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# Developing Genetic Tools for Geobacillus

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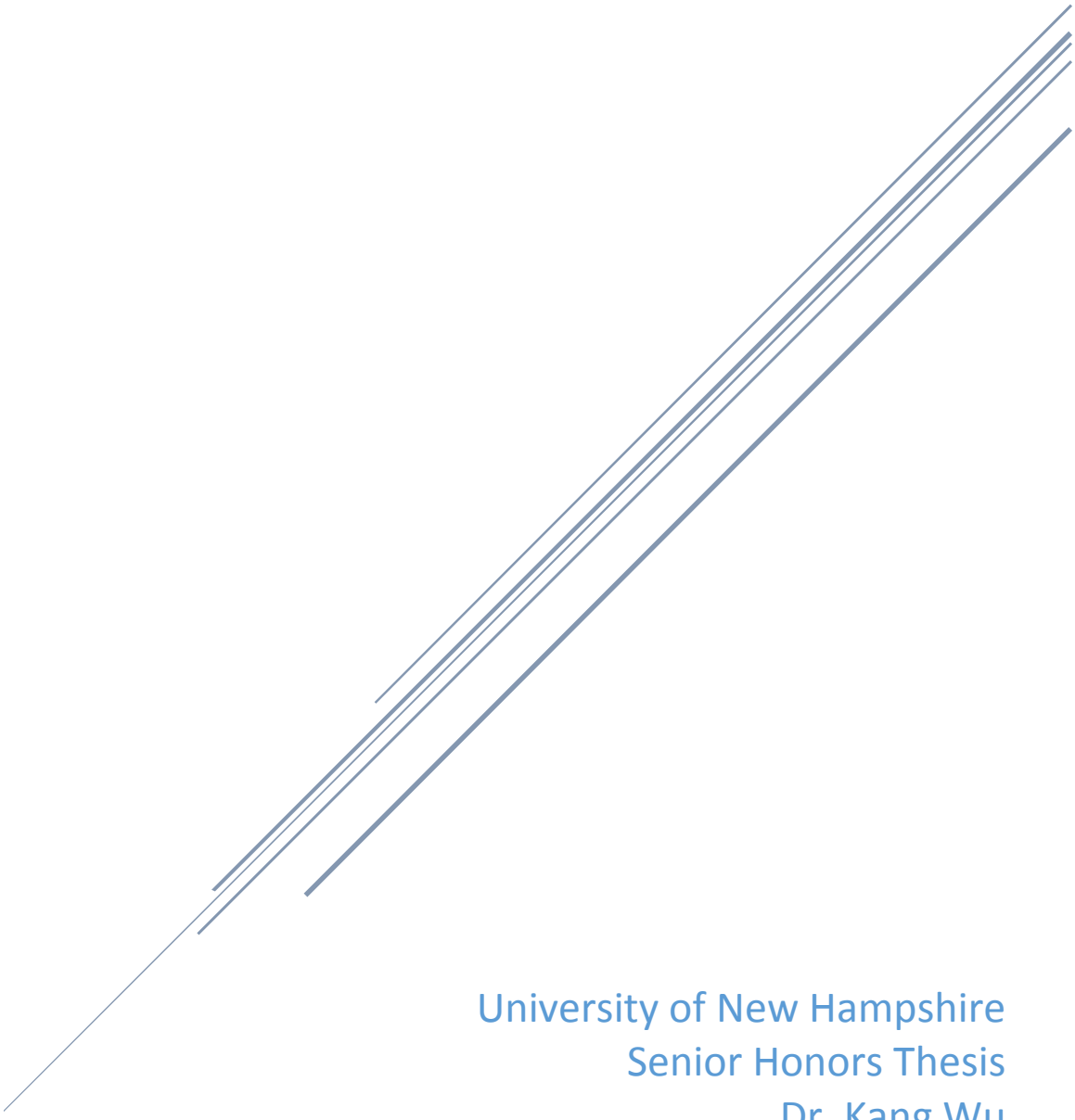

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# Abstract

*Bacillus* and G*eobacillu*s are the primary bacterium used in biotechnology industries due to their ability to excrete extracellular materials such as proteins, enzymes and other byproducts. *Bacillus subtilis*, has a well-characterized competence machinery, giving way to characterized genetic tools. However, a significant problem associated with working with *Bacillus* is the regulation of endospore formation. An alternative to *Bacillus* is *Geobacillus*, a thermophilic bacterium. Thermophiles offer significant advantages over other bacteria as host organisms in biofuel, bioremediation, and biocatalyst processes. However, the genetic tools and competency associated with *Geobacillus* is still unknown. Through, genetic engineering the ability of *ComK* to induce competency in *Geobacillus* is being studied. By inducing competency in this species biotechnology industries can be revolutionized.

# Introduction

## Motivation

In the late 1990's early 2000's a new taxon of bacilli received the name *Geobacillus*, a gram-positive, aerobic, endospore-forming thermophile. *Geobacillus* was discovered in water surrounding oil fields in Uzen, Siberia and China. The water was blackened with hydrocarbons, slime, and silt with temperatures ranging from 55-74$^{o}$C (1).

Many species of *Geobacillus* have demonstrated great potential for bioremediation due to their ability to degrade hydrocarbons which is suggested from their discovery from oil fields. Some species have also been found to have high heavy metal tolerance making them applicable in bioremediation (2). This new taxon has further potential as an industrial host for fuel and enzyme production. Having a high solvent tolerance some species can be good candidates for alcohol production. In biotechnological processes *Geobacillus* offers great potential because of its ability to produce thermostable enzymes and secrete proteins into extracellular medium. Protein purification costs from culture medium is significantly less than that from intracellular production.

Currently over twenty U.S. Patents exist for *Geobacillus* products and processes, demonstrating the taxon's potential in industry (1). To improve these industrial applications and develop more, genetic engineering is required. Currently transformations are performed by protoplast transformation or electroporation. However, both methods of transformation are inefficient and a genetic toolkit is needed to increases the efficiency of transformations. In the development of this toolkit a novel method of transformation is being designed for *Geobacillus*.

## Natural Competence

Natural Competence refers to a cells ability to take up exogenous DNA from the environment and undergo efficient genetic transformation. *B. subtilis*, a close relative to *Geobacillus*, contains one of the only competence machinery that has been characterized (3). It has been found that the competence machinery for *B. subtilis* is a complex system involving around 100 genes, however the master regulator is the *comK* gene (3). For exogenous DNA to be processed and incorporated into the genome *comK* is responsible for the expression of all the other identified competence genes (4). The expression of *comK* is regulated by nutritional signals and cell-cell signals and cannot be linked to the expression of a single transcription factor (4). If the overexpression of *comK* can be induced thus inducing natural competence it could be used as an efficient means of genetically engineering *Geobacillus*.

## Inducible Promoters

A key component in genetic engineering is the regulation of gene expression. Inducible promoters are most commonly used in controlling gene expression. The presence or absence of biotic or abiotic factor induces the activity of the promoter, in turn controlling the expression of the genes operably linked to them (5). Abiotic factors are often difficult to control making chemically activated promoters more common for genetic engineering purposes.

Promoters operate by controlling the transcription of genes, the first step in protein production. In prokaryotes, such as *B. subtilis* and *Geobacillus*, two methods for regulating transcription occur: positive control and negative control. In positive control transcription is turned on when the regulatory protein interacts with the promoter. When the activator is not present the genes are off and only turned on when the activator is present (6). The more common mode of transcription control in prokaryotes is negative control. In this case the expression of the gene is always turned on until a repressor binds to an operator inhibiting RNA polymerase from initiating transcription (6). One of the most widely characterized models of this negative control is the lac operon from *E. coli*.

## Purpose

To successfully utilize *Geobacillus* in industry a toolkit for genetic engineering is required. To develop this a transformation system must be created. The transformation system being developed inducibly expresses *comK*, the master regulator for natural competence. By inducibly expressing *comK* genetic transformation can be controlled using the concept of inducible promoters.

# Theory

To determine if natural competence can be induced in *Geobacillus* a plasmid must be constructed containing an inducible promoter and the *comK* gene. This plasmid can then be transformed into *Geobacillus* to determine in *comK* can actually be inducibly expressed. However, because genetically engineering *Geobacillus* is relatively new and the mechanism for natural competence is unknown, inducing natural competence in *B. subtilis* will first be examined. *B. subtilis* is a close relative and can be used as a means of understanding inducing natural competence then translating back to *Geobacillus*.

The plasmid to be constructed to examine inducing natural competence in *B. subtilis* is shown in Figure 1. The plasmid takes an already existing plasmid containing an IPTG inducible promoter and antibiotic resistant gene ampR and inserts the comK gene. The ampicillin resistant gene is used to screen for colonies containing the plasmid of interest. Colonies containing the plasmid will survive in antibiotic supplemented media which colonies not containing the plasmid would not be able to survive in.

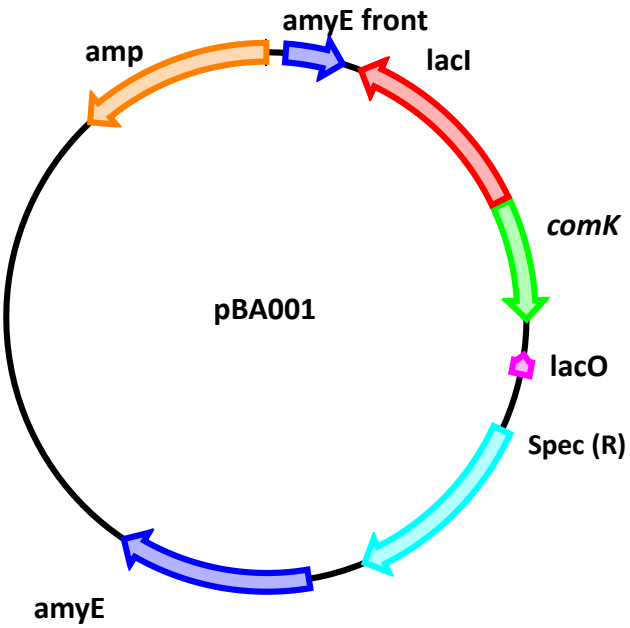


*Figure 1: Plasmid map for pBA001 to determine if comK can be inducibly expressed.*

# Methods and Materials

## Plasmid Construction

To construct the plasmid shown in Figure 1 the existing plasmid pDR111 must be isolated from a mutant strain of *B. subtilis* and the *comK* gene from *B. subtilis.* Following isolation of the plasmid and vector the two can be cloned to form pBA001.

Genomic DNA was isolated from an overnight culture of *B. subtilis* using protocol *Preparation of Genomic DNA from Bacteria*. This gDNA became a template for the primers designed by Dr. Kang Wu, 1015F and 1016R. A polymerase chain reaction (PCR) was performed with these primers, *B. subtilis* gDNA, and taq green mastermix polymerase with an extension time of 40 seconds. This PCR was performed to isolate and amplify the *comK* gene. Following the PCR, Zymo Research™ DNA Clean and Concentrator kit was used to recover ready to use *comK* DNA. To isolate pDR111 from an overnight culture of the mutant *B. subtilis*, Zymo Research Plasmid Miniprep-classic kit was used. This plasmid will be the backbone for the pBA001 being constructed.

To clone the vector and the backbone each must first be digested to create sticky ends. The sticky ends will allow the vector to be ligated to the backbone in the proper location and orientation. The backbone was double-digested using HindIII-HF and NheI-HF, making two sticky ends for the vector to be ligated to. The same digestion was performed using HindIII-HF and NheI-HF and the vector. The protocol for the enzymes was followed and the mixtures incubated at 37°C for four hours. Figure 2 shows a map

of pDR111 with all of the restriction enzyme coding regions. When pDR111 is digested using HindIII-HF and NheI-HF only 14bp are removed from the overall plasmid. When the vector is digested with these enzymes the PCR product recovered is cut before and after the coding sequence for the *comK* gene.

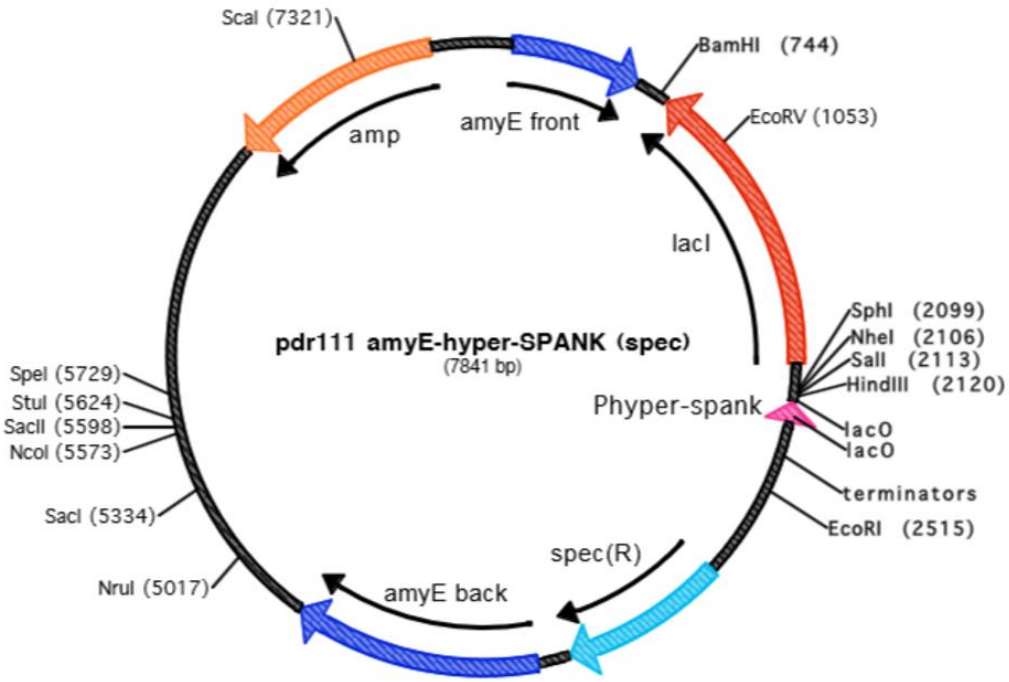


*Figure 2: Plasmid map for pDR111 with restriction enzyme coding regions shown.*

To recover the linearized plasmid and digested vector gel recovery was performed using a 0.8% agarose gel and Zymo Clean Gel Recovery kit. Once the two pieces were recovered they could be ligated together. Following ligation the backbone and vector should be combined to form the plasmid given by Figure 1.

## Transformation and Screening

Transformations using *E.coli* Dh5α are easily performed and efficient. To determine if the construction of pBA001 was successful the plasmid was first transformed in *E.coli*. To perform this transformation *E.coli* Dh5α cells were made chemically competent following *The Inoue Method for Preparation and Transformation of Competent E. Coli: "Ultracompetent" Cells* protocol. The same protocol was followed for the heat shock transformation using these chemically competent cells and pBA001.

To screen for the presence of the plasmid the transformed *E.coli* Dh5α cells were plated on ampicillin supplemented LB plates. Colonies that grew indicate the backbone is present as it contains the ampicillin resistant gene. However, it is possible *E.coli* Dh5α was only transformed with pDR111 so the colonies must be screen for the *comK* gene. This was done performing a colony PCR with the primers 1015F and 1016R which were used to isolate and amplify the *comK* gene from *B. subtilis* gDNA.

## Results

When the transformed *E.coli* Dh5α was screened using antibiotics, there was confirmation of the presence of the ampicillin resistant gene. This indicates the *E.coli* Dh5α cells were transformed using either pDR111 or pBA001. To ensure the transformation was with the proper plasmid the colony PCR was performed as an additional screening method. The gel displayed a band indicates a DNA fragment of 600bp which corresponds to the PCR fragment for the *B. subtilis* comK gene. Had the plasmid not contained the gene, no fragments would have been present. Based on the screening performed the plasmid pBA001 was successfully constructed and transformed into *E.coli* Dh5α.

## Future Work

Although substantial progress has been made, a significant amount of work needs to be performed to construct a genetic tool kit for *Geobacillus*. To determine if naturally competence can be induced by over expression of the comK gene pBA001 must first be transformed into *Bacillus subtilis*. Following this transformation the comK will attempted to be induced to perform further genetic transformations. If this is successful a similar procedure for *Bacillus subtilis* will be followed for *Geobacillus*. The comK gene for *Geobacillus* will be isolated and ligated with the plasmid pKW1011, which contains an inducible promoter.

## Acknowledgements

1. Dr. Kang Wu
2. Tony Castagnaro

## Work Cited


(1) Zeigler, Daniel R. Bacillus Genetic Stock center Catalog of Strains. 7th ed. 3 vols. The Genus Geobacillus.

(2) Poli, Annarita, Ida Romano, Gaetano Caliendo, Giancarlo Nicolaus, Pierangelo Orlando, Antonio De Falco, Licia Lama, Agata Gambacorta, and Barbara Nicolaus. "Geobacillus toebii subsp. decanicus subsp. nov., a hydrocarbon-degrading, heavy metal resistant bacterium from hot compost." The Journal of General and Applied Microbiology 52 (2006): 223-34.

(3) Kovács, Ákos T., Wiep Klaas Smits, Aleksandra M. Mirończuk, and Oscar P. Kuipers. "Ubiquitous late competence genes inspecies indicate the presence of functional DNA uptake machineries." Environmental Microbiology 11 (2009): 1911-922.

(4) Solomon, Jonathan M., and Alan D. Grossman. "Who's competent and when: Regulation of natural genetic competence in bacteria." Trends in Genetics 12 (1996): 150-55.

(5) "Chapter 3." Inducible promoters. 12 May 2014 <http://www.patentlens.net/daisy/promoters/271.html>.

(6) "Prokaryotic promoters." Prokaryotic promoters. 12 May 2014 <http://www.patentlens.net/daisy/promoters/239/g1/1345.html>.