence from GFP was measured over time. The fluorescence levels were normalized to OD600. Error bars represent standard error of the mean from 3 replicate wells. Two-way ANOVA with Tukey's multiple comparisons shows that B12 +pEP011 +xylose is significantly different from the 3 control groups after 75 minutes.

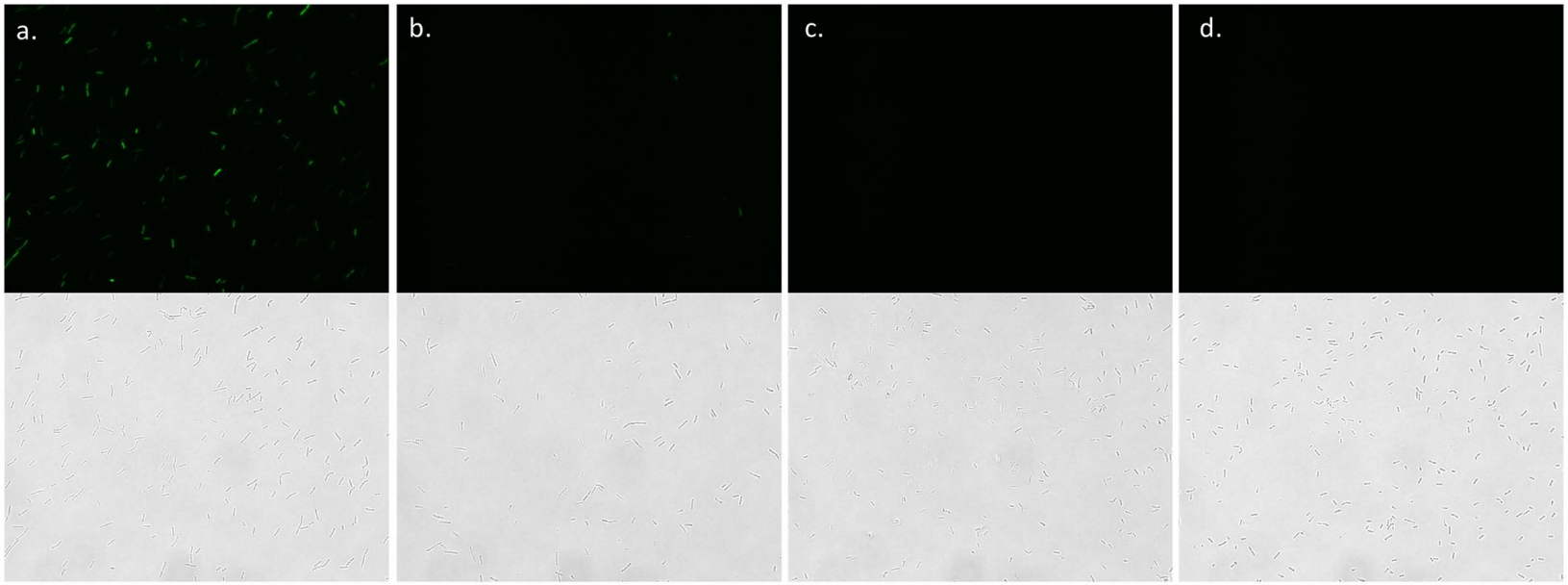

Figure 2-3: Cells with plasmid fluoresce after induction with xylose. Fluorescence (top) and bright field (bottom) images of B12 with pEP011 (a & b) and B12 wild type (c & d) with (a & c) and without (b & d) xylose induction.

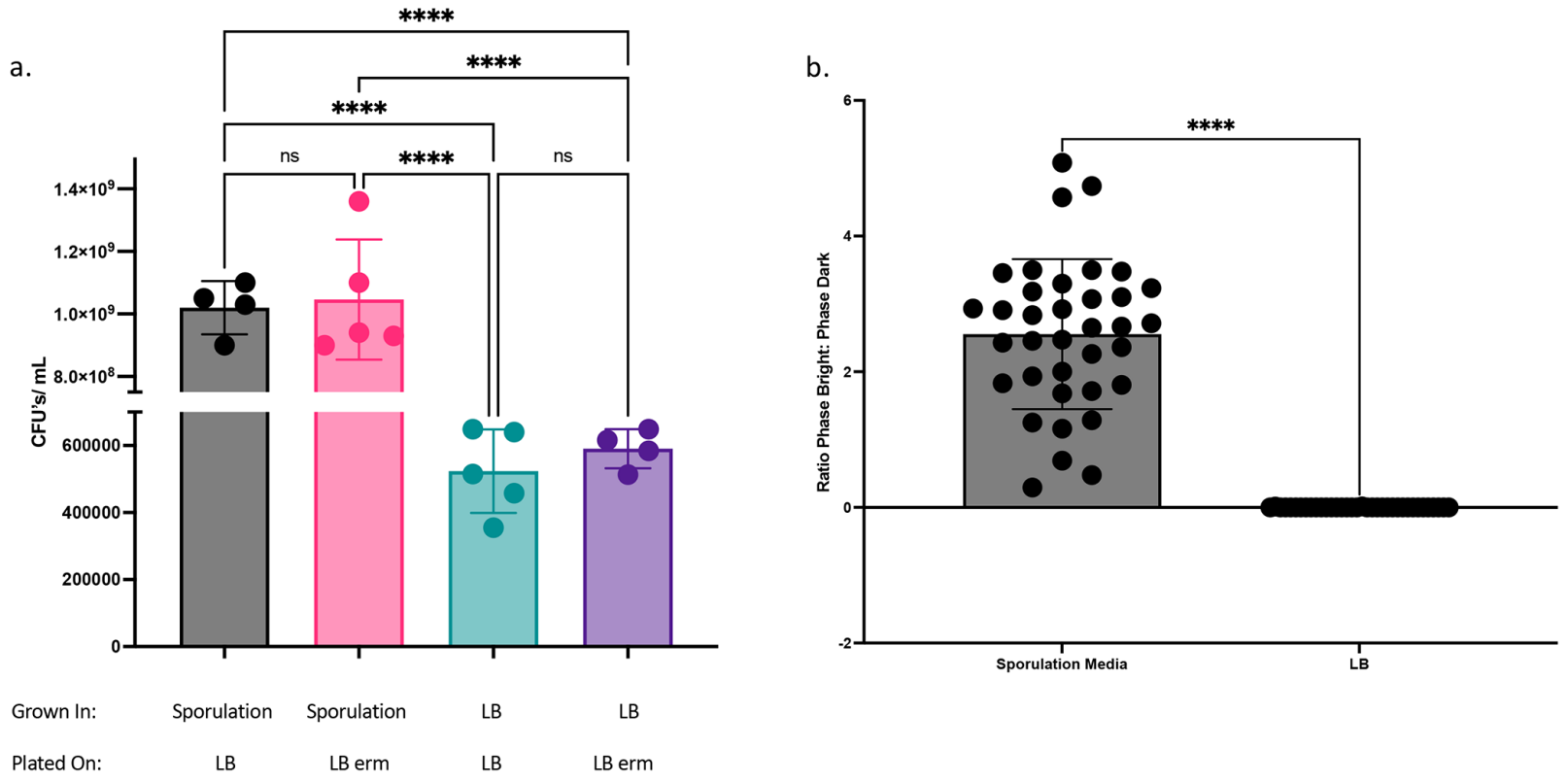

Figure 2-4: Sporulation does not impact the proportion of plasmid containing cells A) Colony forming units of heat-treated cells when grown on LB or LB erythromycin/ lincomycin after 24 hours of growth in either sporulation medium or LB. One- way ANOVA with Tukey's multiple comparisons, **** p-value<0.0001, ns p-value >0.984. This is a representative figure for 1 of 3 biological replicates. B) Proportion of phase bright to phase dark cells. Student's T-Test **** p<0.0001. Three biological replicates.

environmental isolates in our collection that represented multiple environments (soil, aquatic, detergents). We included *B. subtilis* Δ*comK* to ensure that DNA uptake occurred through conjugation rather than natural competence. We observed that multiple species within the genera *Bacillus* and *Preistia* uptake the plasmid pEP011 from *E. coli* EKP23 as the conjugal donor. All strains were successfully conjugated, except for *B. licheniformis, B. pumilus* MTCC6033, and *Bacillus siamensis* NB9, perhaps due to differences in restriction- modification systems or antibiotic sensitivity. After xylose induction, strains expressed GFP at varying levels (Figure 2-5). For example, *B. cereus* exhibited the greatest level of GFP fluorescence, while *B. subtilis* Δ*comK* exhibited the lowest expression (Figure 2.5c), and environmental isolate NB6 had the longest lag before detectable GFP expression (Figure 2-5b).

To elucidate why strains expressed GFP differentially, we performed qPCR to quantify plasmid copy number. We normalized the amount of *gfp* to *ftsZ*, a single copy chromosomal gene, to determine the relative copy number of the plasmid. While we observe that *B. pumilus* B12 fluoresces highly and robustly expresses *gfp*, this does not seem to be the case in *Bacillus sp.* 125, which despite exhibiting equally high levels of fluorescence does not have equally high relative copies of *gfp* (Figure 2-6). The number of *gfp* DNA copies (plasmid number) per *ftsZ* (cell number), do not correlate with the trends in GFP fluorescence levels at 6 hours. Thus, copy number of the plasmid is insufficient to explain GFP intensity alone. Strain variability in GFP fluorescence is therefore likely due to a combination of cellular attributes including varying copy

number, strain specific responses to the xylose- inducible promoter and GFP expression (translation) efficiency.

To explore the use of this backbone as a reporter for native gene expression, we generated transcriptional fusions for two genes expressed by differentially during stationary phase, *aprE* (pEP024), *spoIIGA* (pEP036) (Figure 2.1b & c). We observe that pAprE induces high GFP expression when grown in minimal media supplemented with mannitol but not in DIFCO sporulation media or LB. We observe moderate GFP expression induced by pSpoIIGA when grown in minimal media both statically and with shaking but only in DIFCO sporulation media with shaking (Figure 2.7). Initial fluorescence observed in LB is due to autofluorescence of LB. The diminished relative GFP expression by pSpoIIGA is indicative of a less highly induced gene and/or a smaller subset of the population expressing the gene.

Conclusions

We demonstrate a single protocol that is widely applicable to *Bacillus* spp. The origin of transfer present on this backbone is promiscuous, so this protocol likely can be utilized with other plasmid- mediated gene disruption systems (147, 148). This backbone (pEP011) offers flexibility because it can be further modified to construct transcriptional fusions (pEP024 and pEP036) or overexpress genes with a strong promoter, thus making it a versatile tool. We show that biparental conjugation as a mode of plasmid introduction begins to close the gap between easy to genetically manipulate lab strains and difficult to modify environmental isolates

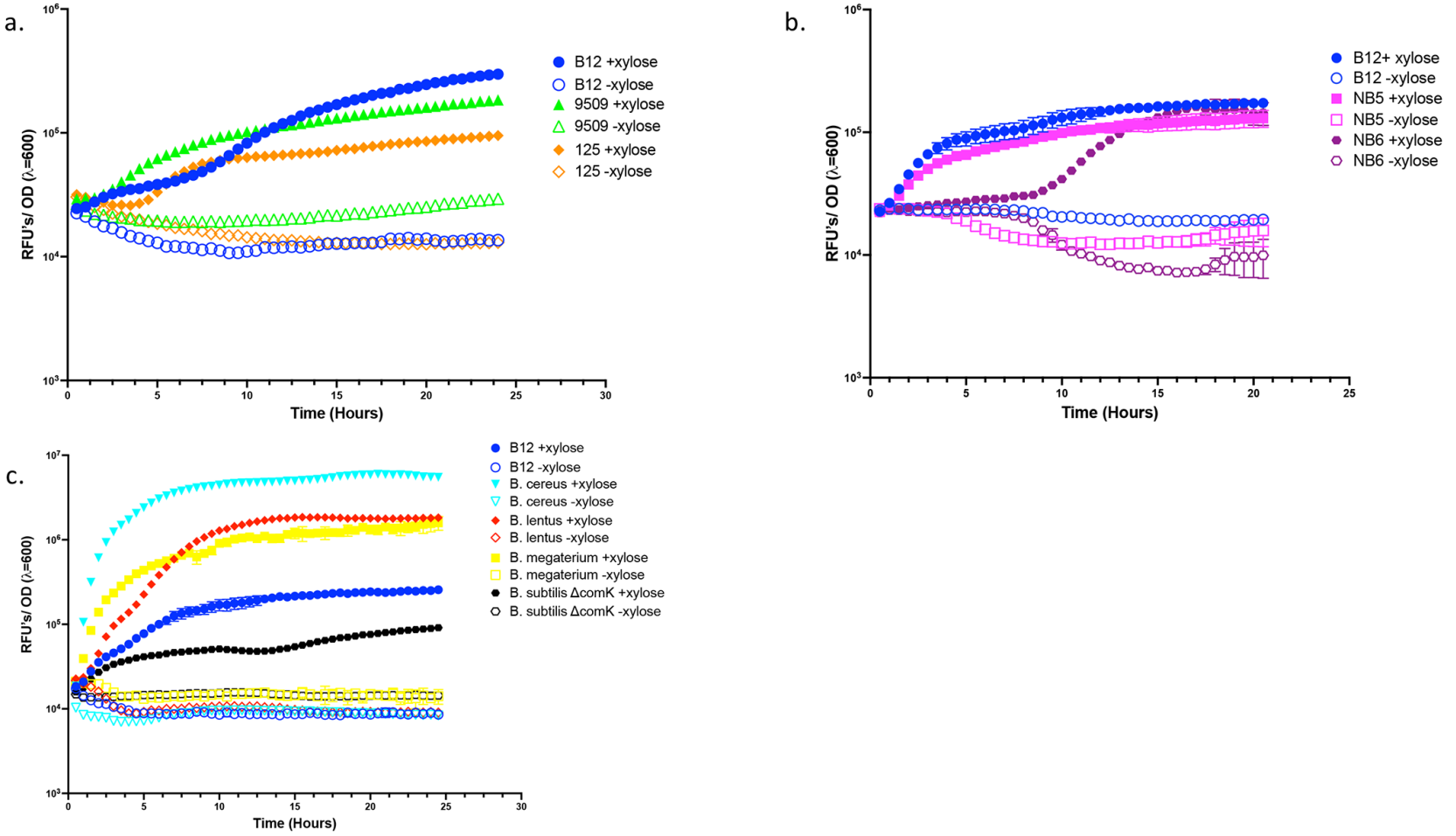

Figure 2-5: *Bacillus spp* express GFP after xylose induction. Measurement of GFP fluorescence over 24 hours normalized to optical density (λ=600 nm). A) *Bacillus pumilus* strains. B) Environmental isolates. C) Representatives of other *Bacillus* and closely related clades. Error bars represent standard error of the mean from 3 replicate wells.

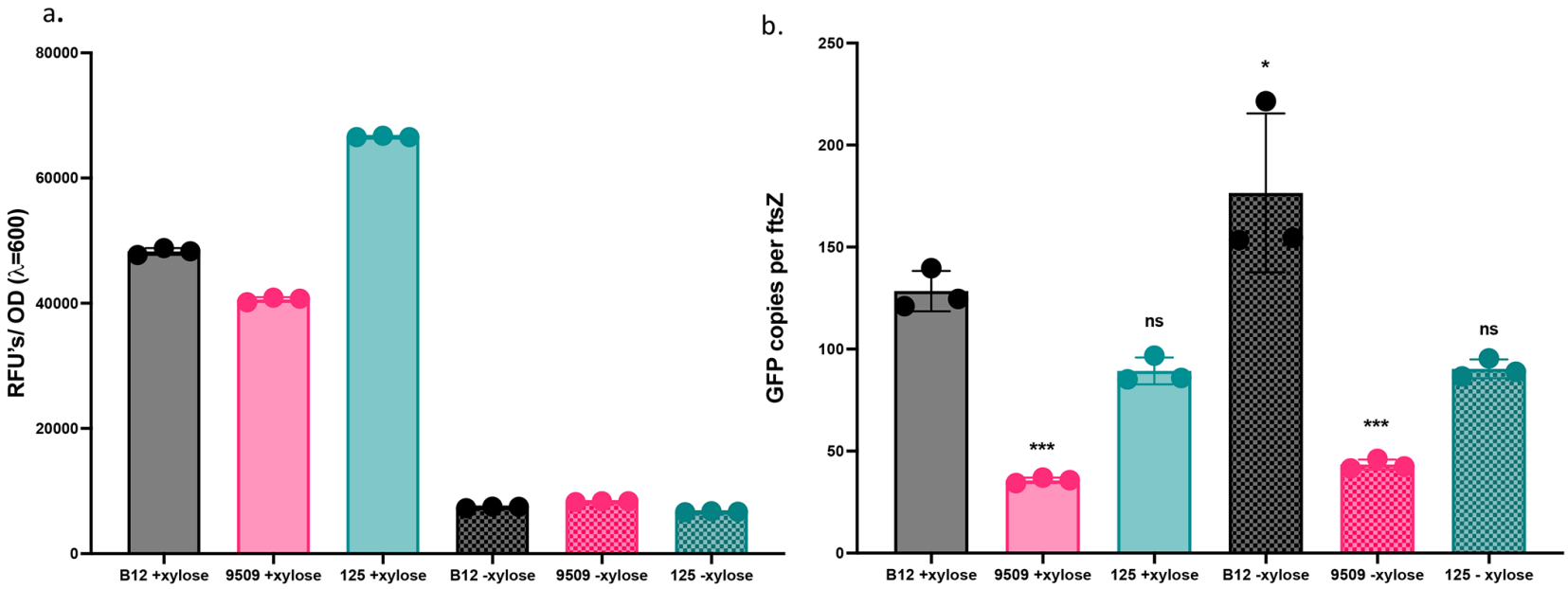

Figure 2-6: Differential expression is partially explained by plasmid copy number. A) Normalized fluorescence after 6 hours growth with or without induction. Significance determined by one-way ANOVA with Tukey's multiple comparisons, **** p-value<0.0001 compared to B12+xylose B) qPCR of GFP normalized to cellular *ftsZ* for xylose induced and uninduced cells. Significance determined with one-way ANOVA with Tukey's multiple comparisons, *** p-value<0.001, * p-value<0.05, compared to B12 +xylose. Error bars represent standard deviation. Figures are a representative figure of two biological replicates, each with three technical replicates.

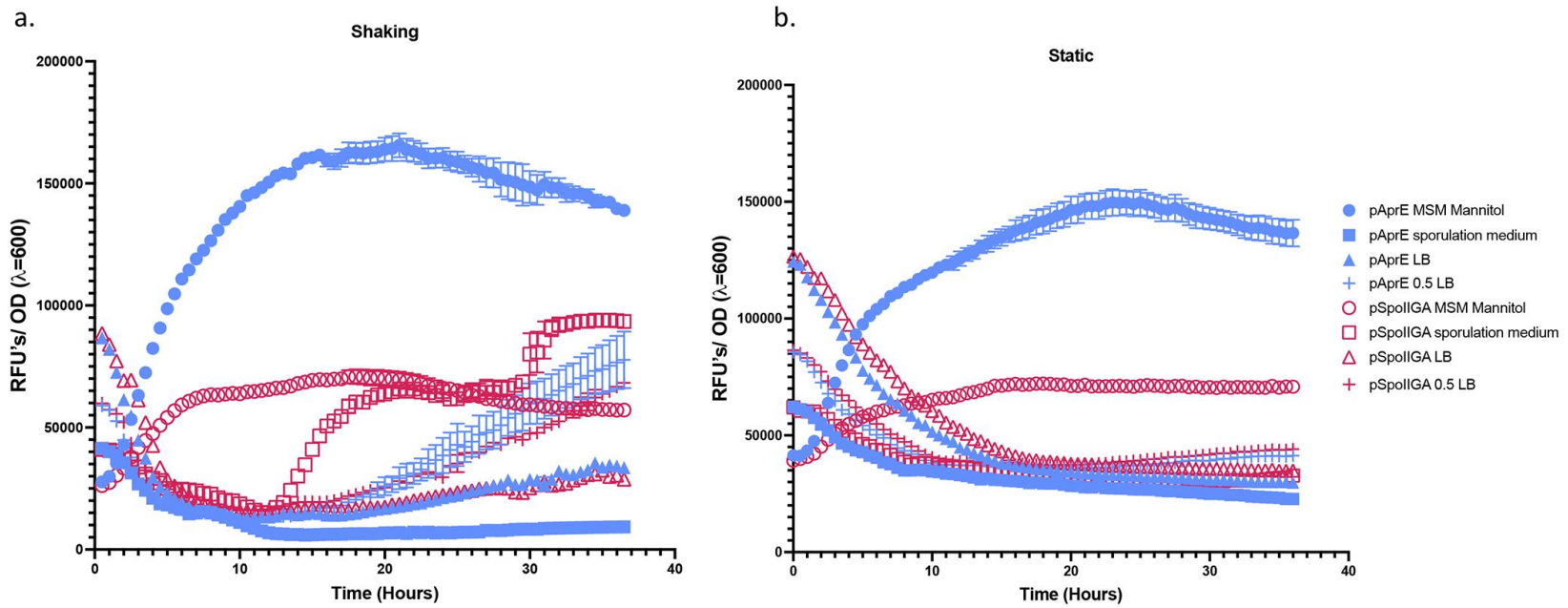

Figure 2-7: Native promoters activate GFP differentially. Measurement of GFP fluorescence over 36 hours normalized to optical density (λ=600 nm). A) 96- well plates incubated with shaking. B) 96- well plates incubated statically. Error bars represent standard error of the mean from 3 replicate wells.

Appendix

Table 2-1: Strains

Species	Strain name	Genotype	Source
E. coli	EZ180	Δ*dapA*, RK4 integrated	Zinser lab. Derived from WM3064 (142, 143)
E. coli	EKP23	EZ180 + pEP011	This work
Bacillus pumilus	B12	Wild Type	(Bonifer et. al 2019)
Bacillus sp.	9509	Wild Type	Lebeis Lab
Bacillus sp.	125	Wild Type	Lebeis Lab
Bacillus pumilus	MTCC6033(149)	Wild Type	Loewen lab
Bacillus subtilis	BKK10420	Δ*comK*	BGSC
Preistia megaterium	QM B1551 (BGSC 7A16)	Wild Type	BGSC
Bacillus licheniformis	ATCC 14580T (BGSC 5A36T)	Wild Type	BGSC
Bacillus cereus	ATCC 13472 (BGSC 6A10)	Wild Type	BGSC
Bacillus lentus	Gibson 165 (BGSC 60A1)	Wild Type	BGSC
*Bacillus stratosphericus**	NB5	Wild Type	This work
*Prestia megaterium**	NB6	Wild Type	This work
*Bacillus siamensis**	NB9	Wild Type	This work

*Top BLAST hit with 16S sequence as of 2022-8-12

Table 2-2: Plasmids

Plasmid	Description	Source ID and Reference
pECE743	XylR-P*xylA*, RFP, ori1030, amp^r, mls^r	BGSC ECE743 (130)
pECE750	GFPmut1, cm^r	BGSC ECE750 (130)
pJOE9734.1	oriT, Kan, pManPA- cas9	BGSC ECE722 (132)
pEP004	oriT, XylR-P*xylA*, RFP, ori1030, amp^r, mls^r	This Work
pEP011	GFP, oriT, XylR-P*xylA*, ori1030, amp^r, mls^r	This Work
pEP024	GFP, oriT, pAprE, ori1030, amp^r, mls^r	This Work
pEP036	GFP, oriT, pSpoIIGA, ori1030, amp^r, mls^r	This Work

Table 2-3:Primers. Cut sites denoted by orange text.

Primer name	Element, Cutsite	Sequence
JCO70	Screening GFP insertion	ATGAGCAAAGGCGAAGAAC
JCO71	Screening GFP insertion	CAGTTCATCCATGCCATGT
EPO 61	oriT, *Pci*I	AAAAACATGTGCTTGGTTTCATCAGCC
EPO 62	oriT, *Pci*I	AAAAACATGTCTGCTTCGGGGTCATTATAG
EPO92	Screening transconjugants	GTCTCATGAGCGGATACATAT
EPO93	Screening transconjugants	CCATATGTTGCATCACCTT
EPO114	pAprE, *aatII*	AAAAGACGTCGCATTTCGGGTATCGAATG
EPO110	pAprE, *xbaI*	AAAATCTAGACTTATTTCAGAATAATCATCCG
EPO165	pSpoIIGA, *aatII*	AAAAGACGTCCGATATTCTTTCTGTTTCATACG
EPO166	pSpoIIGA, *xbaI*	AAAATCTAGAGTTTGTACCAGTATAAAACAGTC
qEPO61	*ftsZ* (qPCR)	GACATGGTCTTTGTGACAGCC
qEPO62	*ftsZ* (qPCR)	GTGAAAGGACGAGTCACAACTC
qEPO117	*gfp* (qPCR)	CAATGCCGGAAGGCTATG
qEPO118	*gfp* (qPCR)	GCGATTGACCAGTGTATCGC
27F	16S sequencing	AGAGTTTGATCMTGGCTCAG
1522R	16S sequencing	AAGGAGGTGATCCANCCRCA

CHAPTER 3 DESCRIBING THE CIS-ACTING REGULATORY REGION OF *BACILLUS PUMILUS* B12 APRE AND ITS REPRESSION BY INOSINE

Publication Statement

This chapter is intended for future publication.

Site directed mutagenesis was performed by Aeric (Yue) Zhou and Jemma McLeish. Primers were designed by Elise Phillips, Aeric (Yue) Zhou. Promoter fragments were cloned and conjugated in *Bacillus* by Elise Phillips, Ainsley King, Kailani Reynolds and Leonard Weaver. Time resolved fluorescence experiments were performed by Elise Phillips, Ainsley King and Mikayla Mangrum. Mass Spectrometry was performed by the Michigan State Mass Spectrometry and Metabolomics core. Manuscript was written and research plan was developed by Elise Phillips with advice and review from Todd Reynolds.

Abstract

Extracellular proteases enable organisms to break down large (and complex) molecules, including plastics. *Bacillus pumilus* B12 degrades the bioplastic polylactic acid (PLA) by producing the alkaline serine protease, AprE. Outside of model *Bacillus* species it is largely unknown how these proteases are regulated. We sought to understand the regulation of AprE in *Bacillus pumilus* B12 to better understand when this PLA degrading protein is produced. Through plasmid- based transcriptional- fusions to GFP, we measured *aprE* expression to define its upstream regulatory region in *B. pumilus* B12. We primarily observe trends similar to those expected based on the known *B. subtilis* transcription factor binding sites. However, some segments of the promoter provide

indications of undefined activator binding sites that may be useful to exploit in the future to improve expression. Previous work has reported repression of *B. pumilus* B12 PLA degradation through adenine addition, and we show that this occurs through repression of *aprE*. The *aprE* gene is more strongly repressed by inosine than adenine, but the transcription factor through which this repression occurs remains unknown.

Importance

Understanding how bioplastic degrading proteins are regulated will inform future waste management decisions, including growth condition optimizations. *B. pumilus* B12 has been shown to degrade the biodegradable bioplastic polylactic acid (PLA) with the protein AprE, but it is unknown how its regulation compares to *aprE* produced by *B. subtilis* or *B. licheniformis,* which are fairly well studied and may help identify ideal conditions for native protease expression. Our observations of potential activator binding regions may enable discovery of a new activator.

Introduction

Extracellular proteases are crucial for many microbes to access more recalcitrant nutrient pools because they break large molecules into smaller monomers and oligomers capable of being transported into the cell. Proteases have important biotechnological applications and are widely produced industrially, especially by *Bacillus* species. A few of their uses include laundry detergent, leather processing and plastic degradation (51).

Serine proteases are a large group of enzymes that have been observed to have activity against plastic waste(35, 36). This ability is largely attributed to enzyme promiscuity and the plastic polymer's modest structural similarity to certain amine groups. The primary extracellular protease produced by *Bacillus* spp. is an alkaline serine protease called subtilisin, referred to as AprE in *B. subtilis* (81, 82). We have observed that *B. pumilus* B12 is capable of degrading the widely used bioplastic, polylactic acid (PLA), by producing a subtilisin homolog (49). In this work, we sought to determine whether *B. pumilus* B12 *aprE* expression is subject to similar regulatory mechanisms as other *Bacillus* species because limited work has been done to describe *aprE* regulation outside of *B. subtilis* (83) and there is little homology between the promoter nucleotide sequence of the two species.

aprE expression occurs natively during what is known as 'transition state', the period after vegetative growth and before sporulation (85, 90). In *Bacillus* species, *aprE* is known to be directly regulated by the transcription factors AbrB, ScoC, SinR, DegU, CodY and Spo0A(83–88). Of these, only DegU and potentially Spo0A are activators, while the rest are repressors. These transcription factors are also subject to regulation, and some of them create feedback loops with each other. This multilevel regulation ensures that these relatively expensive enzymes are produced under the appropriate conditions, yet this poses a hurdle for high protease expression using unmodified organisms. Thus, it is useful to describe the regulation for plastic degrading extracellular proteases, like *aprE* in *B. pumilus* B12, for future optimization. In *B. subtilis*, protease production was optimized by uncoupling the timing of *aprE* expression from transition

phase and enabling high *aprE* expression during exponential growth by generating a triple knockout mutant in *B. subtilis* of the repressors *codY*, *scoC*, and *abrB* (86). This mutant can therefore produce subtilisin under conditions amenable to industrial applications. However, for environmental applications, it is useful to explore the regulation of *aprE* in response to extracellular stimuli in more nutrient limited conditions because of the challenges associated with releasing genetically modified organisms into the environment.

Previous work by our lab has shown that *Bacillus pumilus* B12-mediated PLA degradation can be modulated by nutrient addition(111). Specifically, we observed that adenine causes a significant decrease in PLA degradation, but it was not observed if this is due to regulation of *aprE*. Nucleosides and nucleotide triphosphates (NTP's) are known to be involved in some stationary phase processes and determining cell fate in other *Bacillus spp.*, but a direct connection to *aprE* regulation has not been described. For example, inosine and to a lesser extent adenosine, guanosine, uridine and guanidine have been observed to induce sporulation in *B. subtilis* (105). Additionally, *aprE* transcription factors respond to changes in NTP concentrations. Reduced GTP results in decreased repression of genes by CodY(107), which enables CodY repressed genes, like *aprE* to become activated in nutrient limited conditions. Further, GTP homeostasis and CodY activity are regulated by (p)ppGpp levels (109, 110). Through the use of transcription fluorescence reporters, we observed sections of the upstream regulatory region that impact *aprE* expression and gain evidence for SinR and to a lesser extent CodY and

Spo0A regulation under nutrient limited growth conditions. Additionally, we began to explore the mechanism of adenine and inosine- based control of *aprE*.

Results

B. pumilus and B. subtilis PaprE Share Limited Homology

To begin understanding the cis-regulation of *aprE* in *B. pumilus*, we compared the upstream region to the well characterized organism *B. subtilis* to look for DNA homology that would imply similar regulation. We observe approximately 28% similarity between *B. pumilus* B12 and *B. subtilis* PY79(150), when comparing the ~600 bp upstream of the online promoter predicting tool bprom (151) predicted -10 site of the *aprE* promoter. Despite this dissimilarity, we observe DNA sequences with similarity to published consensus sequences for transcription factor binding sites (Table 3-1), specifically CodY, ScoC, SinR and Spo0A.

Promoter length impacts AprE expression

In order to define the minimal promoter length and gain an understanding of how far upstream of the start site that regulation is controlled, we utilized a "promoter-bashing" approach. We utilized plasmid- based methods because we have not yet developed genetic tools to disrupt the genome of *B. pumilus* B12, but have an effective conjugation system (61). We first generated a series of upstream regulatory region (URR) fusions with a GFP reporter gene that progressively decreased the URR length by approximately -100 to -600 base pairs upstream of the predicted -10. With these reporters, we observe a few clusters of altered GFP expression levels (Figure 3-1A). The

lowest expression is observed at -100, while the highest (and most variable) is the -500 bp construct.

A narrower series ranging from differences of 5 to 50 nucleotides allowed us to more finely map this upstream regulatory region and correlate it with the predicted transcription factor binding sites. Between -500 and -600 bases, the -530 bp construct has significantly reduced expression, the -500 bp exhibits high expression, and -550, -580 and -600 are all lower than the -500 bp construct and statistically similar to one another (Figure 3-1C). This suggests an operator between 550 and 530 bp upstream and influence the other regions, but there are activator sites on either side of this repressor. From -100 to -480 bp upstream, the -150 and -200 bp constructs fluoresce significantly higher than -100 bp and significantly lower than constructs between -250 and- 600 bp (Figure 3-1D). The -250, -300 and -440 bp constructs also fluoresce significantly less than the -340, -400, -480 and -600. This again points to interactions of both repressors and activators.

We observe minimal GFP expression with the -35 bp promoter, yet is somewhat above the media only background. Increased GFP expression is observed from -45 bp upstream, and reduced expression is observed with the -75bp construct. The -45, -50 and -55 bp UPP seem to maintain expression at a constant level, in contrast to most of the other constructs, and their expression is comparable to the 100 bp construct (Figure 3-1E).

Table 3-1: Predicted Transcription factor binding sites based on homology

Transcription Factor	**Published Consensus Sequence**	**Sequence in B12**	**Location in B12 *aprE* Upstream Regulatory Region**	**Mutated in this work**
CodY	AATTTTCWGAAAATT(96)	attattctgaaataa	0- -15	Yes
ScoC	RATANTATY (95)	aataatatt	-307-316	Yes
ScoC	RATANTATY (95)	Aataat (RATANT)	-46-52	No
SinR	GTTCTCT (152) GTTCTCA (88) GNCNCGAAATACA(153)	gttcttt	-338-345	Yes
Spo0A	TGNCGAA (83)	tgacgaa	-491-498	No

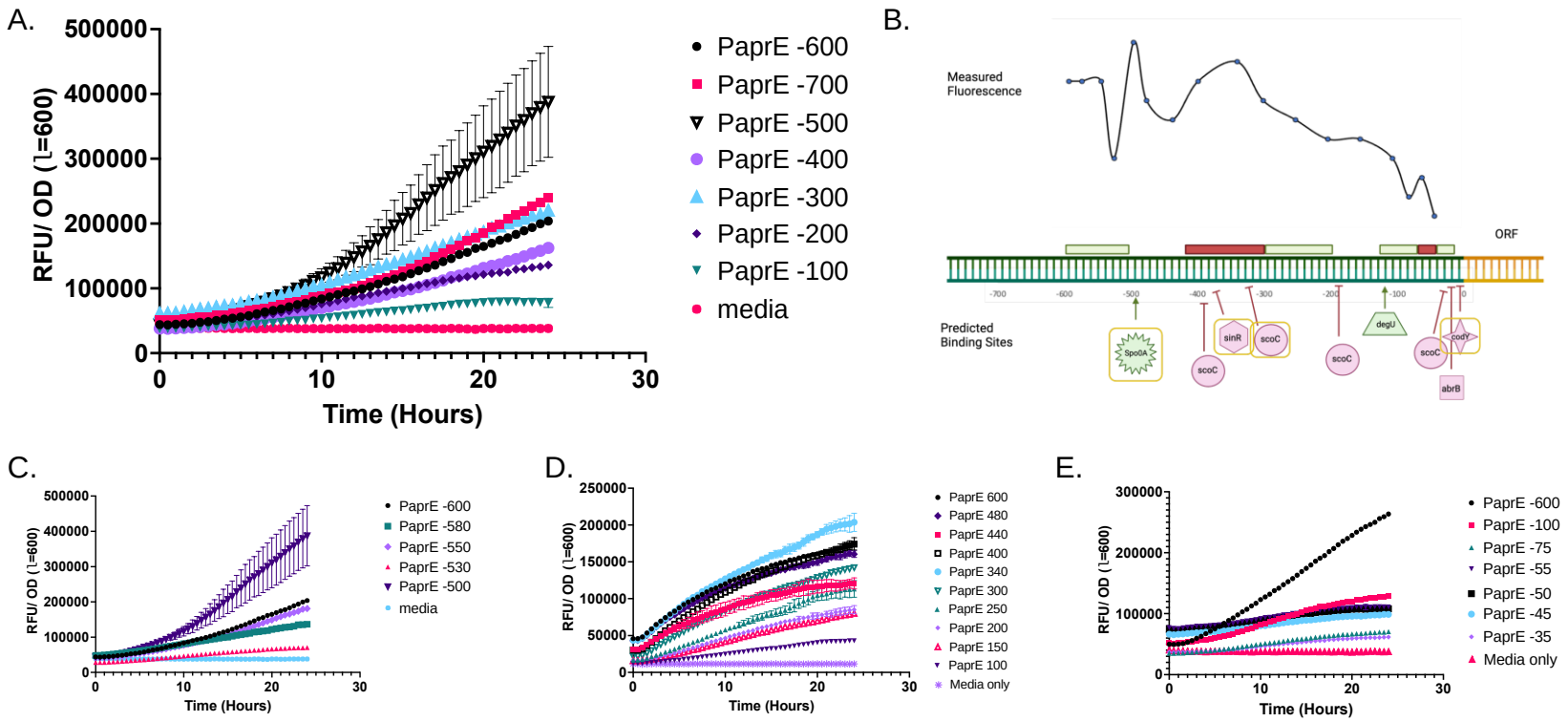

Figure 3-1: Different promoter constructs cause variable fluorescence. A. Normalized fluorescence of 100 base pair truncations of the *aprE* promoter. B. Diagram of relative fluorescence levels over the length of the promoter. C. Normalized fluorescence of promoters less than 100 bases upstream. D. Normalized fluorescence of promoters between 500 and 600 bases upstream.

Mutating transcription factor binding sites has variable impact on expression

We then mutated predicted transcription factor binding sites to correlate the expression trends we observed through promoter bashing to specific transcription factors. We first mutated parts of the predicted CodY operator. CodY is a very pleiotropic regulator which has hundreds of genes attributed to its regulon, including *aprE*(154) and its activity is regulated by nucleotide triphosphate concentrations(107). A 15 nucleotide 'consensus' CodY- DNA binding site has been described in *Lactococcus.* A variation on this consensus sequence seems to be utilized in *Bacillus subtilis* as many genes that are CodY regulated have a DNA sequence that matches 10-13 out of 15 bp motif. In *B. subtilis* a CodY binding site has been described with 5 mismatches (bases not matching the consensus) (96) and we observe 6 mismatches in *B. pumilus*, but 8 of the matches are in the center. This CodY binding site overlaps with the putative -10 site. We used site directed mutagenesis to change the nucleotides that were highly impactful on gene expression for *B. subtilis* CodY regulated genes. We mutated the bases to cytosines because in *B. subtilis* cytosines were never present in those sites. To avoid mutating the RNA polymerase binding site, only the two highly conserved bases (GA) directly downstream of the -10 site in the CodY binding site were mutated (Figure 3-2A &B). Mutating G to C had a limited effect on expression, while the A to C and the double GA to CC decreased expression and resulted in significantly decreased expression (Figure 3-2B). All mutants were still subject to inosine repression.

Another strong repressor of *aprE* is ScoC, which can compensate for mutated CodY in some contexts(86).We mutated a predicted ScoC binding site located at -307 to -

316. This site has a more complete version of the consensus sequence than the downstream putative ScoC binding site. Altering the ScoC binding site from the consensus aataatatt (ratantaty) to the non-complementary bases ccgccgcgg had no significant impact on GFP expression compared to the -300 and -600 bp constructs, but was significantly lower than the -340 bp construct (Figure 3-2 C& D), indicating a limited effect. Expression of GFP with this mutant was further reduced by the addition of inosine.

In contrast, mutating the SinR binding site located between -338 and -345 bp upstream increased GFP expression relative to the -390 and -600 base pair URR fusions, to levels similar to that of the -340 base pair construct (Figure 3-2 E&F), indicating that SinR plays a more important or at least non-redundant role.

Nucleosides repress aprE

We observe reduced *aprE* expression with the addition of adenine, adenosine and inosine (Figure 3-3A). Adenosine and inosine are statistically significantly different (p-value ≤ 0.05) from the control cells grown in mannitol alone from 3.5 hours through 22.5 and 24 hours respectively, with inosine causing a more pronounced repression. The addition of adenine causes a modest difference in expression and is statistically significantly different (p-value ≤ 0.1) between 8.5-19 hours. Inosine causes repression of GFP expression with concentrations ranging from 0.05 mg/ mL to 0.2 mg/ mL (Figure 3-3 B) and it was confirmed that *aprE* transcription is statistically significantly reduced after 6 hours with the addition of 0.1 mg/mL using RT-qPCR (Figure 3-3 C). We observe that with each of our upstream regulatory region-GFP constructs except for the 55 bp

construct, there is a significant reduction in expression when we add 0.1mg/mL inosine (Figure 3-4 A-H).

Since there was not an obvious difference in inosine- controlled regulation for the majority of sites within the *aprE* upstream regulatory region, one possibility is that the decrease in transcription is more universal. Thus, the transcriptional down regulation observed with inosine addition might not be restricted to *B. pumilus* or regulation of *aprE*. When the *aprE* promoter from *B. subtilis* PY79 is fused to GFP and expressed in *B. pumilus* B12 we observe high expression, but it is significantly repressed by the addition of inosine (Figure 3-5A). However, when this reporter is expressed in *B. subtilis* PY79 we observe almost no fluorescence, which is partly attributable to worse growth of *B. subtilis* PY79 in these conditions, but based on the magnitude of fluorescence reduction there is likely an additional factor. Under these growth conditions, inosine also represses *B. pumilus spoIIGA* expression (Figure 3-5B).

The reason for this more universal regulation is not clear, but one potential explanation is that intracellular nucleotide based signaling and energy molecules are impacted by supplementation with nucleotide derivatives. Using mass spectrometry, a slight increase in ppGpp levels is present in cells incubated with inosine compared to those incubated with mannitol alone or with adenosine (Figure 3-5 C), but no significant differences were observed for ATP, GTP, c-di-AMP, or c-di-GMP (data not shown). (p)ppGpp is involved in the stringent response as well as GTP homeostasis and purine synthesis in Bacillus.

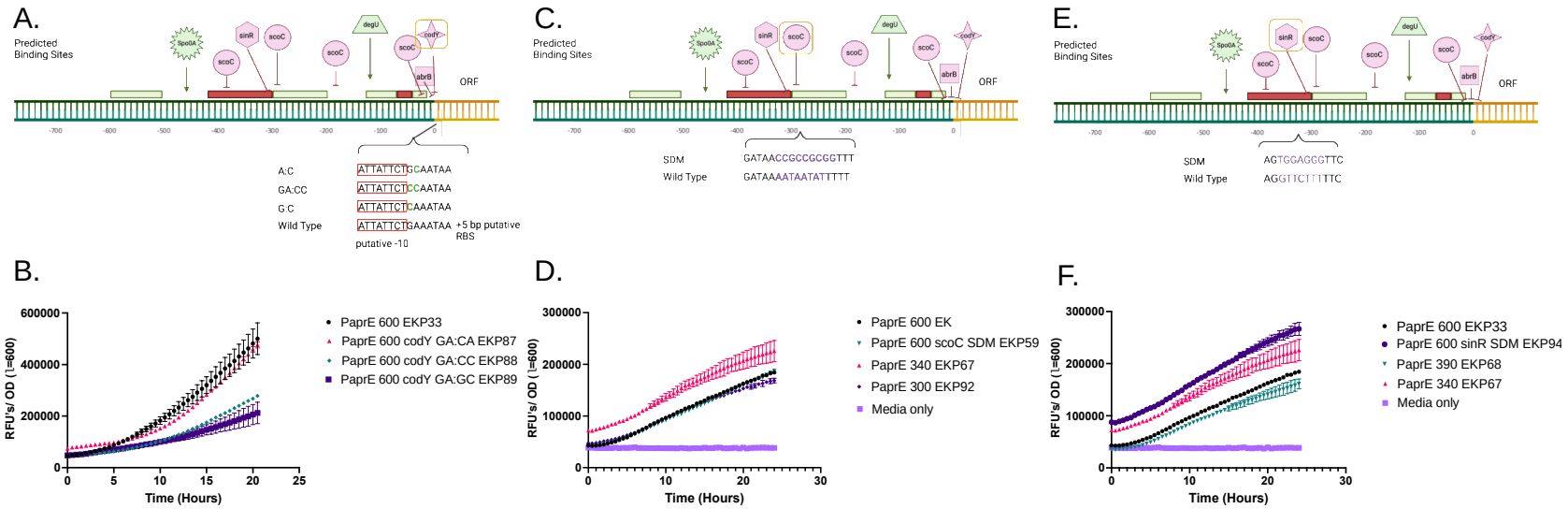

Figure 3-2: Mutated transcription factor binding sites alters expression. A, B. Mutated CodY binding site. Predicted -10 promoter sequence boxed in red. Mutated bases marked in green. C, D. Mutated ScoC binding site. Mutated bases marked in purple. E,F. Mutated SinR binding site. Mutated bases marked in purple. A,C, E. Map of the upstream regulatory region and mutated bases. B, D, F. Relative fluorescence normalized to cell density of upstream regulatory region GFP fusions.

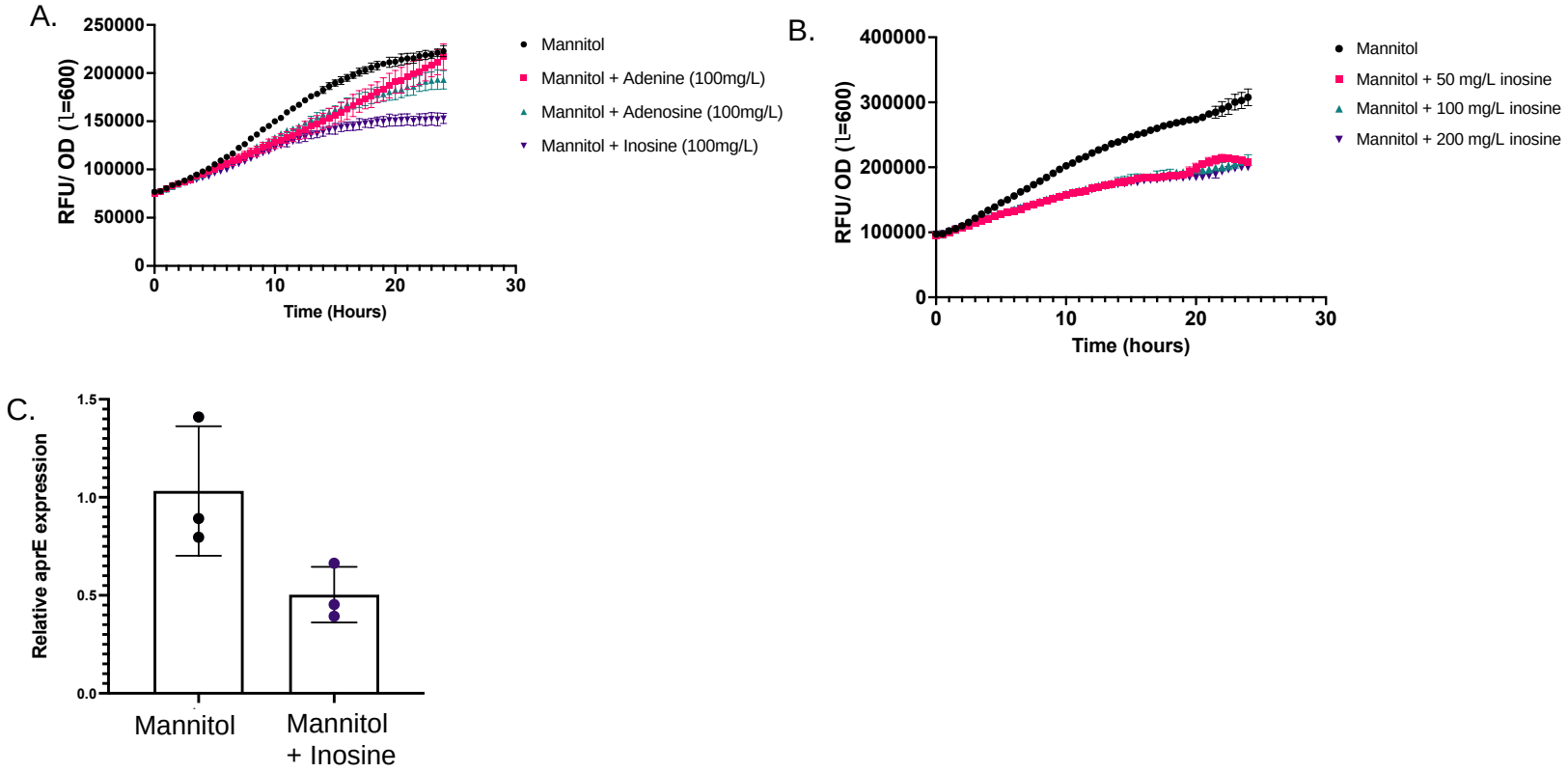

Figure 3-3: Purines repress *aprE* expression, but inosine causes the most repression. A. Relative fluorescence normalized to cell density (OD_{600}) with adenine, adenosine and inosine. B. Relative fluorescence normalized to cell density (OD_{600}) with inosine concentration gradient. C. qRT-PCR *aprE* expression at 6 hours. Normalized to *ftsZ* using Livak's method(155).

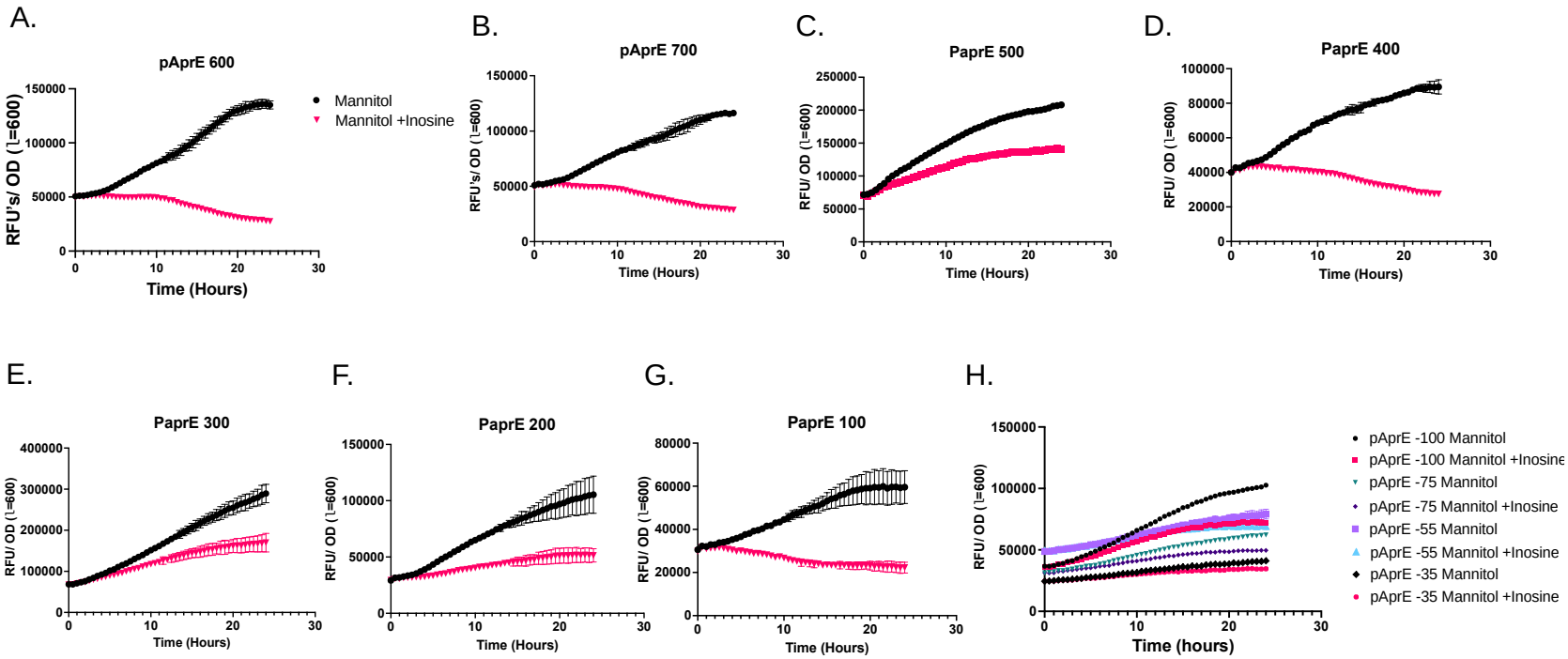

Figure 3-4: Inosine reduces *aprE* expression for all promoter fragments. A. 600 bp promoter. B. 700 bp promoter. C. 500 bp promoter. D. 400 bp promoter. E. 300 bp promoter. F. 200 bp promoter. G. 100 bp promoter. H. Promoters less than 100 base pairs.

Discussion

Despite limited overall DNA homology between the upstream regions of *aprE* in *B. pumilus* B12 and *B. subtilis*, conserved transcription factor binding sites confer similar overall regulation. This idea is corroborated by Zhou *et. al* who describe *aprE* regulation in *B. licheniformis*(83). Using a plasmid-mediated GFP expression reporter we measured expression of *aprE* with varying promoter lengths. Despite overall sequence dissimilarity between the *aprE* upstream regions of *B. subtilis* and *B. pumilus*, similarities in the regulation of *aprE* in the two species are apparent except in a few cases. These differences present opportunities for exploration, especially for increased expression. Between -200 bp upstream and -340 bp upstream we observe reduction in expression indicative of two activator binding sites, but based on *B. subtilis* there are no predicted activators in this region. This suggests a unique or yet to be described *aprE* activator.

We also observed changes in expression in our mutated CodY binding site expression vector, and individual mutations had differing effects. Mutating the A to a C significantly decreased expression but mutating the G to C did not have a significant impact. The double GA to CC mutation was statistically similar to the A to C mutation, so was likely driven by that single mutation. This suggests that the guanine may not be as important to CodY binding in *B. pumilus* as in *B. subtilis*. Interestingly, our mutation causes reduced rather than increased fluorescence, suggesting that we may have improved CodY binding through this mutation, which is unexpected based on work done in *B. subtilis*. Due to the location of this binding site however, we cannot rule out that the mutation may also impact RNA polymerase binding instead of CodY binding

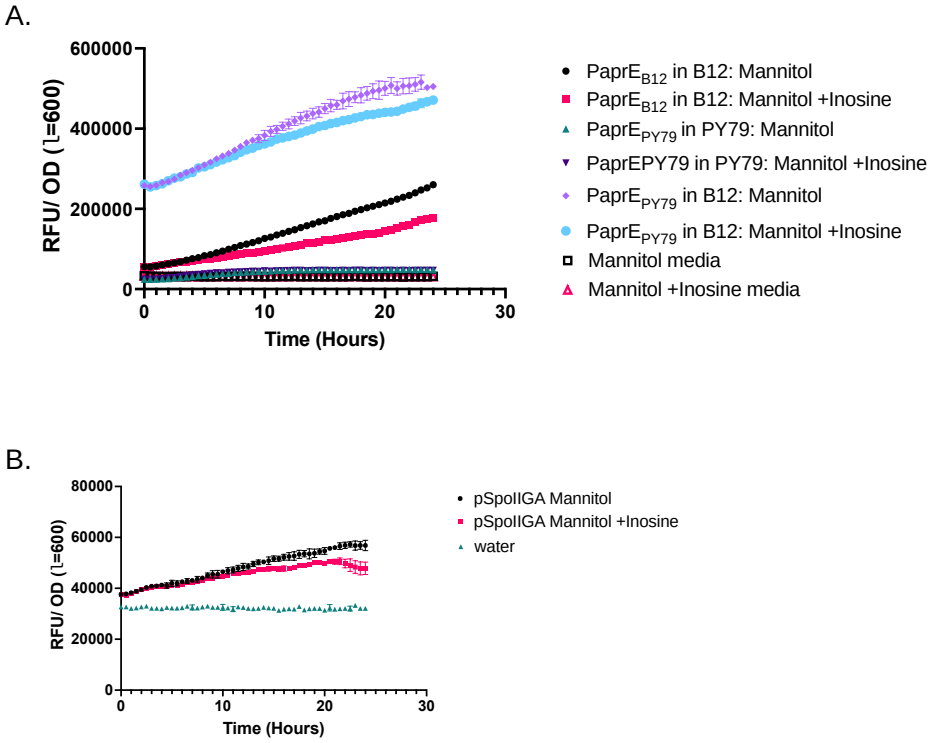

Figure 3-5: Inosine impacts additional targets. A. Relative fluorescence of PaprE from *B. subtilis* PY79 expressed in *B. pumilus* and *B. subtilis* show different levels of expression. PaprE^{PY79} expressed in *B. pumilus* is highly fluorescent and repressed by inosine. B. Relative fluorescence of *B. pumilus* B12 expressing GFP from PspoIIGA. PspoIIGA is repressed by inosine. C. The amount of ppGpp per milligram protein is higher when cells are incubated with inosine supplementation

The mutations in the predicted SinR binding site suggest that *aprE* regulation by SinR is similar in *B. pumilus* and *B. subtilis*. Mutating the ScoC binding site between -340 and -300 bp did not significantly impact expression. While it may be expected that mutating this binding site would increase expression, we observe that its expression is similar to the -300 base pair fusion, so has limited effect. However, it has been described that ScoC deletion has a limited effect on *aprE* expression when CodY is activated(86), so our growth conditions may not be optimal to measure the influence of ScoC on *aprE*. ScoC is however predicted to have multiple binding sites in *Bacillus subtilis*, and perhaps these different sites interact or regulate expression under different conditions. We observe one weak potential ScoC binding site around -46 to -52 bp that has 6/9 matches with the consensus sequence. Developing genetic tools to knockout genes in B12 would enable more confirmation of the influence these transcription factors have on *aprE* regulation.

The reduction in PLA degradation with the addition of adenine observed in Bonifer *et. al* 2019 is likely driven through repression of *aprE* by adenine. We observed the strongest repression of *aprE* when incubated with inosine compared to adenine and adenosine, which seemed to slow the rate of *aprE* expression but by 24 hours GFP expression in minimal media supplemented with mannitol and adenine or adenosine were no different from the mannitol control. This effect could be due to the fact that inosine more directly feeds into purine salvage, while adenine and adenosine require additional steps to be converted to hypoxanthine(156). The stronger effect of inosine in regulating this stationary phase process may also be reflected in the relative efficacy of inosine compared to adenosine as a sporulation inducer, where inosine is 35% more effective

(105). We hypothesize that inosine represses a pleiotropic regulator, such as CodY or Spo0A, and this is why we observe repression for all of our promoter fusions. Inosine repression may also be multifactorial, through reduced transcription rates and transcription factor repression.

Conclusions

Using plasmid-based GFP transcriptional fusions, we have begun to describe the regulation of *aprE* in *B. pumilus* B12. We saw trends consistent with *B. subtilis* transcription factor bindings sites and observed more flexibility in terms of recognition sequences than those reported for ScoC binding. Transcriptomics and untargeted metabolomics may help gain a clearer understanding of the transformations occurring when cells are supplemented with inosine.

Materials and Methods

Bacterial Culturing

For most assays, cells were pre-grown in LB, back diluted in LB to 0.01 OD, and grown for 16 hours. Cells were washed with phosphate buffered saline (PBS), and then inoculated in minimal salts medium (MSM) at 0.2 OD [per liter 1 g $(NH_4)_2SO_4$, 1.5 g KH_2PO_4, 0.5 g K_2HPO_4, 0.2 g $MgSO_4*7H_2O$, 1 g NaCl] supplemented with 1% (wt/vol) mannitol. Adenine, adenosine and inosine were supplemented to 0.1 mg/ mL unless otherwise noted and the media was autoclaved. For cloning, NEB DH5α *E. coli* were

grown in LB ampicillin (100 μg/mL). For conjugation, strains derived from EZ180 were grown in LB ampicillin (100 μg/mL) plus diaminopimelic acid (0.3 mM).

Plasmid Construction and Conjugation

Plasmid construction was performed using NEB DH5α *E. coli*. For promoter inserts greater than 100 base pairs, fragments were cloned into pEP011 using the *AatII* and *XbaI* sites Plasmids were later transformed into chemically competent *E. coli* EZ180 for conjugation. Plasmids and descriptions are found in Table 3-2. Primers used for plasmid construction and verification are found in Table 3-3.

Inserts less than 100 bp

Fragments were amplified using primers listed in Table 2. The thermocycling conditions were as follows: 37°C for 30 minutes, 65°C for 20 minutes, 90°C for 5 minutes, 85°C for 1 minute, 80°C for 1 minute, 75°C for 1 minute, 70°C for 1 minute, 65°C for 1 minute, 60°C for 30 seconds, 55°C for 30 seconds, 50°C for 30 seconds, 45°C for 30 seconds, 40°C for 30 seconds, 35°C for 30 seconds, 30°C for 30 seconds, 25°C for 30 seconds, 20°C for 3 minutes. Successful amplification was determined by gel electrophoresis. Plasmid pEP011 was digested with *AatII* and *XbaI*, and gel purified. 1 ul of each fragment was ligated into pEP011 and transformed into DH5α competent *E. coli*. Single colonies were screened by PCR using primers EPO92 and EPO93. Correct plasmids were then isolated and transformed into EZ180 *E. coli* for conjugation.

Site Directed Mutagenesis

Nucleotides of putative binding sites were mutated using site directed mutagenesis. For CodY, the two highly conserved bases that didn't overlap with the predicted ribosomal -10 were mutated to cytosine which was never found in those positions in a *B. subtilis* CodY binding site survey(96). LAtaq (Takara) was used for amplification and ligation, then reactions were treated with *DpnI* overnight. These reactions were then transformed into NEB DH5α *E. coli* and screened for proper sequences. Plasmid sequencing was performed by Plasmidsaurus.

Plasmids were conjugated into *Bacillus pumilus B12* as described in Phillips 2023(61). Briefly, *E. coli* EZ180 carrying the plasmid of interest was grown to early stationary phase in LB*Amp*DAP and *B. pumilus* B12 was grown to mid-log phase in LB. Cells were combined in equal volumes and spotted onto LB*DAP plates and grown overnight. Cells were then grown for 24 hours in LB erythromycin/lincomycin and plated.

Fluorescence Measurements

Fluorescence was measured every thirty minutes for twenty-four hours in a BioTec Cytation 5 plate reader using λ= 483nm excitation, 513nm emission followed by an absorbance reading at λ=600 nm, without shaking at 30 °C. Relative expression was calculated by dividing fluorescence by optical density. A two- way ANOVA with multiple comparisons was run to compare strains and growth conditions.

Mass Spectrometry Metabolomics

Cells were inoculated into LB from starter cultures at 0.01 OD and incubated for 16 hours. Cells were washed 3 times and then inoculated into 100 mL media at 0.2 OD. Cells were filtered through 0.45 μm cellulose acetate filters (Sartorius), and then washed with PBS 2 times. Cell pellets were frozen at -80 °C. Cell pellets (4 replicates of each condition) were shipped on dry ice to Michigan State University Mass Spectrometry and Metabolomics Core. Nucleotides were extracted using acetonitrile, methanol and formic acid with fluorinated cyclic di- GMP (100nM) as an internal standard. Samples were then normalized to protein concentrations after resolubilizing in 2% SDS and performing a Bradford assay.

RNA extraction and RT-qPCR

Cells were grown statically for six hours at 30 °C in MSM 1% mannitol with inosine supplementation. Cells were vacuum filtered and then flash frozen at -80 °C. RNA was extracted using the hot-phenol chloroform method (157). DNA was removed using TURBO DNase (Invitrogen) following the manufacturer's "rigorous protocol". RNA was reverse transcribed using the High-Capacity cDNA Reverse Transcription Kit according to the manufacturer's protocol (AppliedBiosystems). qPCR was performed using PowerUp SYBR Green Master mix using primers qEPO61,62(61) and qEPO69, 70. *aprE* expression was normalized to *ftsZ* using Livak's method(155)

Acknowledgements

We are grateful to Ben Astles for sharing the primer annealing protocol for our short promoter fragments and Dr. Elizabeth Fozo for identifying ways to improve RNA extraction. Michigan state Mass Spectrometry and Metabolomics core is acknowledged, specifically Anthony Schilmiller, for performing the mass spectrometry.

Appendix

Table 3-2: Plasmids

Plasmid	Characteristics	Source and Reference
pEP011	Conjugation Backbone	Phillips *et. al* 2023(61)
pEP017	PaprE 600	This Work
pEP019	PaprE 700	This Work
pEP022	PaprE 500	This Work
pEP023	PaprE 430	This Work
pEP024	PaprE 250	Phillips *et. al* 2023(61)
pEP025	PaprE 100	This Work
pEP027	PaprE 600 scoC binding site mutated	This Work
pEP028	PaprE 600 sinR binding site mutated	This Work
pEP029	PaprE 200	This Work
pEP030	PaprE 340	This Work
pEP031	PaprE 390	This Work
pEP033	PaprE 150	This Work
pEP034	Bacillus subtilis PY79 PaprE 600	This work
pEP037	PaprE 75	This Work
pEP038	PaprE 55	This Work
pEP039	codY binding site G→ C	This Work
pEP040	codY binding site GA→ CC	This Work
pEP041	codY binding site A→ C	This Work
pEP042	PaprE 35	This Work
pEP043	PaprE 300	This Work
pEP044	pAprE 600 spo0A binding site mutated	This Work
pEP045	PaprE 480* GFP fusion (LWO4 primer)	This Work

Plasmid	Characteristics	Source and Reference
pEP046	PaprE 45 bp* GFP fusion	This Work

Table 3-2 Continued

pEP047	PaprE 50 bp* GFP fusion	This Work
pEP048	PaprE 500 *GFP fusion	This Work
pEP049	PaprE 550 *GFP fusion	This Work
pAS01	PrelA *GFP fusion	This Work

Table 3-3: Primers. Bold purple denotes restriction enzyme recognition sites, italicized red denotes bases mutated with SDM

Primer name	Element, Cutsite/ Mutation, Direction	Sequence 5'-3'
qEPO69	qPCR *aprE* Forward	CAGCGGTATTGAATGGGC
qEPO70	qPCR *aprE* Reverse	CGGTTATTCGCTGTGTCTACAG
EPO92	Screening Forward	GTCTCATGAGCGGATACATAT
EPO93	Screening Reverse	CCATATGTTGCATCACCTT
EPO107	-700 bp, aatII, Forward	AAAA**GACGTC**GAACGTGACTATGGCGACG

Table 3-3 Continued

EPO109	-600 bp, aatII, Forward	AAAA**GACGTC**CACTTCAAGATCGAATGCT
EPO110	xbaI, Reverse	AAAA**TCTAGA**CTTATTTCAGAATAATCATCCG
EPO112	-530 bp, aatII Forward	AAAA**GACGTC**CTGCCATCCATGCCTTACT
EPO113	-440 bp, aatII Forward	AAAA**GACGTC**GGACAGATGGTCAATGGAAG
EPO114	-250 bp, aatII Forward	AAAA**GACGTC**GCATTTCGGGTATCGAATG
EPO115	-100 bp, aatII Forward	AAAA**GACGTC**CAGGTCTACTCTTATTTGCCTATC
EPO127	-200 bp aatII Forward	AAAA**GACGTC**GCACGATTCCTTTTTAATAGAA
EPO128	-400 bp aatII Forward	AAAA**GACGTC**CACAATCTGCTCCTTCTTAAAG

EPO129	-340 bp aatII Forward	AAAA**GACGTC**CATGTCAAAAACAATGATAAA

Table 3-3 Continued

EPO169	-75bp Forward	**C**CTCTATTAAACTGAAAATACAGAATAATCAAACGGATCATTCTAA TAGACTACGGATGATTATTCTGAAATAAG**T**
EPO170	-75bp Reverse	**CTAGA**CTTATTTCAGAATAATCATCCGTAGTCTATTAGAATGATCC GTTTGATTATTCTGTATTTTCAGTTTAATAGAG**GACGT**
EPO171	-55bp Forward	**C**CAGAATAATCAAACGGATCATTCTAATAGACTACGGATGATTATT CTGAAATAAG**T**
EPO172	-55bp Reverse	**CTAGA**CTTATTTCAGAATAATCATCCGTAGTCTATTAGAATGATCC GTTTGATTATTCTG**GACGT**
EPO173	-35bp Forward	**C**CATTCTAATAGACTACGGATGATTATTCTGAAATAAGT
EPO174	-35bp Reverse	**CTAGA**CTTATTTCAGAATAATCATCCGTAGTCTATTAGAATG**GACG** **T**
EPO175	SDM CodY G--> C Forward	GATGATTATTCT*CAA*ATAAGTCTAGATAAGGAGGTC
EPO176	SDM CodY G--> C Reverse	ATCTAGACTTAT*TTG*AGAATAATCATCCGTAGTCTA
EPO177	SDM CodY	GATGATTATTCT*CCA*ATAAGTCTAGATAAGGAGGTC

	GA--> CC Forward	

Table 3-3 Continued

EPO178	SDM CodY GA--> CC Reverse	ATCTAGACTTAT*TGG*AGAATAATCATCCGTAGTCTA
EPO179	SDM CodY A--> C Forward	GATGATTATTCTG*GCA*TAAGTCTAGATAAGGAGGTC
EPO180	SDM CodY A--> C Reverse	ATCTAGACTTAT*TGC*AGAATAATCATCCGTAGTCTA
EPO193	SDM spo0A Forward	CACCTCACTAGC*ACACACA*CATATGGGTATTAAGGATACA
EPO194	SDM spo0A Reverse	AATACCCATATG*TGTGTGT*GCTAGTGAGGTGAGTAAGGCA
EPO195	-50 bp Forward	**C**TAATCAAACGGATCATTCTAATAGACTACGGATGATTATTCTGAA ATAAG**T**
EPO196	-50 bp Reverse	**CTAGA**CTTATTTCAGAATAATCATCCGTAGTCTATTAGAATGATCC GTTTGATTA**GACGT**

EPO197	-45 bp Forward	**C**AAACGGATCATTCTAATAGACTACGGATGATTATTCTGAAATAAG **T**

Table 3-3 Continued

EPO198	-45 bp Reverse	**CTAGA**CTTATTTCAGAATAATCATCCGTAGTCTATTAGAATGATCC GTTT**GACGT**
EPO199	-500 bp, aatII Forward	AAAA**GACGTC**CCTCACTAGCTGACGAAC
EPO201	-550 bp, aatII Forward	AAAA**GACGTC**CTAATCGTAGCACATGGTG
LWO1	-580 bp aatII Forward	AAAA**GACGTC**GAAGGTATCGAAAAAGTGAG
LWO5	-460 bp aatII Forward	AAAA**GACGTC**GAATGCCTGCTTAAATTATGTG
YZO107	SDM scoC Forward	CCTGTTTTCATAACAGGTTCTTTTTCATGTCAAAAACAATGATAA*CC* *GCCGCGG*TTTTTATATCGAAATTCGAAATAGATGATA
YZO108	SDM scoC Reverse	ATAGGTAGAAAAGGCTATCATCTATTTCGAATTTCGATATAAAAA*C* *CGCCGCGG*TTATCATTGTTTTTGACATGAAAAAGAACC
YZO 127	SDM sinR Forward	CTTAACACAATCTGCTCCTTCTTAAAGAACCTGTTTTCATAACAG *TGGAGGG* GTTCATGTCAAAAACAATGATAAAATAATAT

YZO128	SDM sinR Reverse	TTTCGATATAAAAAAATATTATTTTATCATTGTTTTTGACATGAA *CCCTCCA* CTGTTATGAAAACAGGTTCTTTAAGAAGGA

CHAPTER 4 CONCLUSIONS AND FUTURE DIRECTIONS

Development of Genetic Tools for Non-Model *Bacillus*

Through this dissertation, we have developed a genetic tool to introduce DNA into environmental isolates of *Bacillus* and utilized this tool to describe the cis-acting transcriptional regulation of a polylactic acid degrading protein, AprE. While developing our conjugation methodology (Chapter 2), we observed effective conjugation in nine of the twelve strains attempted. The *Bacillus* strains that did not successfully conjugate were not phylogenetically distinct based on 16S rRNA gene classification, as some strains that did not conjugate successfully were the same species as those that did. Thus, there are likely other genetic determinants dictating conjugation efficacy. Initial steps towards including these strains would be performing a minimum inhibitory concentration (MIC) assay with the antibiotics used as counterselection to determine whether our concentrations were appropriate. Genomic analysis to compare the genomes of the twelve strains may also elucidate any genes, such as restriction modification systems, that are unique to the three unsuccessful strains. This analysis may, however be strengthened by more strains, so acquiring more strains throughout the genus and testing the conjugation conditions with them may be beneficial.

Our current toolbox is limited to transcriptional and translational fusions due to the architecture of the vector backbone. Further development of rapid genetic tools in *B. pumilus* B12 will enable elucidation of unique mechanisms involved in AprE regulation, as well as other phenotypes of interest. For example, by developing and utilizing a

plasmid-based transposon mutagenesis (158) we may be able to identify activator mutants to improve degradation and identify novel genes involved in AprE regulation. Further, for more targeted mutations, adapting plasmids with CRISPR-Cas (159) or making integrative plasmids, we would be able generate knockouts and other mutants. Many researchers in *B. subtilis* utilize the amylase (*amyE*) locus to integrate ectopic plasmids, but this gene is not present in all *Bacillus* species (including *B. pumilus* B12), so it would also be valuable to identify a more widely distributed non- essential gene to use for other strains, or for those interested in studying amylase production.

Regulation of *B. pumilus* AprE under PLA degrading conditions

This body of work uniquely studies the transcriptional regulation of bioplastic degrading proteins *in vitro* and primarily focused on repressors of PLA degradation. Our *in vitro* approach is appealing because it reduces the unknown variables associated with biostimulation studies that use mesocosms or *in situ* conditions. I*n vitro* studies constrain the number of unknown variables and offer a better opportunity to study multiple variables or combinations of variables than typically available while doing field work. Additionally, focusing on individual strains may help explain some of the variation observed in environmental studies. For example, while some studies observe an increase in plastic mulch degradation by adding nitrogen (organic and inorganic), others observe reductions in degradation(160–162). However, follow up work will need to be done to see whether findings from this type of study translate into more complex systems. For example, multiple strains could then be explored together in various combinations to

improve degradation and more adequately mirror complex communities found in nature, or small- scale soil experiments.

Future work should focus on identifying factors that improve AprE expression, including transcription factors and media supplementation. Performing a protein pulldown by crosslinking with the promoter regions between -340 and -250 may help identify activating transcription factors, as we observed reductions in GFP expression in these regions, consistent with the removal of activator binding sites. This will not only provide targets for improved PLA degradation, but also provide fundamental information as only two direct AprE activators have been identified in *Bacillus* so far. It would also be valuable to determine whether Spo0A directly induces AprE in *B. pumilus* B12 as this has been shown in *B. licheniformis,* but not *B. subtilis.* We observe a putative Spo0A binding site in the upstream region (-491-498), and do observe a drop in GFP expression when we compare the -500 and -480 bp constructs. This binding site is considerably further upstream than that described in *B. licheniformis* and we only observe one binding site instead of two, which may prevent Spo0A from being able to regulate the gene, as it is thought that the two binding sites allow it to bend the DNA. The high expression observed when *B. pumilus* B12 expressed GFP with the *B. subtilis aprE* promoter has interesting implications for heterologous expression, and could be further disentangled by generating chimeric promoters. It could also be interesting to mine *Bacillus* genomes to find a strain that produces a version of AprE nearly identical to *B. pumilus* B12 that degrades PLA, but has an upstream regulatory region more similar to the highly

expressing *B. subtilis* PY79, so that we have a highly expressing PLA degrading strain without doing any genetic modifications.

Further work towards identifying supplements that improve protease expression will be important for addressing environmental PLA waste. Our lab has previously shown that at certain increased concentrations of potassium and phosphate there is improved PLA degradation, so it would be nice to confirm that this is occurring through AprE expression, like we have done with adenine. While it is likely that this is the case, if AprE is not upregulated, that means there may be additional enzymes involved in PLA degradation by *B. pumilus* B12. If there are two enzymes, it will be interesting to see whether one is more efficient and under what conditions the most degradation occurs. In complex media, other groups have seen that supplementation with yeast extract, peptone, magnesium sulfate and potassium chloride improve AprE production (protease activity) (163, 164). Media modification to upregulate activators is another potential avenue for improving regulation.

To more completely define the mechanism of inosine repression, a few follow up experiments should be performed. While we do not necessarily want to repress PLA degradations in many situations, it is important to fully understand conditions that repress AprE so that they can be avoided or applied in very specific situations. Further, describing conditions of repression will help those trying to respond to situations when degradation is not occurring as expected. The observed increase in ppGpp suggests a potential role for the stringent response (and RelA) in inosine's repression of AprE. Potentially, the stringent response is being activated and the widespread transcriptional

changes that occur during the stringent response causes the repression of AprE. Norvaline (a non-canonical amino acid) has been shown to induce the stringent response in exponentially growing cells, so perhaps incubation with norvaline would cause a similar repression of AprE to that observed when inosine is added. Further, exploring the transcriptional response to inosine over time would help identify the ways *B. pumilus* B12 is reacting to the presence of inosine. Ideally, this would be done through RNA sequencing because it is non-targeted, but smaller targeted experiments using RT-qPCR to look at individual transcription factors, especially CodY and Spo0A would probably be able to provide clues about what is occurring. Studying the impact of inosine on *Bacillus* phenotypes is also interesting from the perspective of fundamental research because a minimal body of work describes how *Bacillus* responds to exogenous inosine in different growth phases. Specifically, one study described the role of inosine in inducing sporulation, but no mechanism has been described and no one has described how inosine impacts transition phase and early to middle stationary phase. Additionally, inosine has been described as a germinant after sporulation and can be utilized in purine salvage. Inosine is clearly a signal that *Bacillus* species respond to and could be more well described.

Concluding thoughts

While identifying new organisms capable of plastic and bioplastic degradation is a crucial first step towards reducing (bio)plastic waste, it is essential that we work towards understanding the conditions that influence the efficiency of degradation. Without a thorough conception of conditions that influence degradation, applications of

(bio)plastic degrading organisms are likely to face inconsistent outcomes that will delay widespread implementation. Thus, this dissertation provides information that may be useful in trying to adopt *B. pumilus* B12 as bioaugmention for PLA degradation.

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APPENDICES

A Comparison of Closely Related *Bacillus* isolates

Publication statement: This is not intended for future publication

Author contributions: Sahar Hasim performed the atomic force microscopy.

Introduction

Within the genus *Bacillus* some members are capable of PLA degradation while others are not. We sought to understand whether *Bacillus* isolates within the same JGI "clique" are capable of PLA degradation.

Materials and Methods

Bacterial Culturing for AFM

Bacillus species were inoculated into minimal salts media 1% glucose at 0.1 OD from overnight cultures. 1 mL of culture was aliquoted into PLA vials (50 mg/mL) and incubated at 30 °C for 48 hours. Culture was then aspirated and plastic films were washed 5X with PBS. The films were then dried under nitrogen for shipment.

Pangenome comparisons

Genomes were compared using Anvi'o (165) as described in the 2020 tutorial. The genomes were downloaded from RAST (*B. pumilus* B12), JGI (*B. sp.* 125, *B. sp.* 27A and *B. sp.* 9509) or NCBI (*B. pumilus* MTCC6033).

Alignment of AprE

The 800 bases upstream of *aprE* through the stop codon were aligned using MegAlignPro using MUSCLE's default parameters.

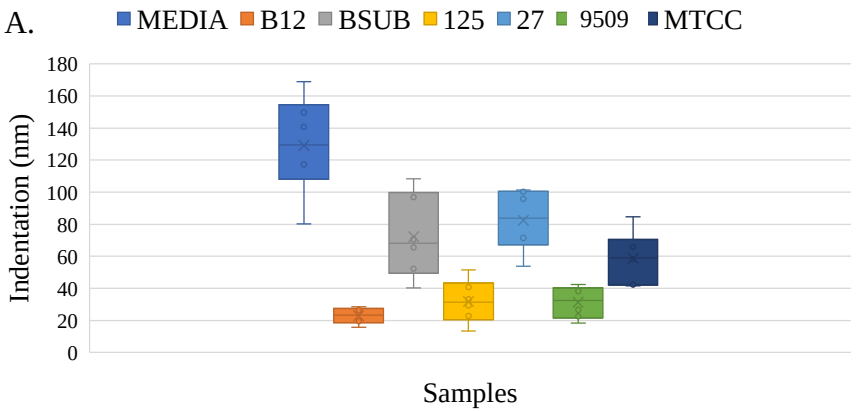
Results and Discussion

In addition to *B. pumilus* B12, *B. sp.* 125 and *B. sp.* 9509 cause an increase in the ability of an AFM probe to indent the PLA surface, which suggests that they degrade PLA under our incubation conditions (Figure 4-1a). *B. sp.* 27A, and *B. pumilus* MTCC6033 do not cause degradation under these conditions.

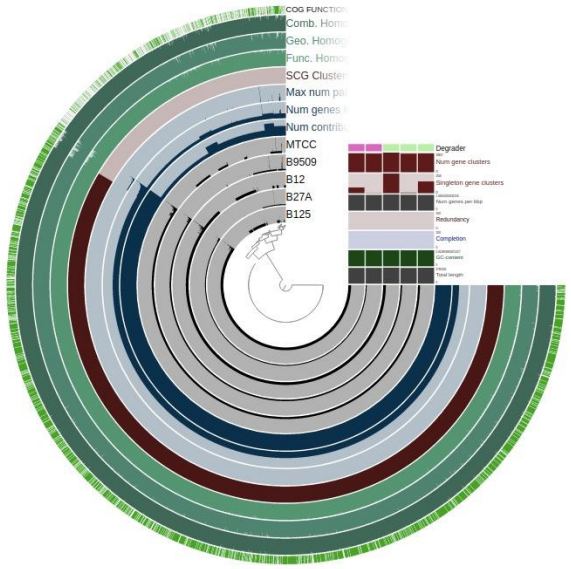
We then compared the genomes of these five strains to identify any genes that may be unique to the PLA degrading strains. By comparing the genomes of *B. pumilus* B12, *B. sp.* 125 and *B. sp.* 9509 (the degraders) to *B. sp.* 27A, and *B. pumilus* MTCC6033 (the non-degraders) we did not observe any genes that all three degraders had that were also absent in the non-degraders (Figure 4-1c). This suggested to us that differences in PLA degradation are due to differences in protein sequence and activity, or regulation.

By aligning the predicted open reading frames of AprE for these five strains, we observe 98% similarity in the DNA sequence (Figure 4-2). The high degree of similarity implies that it is likely that the differences in PLA degradation are occurring at a regulatory level, or the different strains have different abilities to grow in these growth conditions. Similarly, the 800 nucleotides upstream of the predicted open reading frame are 97.6% identical, and none of those mismatches occur in predicted transcription factor binding sites (Figure 4-3). This suggests that differences in PLA degradation are not explainable through sequence analysis of AprE and its promoter region.

A.



B.



C.

COG_FUNCTION	p_yes	p_no
Predicted glycoside hydrolase or deacetylase ChbG, UPF0249 family	0.6667	0
Retron-type reverse transcriptase	0.6667	0
dUTPase	0.6667	0
N-acetylmuramoyl-L-alanine amidase CwlA	0.6667	0
DNA polymerase I - 3'-5' exonuclease and polymerase domains	0.6667	0
Transposase and inactivated derivatives	0.3333	1
NADP-dependent 3-hydroxy acid dehydrogenase YdfG	0.3333	1
Glutaredoxin	0.3333	1
DNA gyrase inhibitor GyrI	0.3333	1
NAD(P)H-dependent FMN reductase	0.3333	1
Transposase InsO and inactivated derivatives	0.3333	1
Thymidylate synthase ThyX	0.6667	0
Predicted restriction endonuclease, Mrr-cat superfamily	1	0.5
Beta-lactamase class A	1	0.5

Figure 4-1: Closely Related *Bacillus* strains have different PLA degradation capabilities. A.B12, 9509 and 125 cause the AFM probe to indent more deeply in the PLA film. B. Pangenome of the 5 strains. C. Enriched genes separated by degraders (yes) and non-degraders (no).

Figure 4-2: ORF Alignment of Closely Related *Bacillus* strains Indicate Minimal Differences in the Protein Sequence

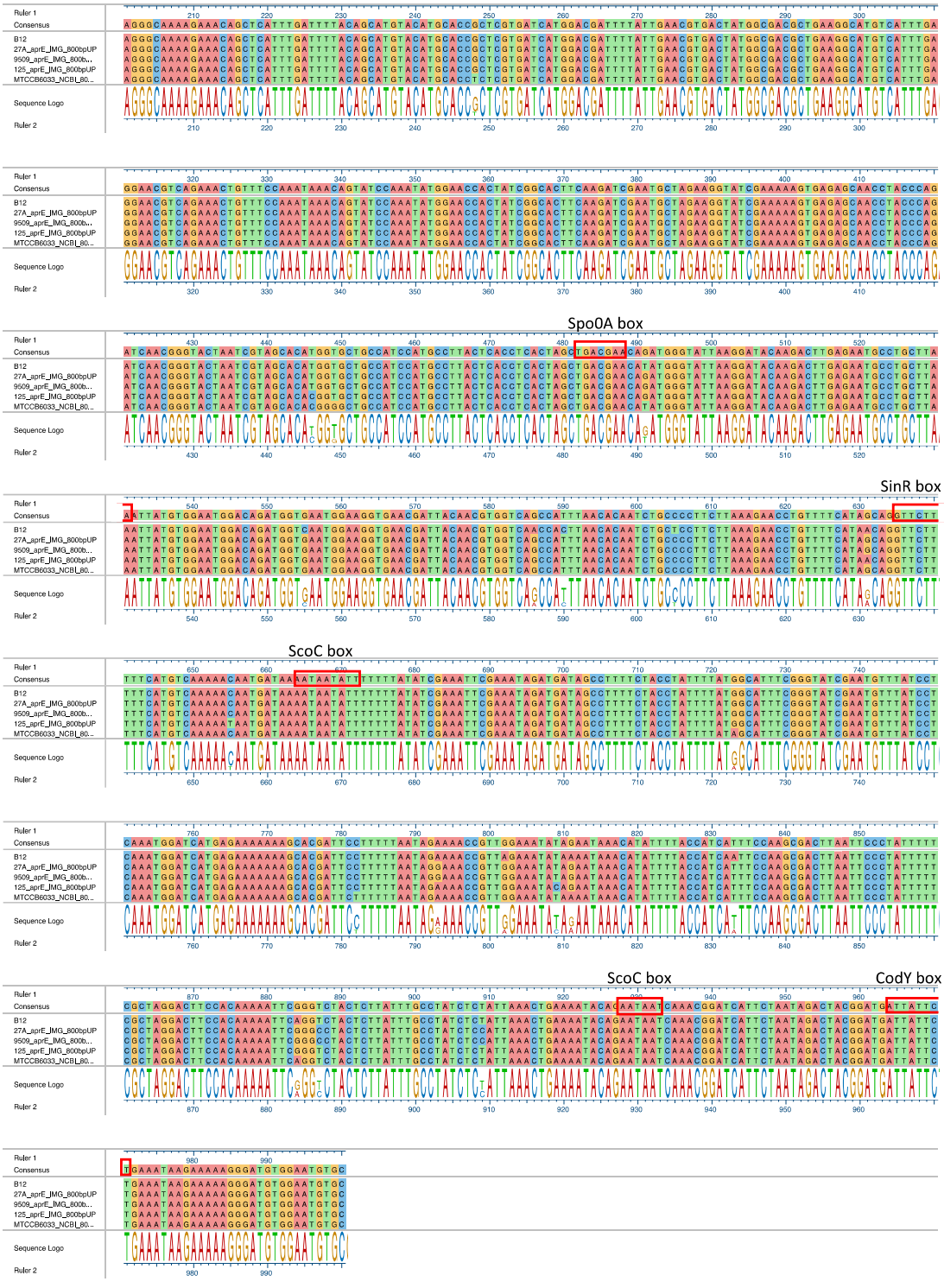


Figure 4-3: Promoter alignment shows high similarity in transcription factor binding sites.

Conclusion

Although these five strains are highly similar at the nucleotide level, they show differences in their ability to degrade PLA. Future work should repeat our PLA degradation AFM and use additional methods to compare these strains. Additionally, basic experiments to look at growth rate in our conditions would be valuable. Finally, using transcription fusions we may be able to see whether AprE is expressed in all of these strains under our growth conditions.

Acknowledgements

Thank you to the Lebeis Lab and Lowen Lab for providing us with strains. Thanks to Bridget O'Bannion for help with running Anvi'o.

Worksheets Developed for Use in a High School Environmental Science Class

Publication Statement: These materials are not intended for publication. They were developed by Elise Phillips after discussions with Todd Reynolds, Melody Hawkins and Rachel Howell.

Intended Use: This set of worksheets was intended for use with high school students that have completed an introductory biology course. The goal is for students to collect a sample (from their school or surroundings) to try to isolate PLA degrading microorganisms. These materials were made for the Spring 2022 semesters of Environmental Science.

Lab Notebook
Identifying Plastic Degrading Microorganisms

Name:_____

Collecting Plastic Degrading Microorganisms
Date:_____

Location Swabbed:

Hypothesis:

Protocol:

Observations

***Bacillus pumilus* B12**
Isolated from: UTIA agriculture plots
Degrades PLA
Observations:

***Bacillus pumilus* 9509**
Isolated from: Duckweed in New Jersey
Degrades PLA
Observations:

***Bacillus subtilis* PY79**
Isolated from: Lab mutant
Does Not Degrade PLA
Observations:

***Bacillus subtilis* WB800**
Isolated from: Lab mutant
Does not produce proteases
Observations:

***Brevunidimonas* 374**
Isolated from: Arabidopsis roots
Does Not Degrade PLA
Observations:

Preparation

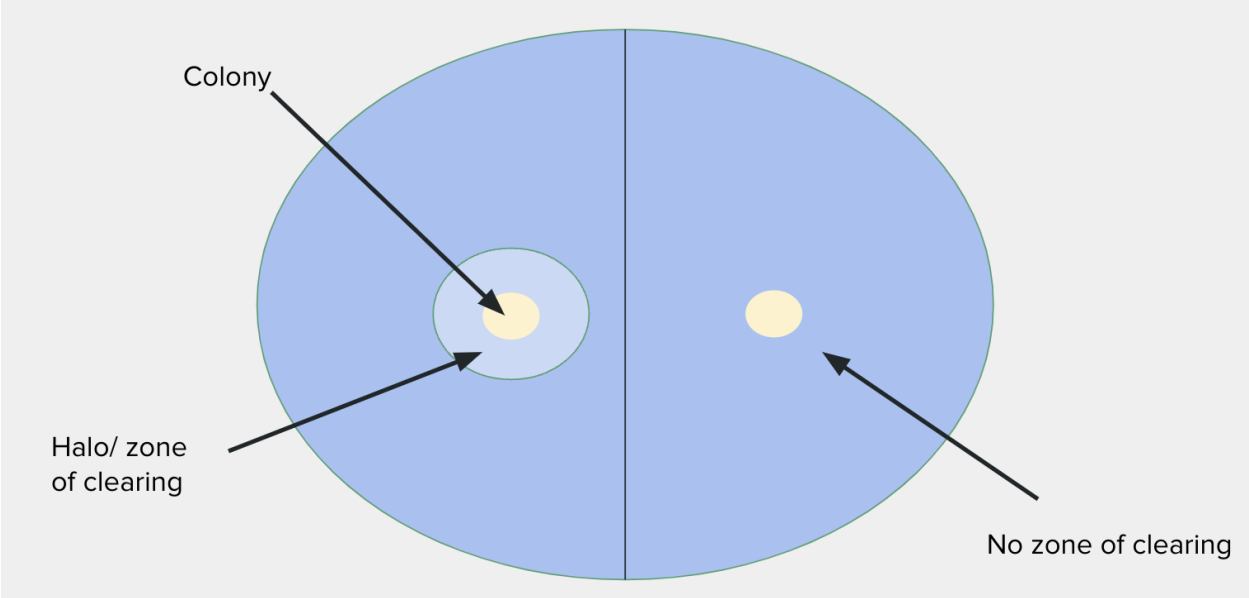
1. Get 3 of each plate (lipase, tributyrin, skim milk).
2. Label each plate with your name, the date.
3. Draw a line down the middle.
4. Write the name of one bacteria on each side of the line.

Inoculation

1. Dip the q-tip in the culture of bacteria
2. Draw a dime-sized circle with the bacteria on the first plate
3. Using a new q-tip every time, add the bacteria to the next two plates
4. Repeat for every bacteria

Data Collection

1. Every ~3 days, measure the diameter of the bacterial colony, then the diameter of the halo



Name:
Assessing Microbial Degradation of Diverse Substrates Using Known Organisms

Plate:

Hypothesis:

Organism:

Organism:

Organism:

Organism:

Table 4-1:Halo Formation Results table

DAY	B12 Colony	B12 Halo	9509 Colony	9509 Halo	PY79 Colony	PY79 Halo	WB800 Colony	WB800 Halo	374 Colony	374 Halo

Name:

Strain 1: Isolated from the greenhouse soil
Strain 2: Isolated from a vending machine

What kind of enzyme do you think each organism is using to break down PLA?
Write Your Hypothesis Here

What experiments could you do to test this?

Which strain do you think will be the best PLA degrader?

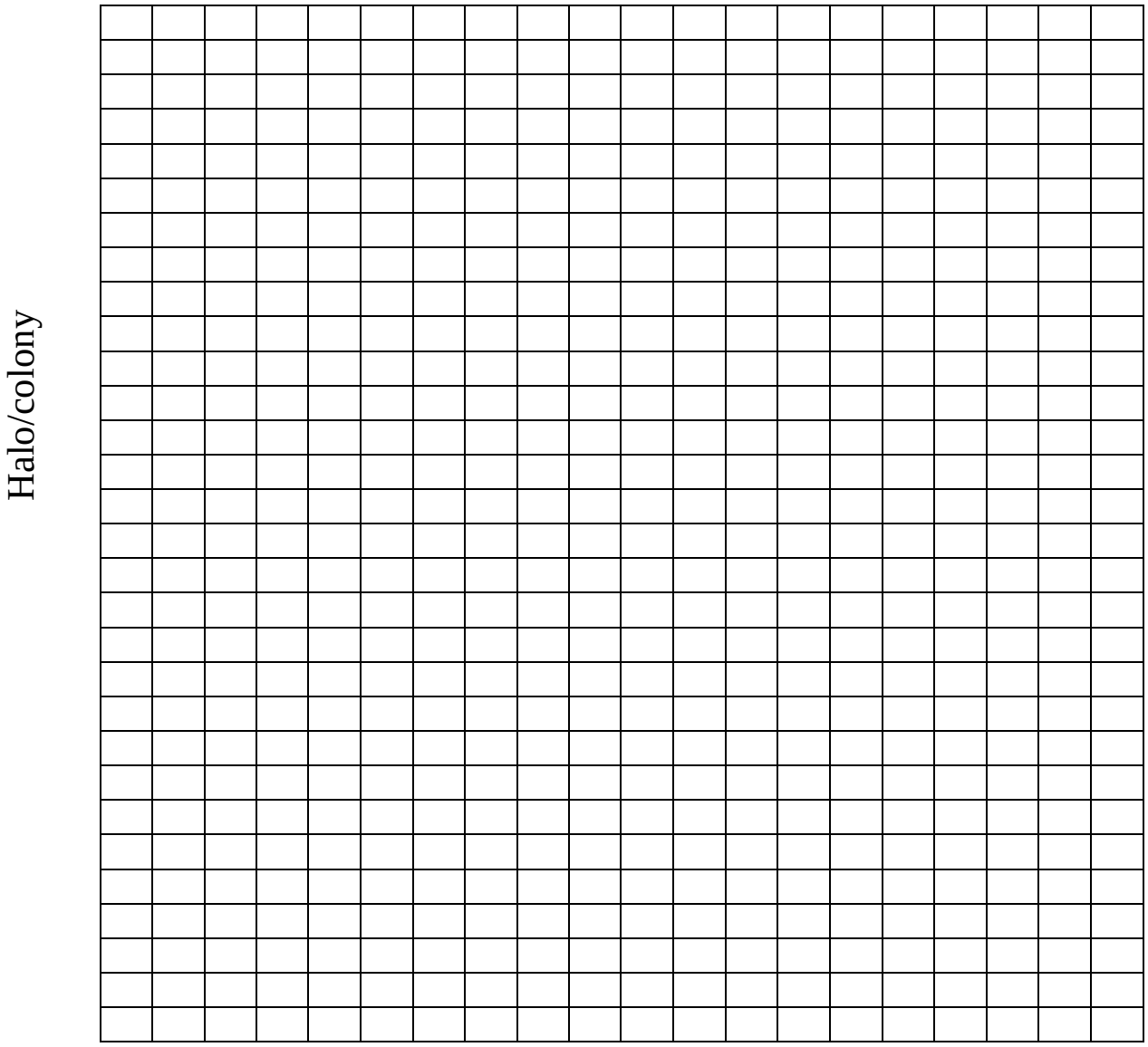
What experiments could you do to test this?

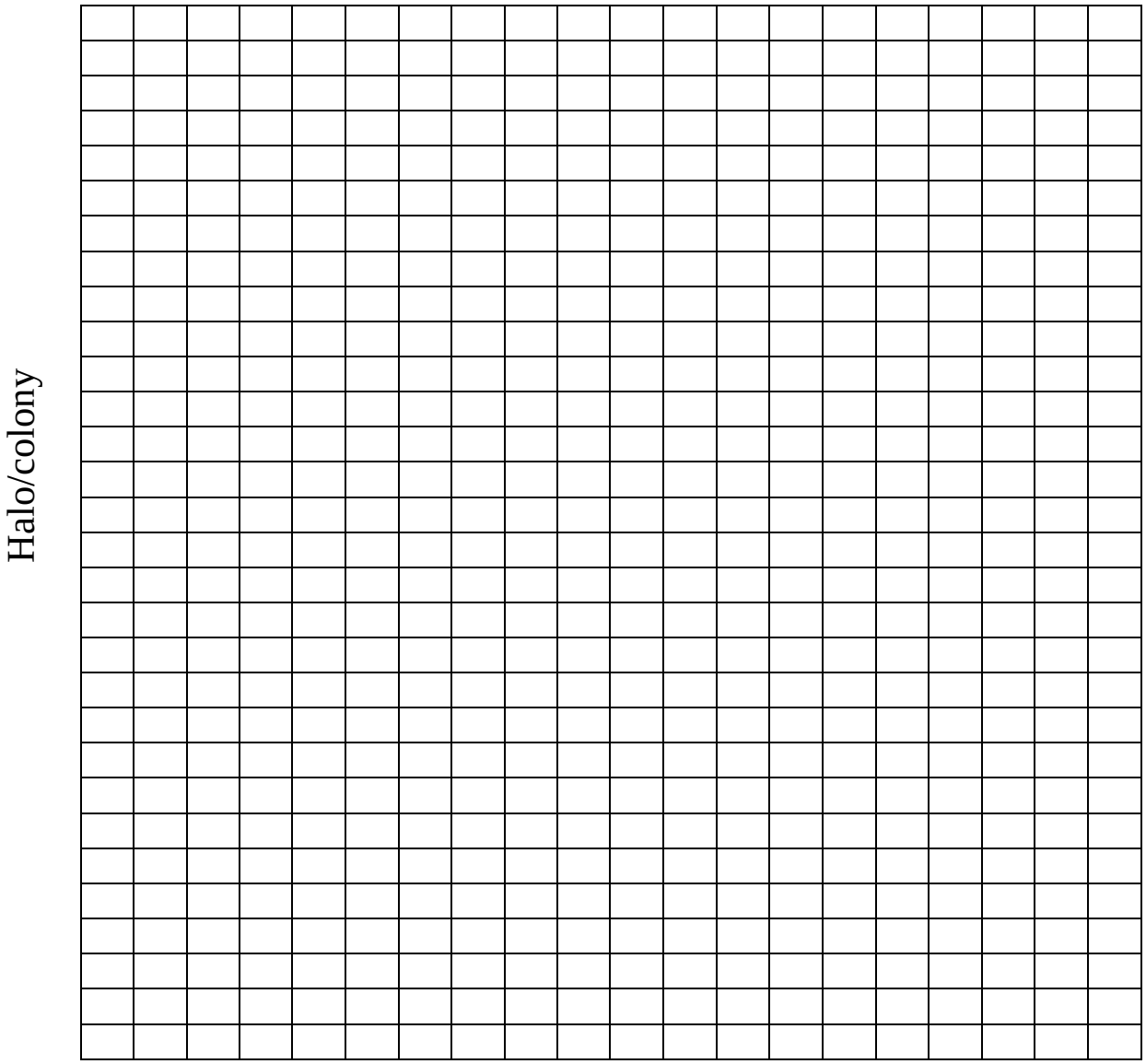
Data Collection!
Strain 1:

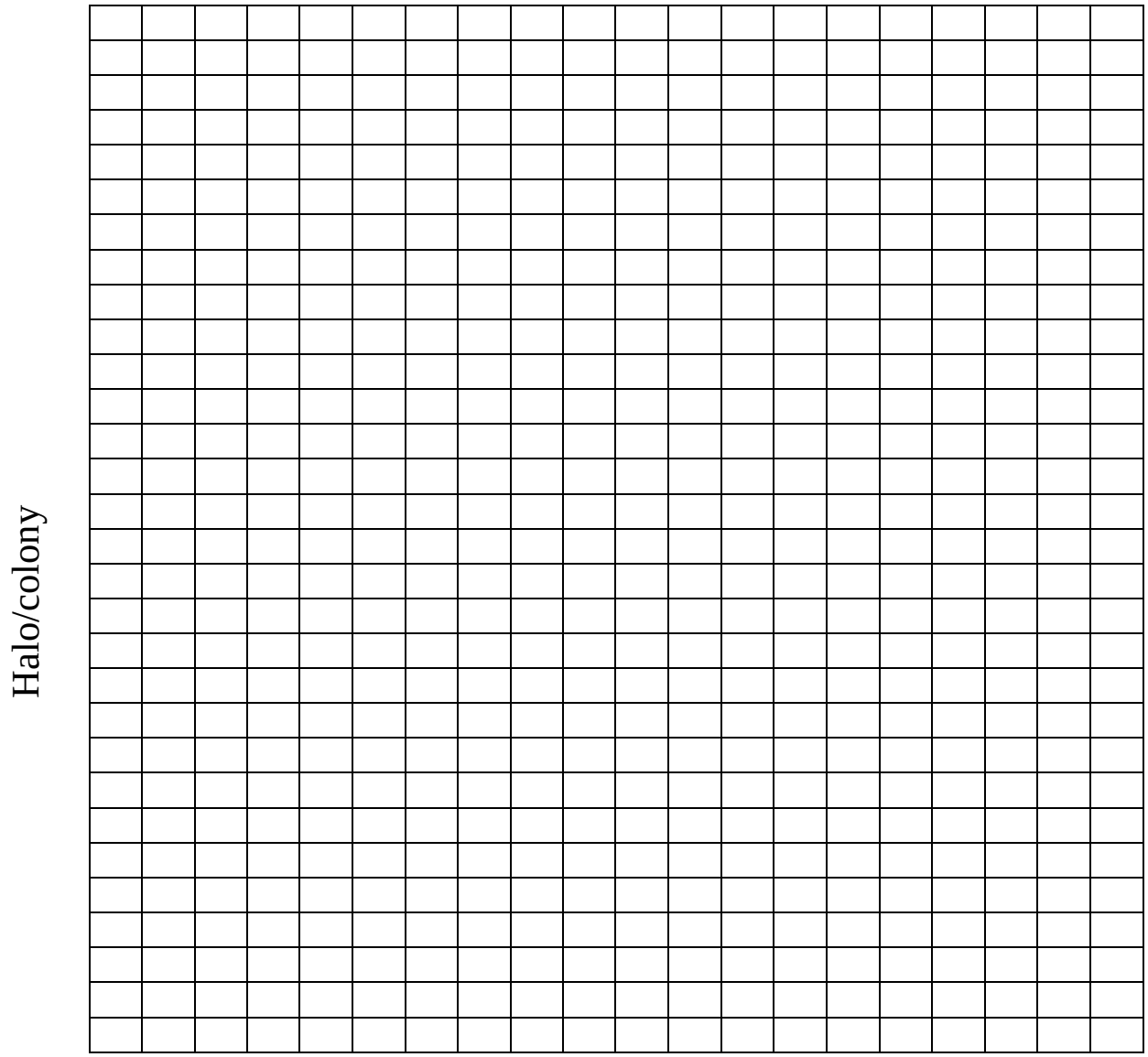
Day	Tribut. colony	Tribut. halo	Ratio halo/ colony	Lipase colony	Lipase halo	Ratio halo/ colony	Milk colony	Milk halo	Ratio halo/ colony

Strain 2:

Day	Tribut. colony	Tribut. halo	Ratio halo/ colony	Lipase colony	Lipase halo	Ratio halo/ colony	Milk colony	Milk halo	Ratio halo/ colony


Halo/colony
Day


Halo/colony
Day


Halo/colony
Day

Based on your observations what enzymes does strain 1 produce?

Based on your observations what enzymes does strain 2 produce?

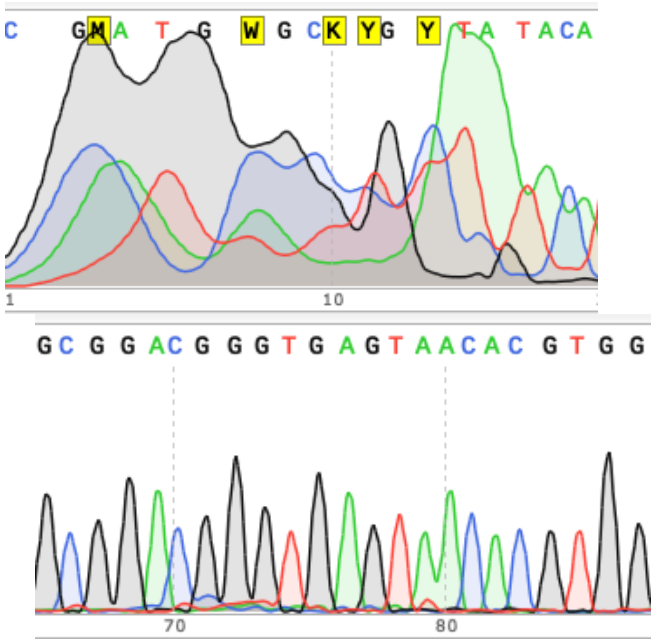
Is one strain better at producing enzymes? What did you observe that makes you thing that?

How does this data change your hypothesis about the best PLA degrading strain?

Identifying Microorganisms with their DNA

Sequence Clean Up

In general, the ends of a sequencing run are not as reliable as sequence in the middle. Because of this, you typically want to trim the ends of a sequence before running it through an alignment tool.

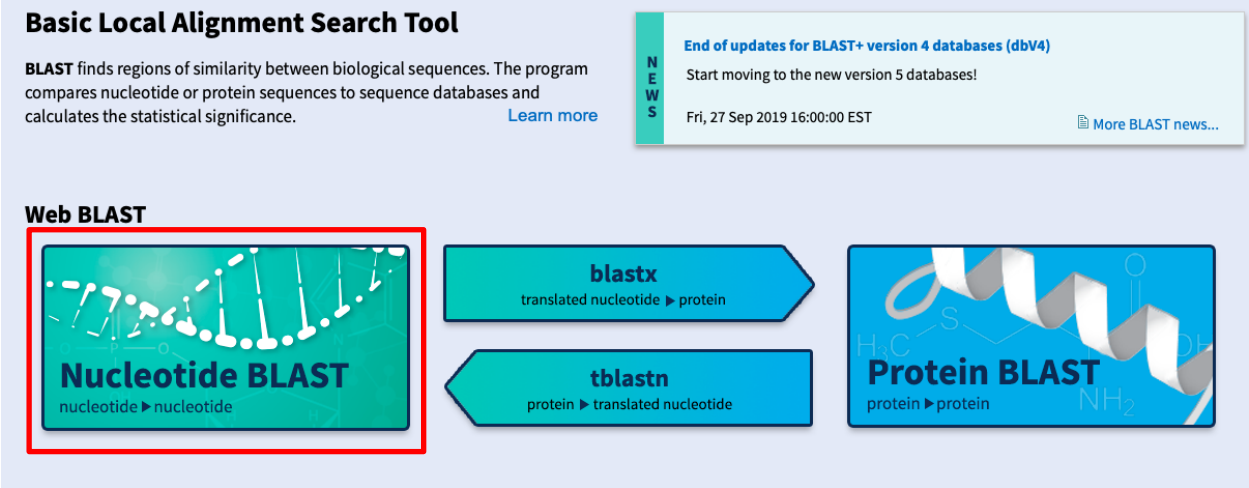


Bad reads Good reads

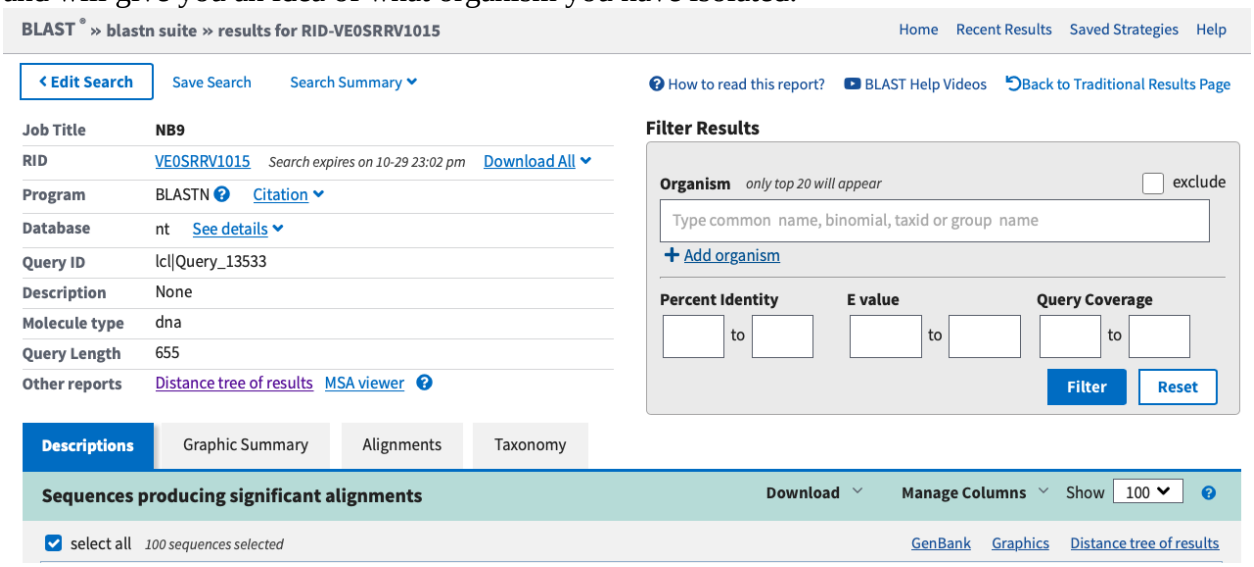
To trim the reads you can either delete the bad reads, or make a second file where you copy in only the good reads.

Sequence Comparison

If you have correctly navigated to NCBI's BLAST you should see this page.



After your sequence is done running you should see a page that looks something like this and will give you an idea of what organism you have isolated.



VITA

Elise K. Phillips grew up in the San Francisco Bay Area. She then moved to a rainy city that she was told you would like and got an undergraduate degree. Elise moved to a different rainy city, climbed some stuff, made a pinata and wrote a dissertation. She graduated from the University of Tennessee, Knoxville in 2023.