

## ABSTRACT

GREENHUT, WILLIAM BRADLEY. Development of an asporogenic mutant of *Bacillus licheniformis* PWD-1. (Under the direction of Dr. Jason Shih)

*Bacillus licheniformis* PWD-1 is a keratin-degrading, spore-forming bacterium isolated from a poultry waste digester. A mutant of *B. licheniformis* PWD-1, named *B. licheniformis* WBG-2, was developed that is deficient in sporulation. The mutation was created using the Splicing by Overlap-Extension PCR method (Gene SOE'ing) to create a 256bp deletion in the *spoIIAC* gene, which encodes an essential sporulation-specific sigma factor. In-vivo gene replacement was accomplished with the use of a temperature-sensitive plasmid that is able to integrate and excise from the *B. licheniformis* chromosome. PCR and DNA sequencing were used to confirm the deletion, while heat-treatment assays and electron microscopy verified the absence of spores. The mutant, while completely asporogenic, is able to express normal levels of keratinase as compared with *B. licheniformis* PWD-1.

DEVELOPMENT OF AN ASPOROGENIC MUTANT  
OF *BACILLUS LICHENIFORMIS* PWD-1

by

William Bradley Greenhut

A thesis submitted in partial fulfillment of the  
requirements for the degree of

Master of Science

MICROBIOLOGY

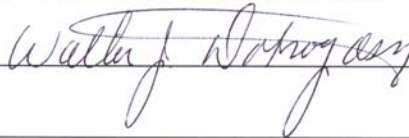
North Carolina State University

2003

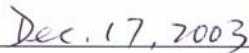
Approved by



Chairperson of Supervisory Committee



Date



## **BIOGRAPHY**

The author was born August 22, 1977 in Pensacola FL to parents Bill and Connie Greenhut. He received his BA in Biology from the University of North Carolina – Asheville in 2000. Upon graduation, he worked as a Q.C. Associate Biologist for Bayer Biologicals in Clayton NC for one year. The author began work on his M.S. in Microbiology at North Carolina State University in 2001. In 2003, the author got married to his college sweetheart, Allison. After graduating, he hopes to work in national defense, pharmaceutical production, or in food microbiology.

## ACKNOWLEDGMENTS

I would like to thank my advisor Dr. Jason Shih for his support and for allowing me freedom in designing my project from choosing the project, the gene, and the method. I would also like to thank him for his faith in my eventual success. Next, I would like to thank my loving family and wife for their emotional support during this challenging period. I would also like to thank my coworkers Brian, Kawan, Rattana, and Nasser for their friendship over the years. Also, a special thanks to Valerie from the N.C. State Electron Microscopy Center for help in preparing and viewing my samples. Most importantly, I would like to thank Dr. J.J. Wang, who is my mentor, and who has given me valuable technical advice and knowledge about succeeding in molecular biology.

## TABLE OF CONTENTS

List of Figures.....	vi
Literature Review	
<i>B. licheniformis</i> Sporulation Mutants are Desirable.....	1
<i>B. licheniformis</i> PWD-1 and Keratinase.....	2
Introduction to Sporulation.....	3
Sporulation $\sigma$ factors.....	4
Sigma F ( $\sigma^F$ ) .....	5
Sigma F activation is coupled to asymmetric cell division and compartmentalized gene expression.....	8
$\sigma^E$ Activation is Dependent on $\sigma^F$ .....	10
$\sigma^G$ Is Also Dependent On $\sigma^F$ .....	12
Disporic Cells.....	12
Sporulation and Proteases.....	15
Splicing by Overlap Extension.....	16
Selection of <i>spoIIAC</i> as the Target for Gene Replacement.....	16
Materials and Methods	
Bacterial Strains and Growth Conditions.....	21
PCR Primers used in the Splicing by Overlap Extension Reaction.....	21
Preparation of Genomic DNA from <i>B. licheniformis</i> PWD-1.....	22
PCR Amplification.....	22
Sub-Cloning in <i>E. coli</i> DH5 $\alpha$ .....	23
Sub-Cloning in <i>B. subtilis</i> DB104.....	24
Transformation of pSPO in <i>B. licheniformis</i> PWD-1.....	25
Gene integration and Excision.....	26
Verification of gene replacement by PCR.....	26
DNA Sequencing.....	27
Heat Treatment Assay.....	27
Proteolytic Activity.....	28
PCR Amplification of <i>kerA</i> .....	29
Electron Microscopy.....	29
Results	
Splicing by Overlap Extension.....	32
Sub-Cloning in <i>E. coli</i> DH5 $\alpha$ .....	32
Sub-Cloning in <i>B. subtilis</i> DB104.....	32
Transformation of pSPO in <i>B. licheniformis</i> PWD-1.....	32

Gene integration and excision.....	32
Verification of gene replacement by PCR.....	32
DNA Sequencing.....	33
Heat Treatment Assay.....	33
Proteolytic Activity.....	33
Electron Microscopy.....	34
Discussion.....	49
Bibliography.....	51
Appendix A.....	58
Appendix B.....	64

## List of Figures

Fig. 1	Morphological Stages of Sporulation.....	17
Fig. 2	Sigma Factor Cascade.....	18
Fig. 3	Biochemical relationships between early sporulation genes and their products...19	
Fig. 4	Splicing by Overlap Extension (Gene SOEing) .....	20
Fig. 5	Nucleotide Sequence of Predicted Overlap Region.....	30
Fig. 6	Gene Replacement Strategy.....	31
Fig. 7	Splicing by Overlap Extension PCR. ....	36
Fig. 8	Plasmid map of PCR2.1- <i>spoIIACA</i> .....	37
Fig. 9	Screening of <i>E. coli</i> transformant by restriction digest.....	38
Fig. 10	<i>PstI</i> digests of <i>spoIIACA</i> and pE194 prior to ligation .....	39
Fig. 11	Plasmid map of pSPO.....	40
Fig. 12	Screening of <i>B. subtilis</i> transformants by restriction digest.....	41
Fig. 13	Screening of <i>B. licheniformis</i> PWD-1 transformants by Colony PCR.....	42
Fig. 14	Screening of <i>B. licheniformis</i> PWD-1 Clones for Gene Replacement by Colony PCR.....	43
Fig. 15	Amplification of <i>kerA</i> from <i>B. licheniformis</i> PWD-1 and WBG-2.....	44
Fig. 16	Graphical view of Sequenced PCR Products.....	45
Fig. 17	Heat Treatment Assay.....	46
Fig. 18	Proteolytic Activity and Cell Growth.....	47
Fig. 19	Electron Micrograph of <i>B. licheniformis</i> PWD-1 and WBG-2 .....	48

## Introduction and Literature Review

### ***B. licheniformis* Sporulation Mutants are Desirable**

*B. licheniformis* is an organism that has been used extensively for the commercial production of protease, amylase, and antibiotics. Most such strains are asporogenic; however most are also randomly mutagenized (Fleming *et al.*, 1995). Carbohydrase and protease production from *B. licheniformis* has been affirmed as G.R.A.S (Generally Regarded As Safe) by the F.D.A. (21 CFR Part 184.1027). Several genetically modified and sporulation-deficient strains of *B. licheniformis* have been granted G.R.A.S. status (<http://www.cfsan.fda.gov/~rdb/opa-gras.html>). The benefits of *B. licheniformis* sporulation mutants can be seen in the following statements regarding *B. licheniformis*' exemption from the E.P.A.'s Toxic Substances Control Act (TSCA) (Section 5(h)(4):

*“A request for comments was made in the Proposed Decision Document for B. licheniformis regarding the recommendation that since the hazards associated with the use of B. licheniformis in fermentation were low, all strains of B. licheniformis should be included in the tiered exemption even though the Proposed Risk Assessment recommended only those strains with a sporulation deficiency of  $10^{-7}$ .”*

*“EPA's standards for minimizing emission specify that liquid and solid waste containing the microorganisms be treated to give a validated decrease in viable microbial populations so that at least 99.9999 percent of the organisms resulting from the fermentation will be killed. Since the microorganisms used in fermentation processes are usually debilitated, either intentionally or through acclimation to industrial fermentation, the small fraction of microorganisms remaining viable after inactivation treatments will likely have a reduced ability to survive during disposal or in the environment.*



*Moreover, industrial companies, in an attempt to keep their proprietary microorganisms from competitors and to reduce the microbial numbers to those permitted by local sanitation authorities, modify the microorganisms to increase the ability of their microorganisms to survive and perform their assigned tasks in the fermentor but decrease their ability to survive in the environment external to the fermentor.”*

Thus, for reasons of waste treatment costs, property protection, and environmental health, sporulation mutants of *B. licheniformis* are desirable. However, the mutation must be stable, render the organism totally asporogenic, and not significantly alter growth or production of the desired substance. The goal of this project was to develop an asporogenic mutant of the strain studied in our laboratory, *B. licheniformis* PWD-1.

### ***B. licheniformis* PWD-1 and Keratinase**

*B. licheniformis* PWD-1 was first isolated from an anaerobic poultry waste digester though shows maximum growth under aerobic conditions (Williams *et al.*, 1990). It was found to be able to grow on feathers as a primary source of energy, carbon, and sulfur (Williams *et al.*, 1990). It also secretes a unique protease which is keratinolytic, degrading a wide variety of substrates including feather.

A novel feature of *B. licheniformis* PWD-1 is its ability to secrete keratinase. Keratinase, an enzyme which degrades keratin, allows this organism to grow on chicken feathers. Keratinase was isolated from *B. licheniformis* PWD-1 and found to have very high proteolytic activity as well as broad substrate specificity (Lin *et al.*, 1992). The optimum pH and pI were found to be 7.5 and 7.25 respectively (Lin *et al.*, 1992). The enzyme has an optimum temperature of 50°C and is stable when stored at -20°C (Lin *et al.*, 1992). The gene encoding keratinase, *kerA*, was sequenced and found to be 97% similar to the gene encoding subtilisin Carlsberg from *B. licheniformis* 6816 (Lin *et*

*al.*, 1995). Northern-blot analysis revealed that *kerA* is expressed in feather medium but not in Nutrient Broth (Lin *et al.*, 1995). A scale-up procedure for the fermentation production of keratinase from *B. licheniformis* PWD-1 was developed (Wang *et al.*, 1999). Keratinase produced from *B. licheniformis* PWD-1 has wide-ranging applications. One use is keratinase-supplemented feed, which improves the growth of broiler chicks (Odetallah *et al.*, 2003). Another application of keratinase is the enzymatic degradation of prion, the causative agent of Mad Cow Disease (Langeveld *et al.*, 2003).

## **Introduction to Sporulation**

Sporulation in *Bacilli* is a common natural process in which the bacterium forms a resilient, highly protected endospore to protect itself from harsh environmental conditions. Such endospores are capable of withstanding the most extreme of conditions and are known to be able to germinate many decades later. Sporulation has been studied for decades as a simple developmental model in prokaryotes. ‘Simple’ is not an accurate description however, since more than 125 genes are involved and the entire sporulation process is not completely understood (Stragier and Losick, 1996). Nevertheless much is known about the process and it seems that the organism has developed complex ways of regulating sporulation at the levels of transcription, translation, post-translational processing, and through control of enzyme activity. However, most of the research on sporulation has involved *B. subtilis*. Less is known about sporulation in *B. licheniformis*, although the two are generally believed to undergo nearly identical sporulation processes.

The process of sporulation begins when nutrient starvation, cell density, or cell-cycle signals are sensed by the cell. It then begins an 8-10 hr differentiation process that leads to the formation of a

dormant spore. The process of sporulation is divided into seven stages based upon morphological progression (Figure 1), and sporulation loci are classified at the morphological stage at which they arrest development (Piggot and Coote, 1976). During stage II, an asymmetric septation event occurs, dividing the cell into prespore and mother-cell compartments. The prespore is then engulfed by the mother cell to form a spore protoplast in stage III. During stage IV, the cortex of the spore is formed, which is a thin peptidoglycan layer outside of the core that includes proteins. Various coat proteins are added on to the outside of the prespore during stage V. The spore is said to mature during stage VI, because it is then that the spore develops resistance to and heat, toxic chemicals, other factors. Finally, in stage VII, the mother cell lyses and the spore is freed. The exact composition of the mature spore has been difficult to characterize. However, using LC/MS/MS over 200 proteins and polypeptides have been identified in *B. subtilis* spores (Kuwana *et al.*, 2002).

Although the exact signal that cause a cell to sporulate are unclear, it is clear that sporulation initiation is controlled by a multi-component phosphorelay composed at least 5 sensor-histidine kinases that donate phosphate to Spo0F, to Spo0B, and finally to Spo0A. Phosphorylated-Spo0A is a transcription factor and the master regulator of sporulation. Among the Spo0A dependent genes are *spoIIA*, *spoIIE*, and *spoIIG* (Hoch, 1993; Stragier and Losick, 1996).

### **Sporulation $\sigma$ factors**

Sigma factors play a vital role in the regulation of gene expression in *B. subtilis*. There are at present 10 known  $\sigma$  factors, with 4 of them being sporulation-specific. Through the cycling of  $\sigma$  factors with different promoter specificities, RNA polymerase is redirected to different sets of genes. This ensures certain sets of genes are expressed at the right time, but also ensures

compartment specific gene expression. The process of sporulation has been shown to be regulated by a “cascade of  $\sigma$  factors” (Losick and Pero, 1981). The temporal and spatial regulation observed in sporulation is regulated by the appearance of unique  $\sigma$  factors, which in turn are regulated at the levels of transcription, gene-rearrangement, and translation (Smith and Mandic-Mulec 1991). The sigma factors are activated in the following order: H, F, E, G, and K (Figure 2).

Sigma F is the first activated in the prespore, which is required for activation of pro- $\sigma$ E in the mother cell. Activated  $\sigma$ E is required for the activation of  $\sigma$ G in the forespore, and  $\sigma$ G is required for the activation of pro- $\sigma$ K in the mother cell. In general, proteins synthesized under the regulation of  $\sigma$ E and  $\sigma$ K will be localized in the cortex or coat. However, proteins synthesized in the forespore under the regulation of  $\sigma$ F and  $\sigma$ G will be localized in the cortex and core (Driks *et al.*, 1999).

### **Sigma F ( $\sigma$ F)**

The first sporulation specific  $\sigma$  factor to be activated is  $\sigma$ F, encoded by *spoIIAC*. Although produced early,  $\sigma$ F is not activated until approximately 30-60 minutes into sporulation (Driks *et al.*, Errington *et al.*, Losick *et al.*, 1992, Margolis *et al.*, 1991). Further,  $\sigma$ F activation appears to be confined to the prespore and not the mother cell compartment (Margolis *et al.*, 1991).

Sigma F is transcribed as part of a three gene operon including *spoIIAA*, *spoIIAB*, and *spoIIAC*. The *spoIIAC* gene is the promoter-distal gene of the tri-cistronic operon. Transcription of *spoIIA* requires the transcriptional activator Spo0A and  $\sigma$ H in vivo (Burbulys *et al.*, 1991, Wu 1989). The requirements of these regulators ensure that  $\sigma$ F is expressed at the correct time.

The *spoIIAC* gene (and its product  $\sigma^F$ ) are highly conserved among related bacteria. *B. licheniformis* and *B. subtilis* produce  $\sigma^F$  proteins that are the exact same size and 91% identical (Yudkin *et al.*, 1989). The coding sequence of *spoIIAC* from *B. licheniformis* also contained 3 stretches of more than 40 consecutive residues identical to those from *B. subtilis* (Yudkin *et al.*, 1989). The 3' region of *spoIIAC* is similar to that of *B. subtilis*, but has 3 elaborate hairpins that presumably function as a rho-independent terminator (Yudkin *et al.*, 1989).

Sigma F depends on regulation at the level of activity, however. The first two genes, *spoIIAA* and *spoIIAB*, encode anti-anti- $\sigma$  factors and anti- $\sigma$  factors, respectively (Schmidt *et al.*, 1990, Duncan *et al.*, 1993). This was based on 4 key pieces of evidence: (i) overexpression of *spoIIAB* inhibits  $\sigma^F$  directed gene expression, (ii) a mutation in *spoIIAB* stimulates  $\sigma^F$  directed gene expression, (iii) a mutation in *spoIIAA* blocks  $\sigma^F$  directed gene expression, and (iv) a mutation in *spoIIAB* relieves the blocks in  $\sigma^F$  directed gene expression caused by a mutation in *spoIIAA* (Schmidt *et al.*, 1990). Sigma F is kept inactive by SpoIIAB, an anti- $\sigma$  factor that binds directly to and inhibits  $\sigma^F$  (Duncan *et al.*, 1993). SpoIIAB is also a protein-kinase, and phosphorylates the product of *spoIIAA* (Min *et al.*, 1993, Diederich *et al.*, 1994, Duncan *et al.*, 1996). Phosphorylated SpoIIAA is inactive (Duncan *et al.*, 1995, 1996). The activation of SpoIIAA occurs through SpoIIIE, a protein-phosphatase bound to the sporulation septum (Arigoni *et al.*, 1995; Arigoni *et al.*, 1996; Barak *et al.*, 1996; Duncan *et al.*, 1995; Feucht *et al.*, 1996). Once dephosphorylated by SpoIIIE, SpoIIAA binds to and inhibits, SpoIIAB. When SpoIIAB is bound by SpoIIAA,  $\sigma^F$  is freed and activated. Mutations in *spoIIIE*, like mutations in *spoIIA*, block sporulation at stage II and prevent activation in  $\sigma^F$  (Barak *et al.*, 1996).

The sequence of events that leads to  $\sigma^F$  activation may depend on the ATP/ADP balance in the cell. SpoIIAB is a dimer that can bind either ATP or ADP (Duncan *et al.*, 1993; Min *et al.*, 1993). The activation of SpoIIAA requires ADP in-vitro (and inactivation requires ATP). The formation of the SpoIIAA-SpoIIAB(ADP) complex is believed to allow  $\sigma^F$  activation by sequestering SpoIIAB so that it cannot phosphorylate SpoIIA or inhibit  $\sigma^F$  (Ho *et al.*, 2003).

Relatively few genes have been shown to depend on  $\sigma^F$  for expression. Among those that do require  $\sigma^F$  are *spoIIIG*, *lonB*, *gpr*, *katX*, and *rsfA*. The *spoIIIG* gene encodes  $\sigma^G$ , a late forespore-specific  $\sigma$  factor. *lonB* encodes a ATP-dependent protease. Although *lonB* mutations do not produce any discernible phenotype, LonB in conjunction with LonA may downregulate the activity of  $\sigma^F$  (Serrano *et al.*, 2001). *gpr* encodes an endopeptidase which depresses the spore's protein reserve (SASPs) upon germination (Sussman *et al.*, 1991). *katX* is a catalase responsible for hydrogen peroxide resistance of the germinating spore (Bagyan *et al.*, 1998). *rsfA* is the first  $\sigma^F$  dependent gene found that may also regulate  $\sigma^F$  transcription (Wu *et al.*, 2000). Though not essential for sporulation, RsfA was found to regulate most  $\sigma^F$  dependent genes (Wu *et al.*, 2000). RsfA bears similarity to leucine-zipper type proteins and colocalizes with DNA, suggesting it's role as a transcriptional regulator (Wu *et al.*, 2000). With the completion of the *B. subtilis* genome sequencing, new techniques could be applied to search for  $\sigma^F$  dependent genes. Using microarray analysis of Spo0A and  $\sigma^F$  mutants, 520 genes were identified whose expression were at least 3-fold dependent on Spo0A but not on  $\sigma^F$  (Fawcett *et al.*, 2000). Approximately 66 genes were identified whose transcript levels were dependent on both Spo0A and  $\sigma^F$  (Fawcett *et al.*, 2000). Besides the known  $\sigma^F$  and  $\sigma^E$  dependent genes, several new genes were discovered, and many are predicted to be involved in fatty acid metabolism. But because  $\sigma^F$  directs  $\sigma^E$  activation, these 66 also included genes dependent on  $\sigma^E$  or  $\sigma^G$ . To help establish  $\sigma^F$  specific genes, a hidden Markov model of

promoter sequences was analyzed, 11 genes were identified to likely be  $\sigma^F$  dependent (Fawcett *et al.*, 2000). Several of the genes found to be under  $\sigma^F$  control have been shown to be located in the prespore compartment. Often this is done with *lacZ* fusions of genes under  $\sigma^F$  control and visualizing gold-conjugated primary and secondary antibodies by electron microscopy.

An intriguing question about  $\sigma^F$  is how it replaces the other  $\sigma$  factors ( $\sigma^A$  and  $\sigma^H$ ) in the early sporulation events. The affinity of RNAP for  $\sigma^F$  was 25-fold lower than  $\sigma^A$  (Lord *et al.*, 1999). At its peak, the concentration of  $\sigma^F$  was twofold higher than that of  $\sigma^F$ . This difference cannot account for the replacement of  $\sigma^A$  by  $\sigma^F$  in the prespore (Lord *et al.*, 1999). The promoter recognition sequence characteristic of  $\sigma^F$  was identified by using random-sequence oligonucleotides. The oligos were used to alter the -10 and -35 regions of a *spoIIQ* promoter-*lacZ* fusion introduced into *B. subtilis* chromosome. A weak -10 consensus of GG/tNNANNNT was found, of which ANNNT is common to all sporulation associated  $\sigma$  factors and  $\sigma^A$  (Amaya *et al.*, 2001). The -35 consensus sequence was stronger, GTATA/T, of which GNATA is also recognized by all sporulation specific  $\sigma$  factors (Amaya *et al.*, 2001). Using the predicted -10 and -35 sequences, the *B. subtilis* genome was analyzed and found to have 193 possible matches (Amaya *et al.*, 2001), but only 1 of 7 analyzed turned out to be  $\sigma^F$  dependent. Thus, there seems to be more to  $\sigma^F$  specificity than just the -10 and -35 regions.

### **Sigma F activation is coupled to asymmetric cell division and compartmentalized gene expression**

It has long been known that gene regulation is coupled to the completion of “morphological checkpoints” in *B. subtilis* spore formation. Recently evidence has been cited that SpoIIE, which is responsible for  $\sigma^F$  activation, is also required for proper asymmetric cell division (Hilbert 2003).

SpoIIE is an integral membrane protein with 10 membrane-spanning segments and a PP2C-like phosphatase domain in its tail (Wu *et al.*, 1998; Arigoni *et al.*, 1999). It is believed that SpoIIE is the critical molecule that couples asymmetric cell division and compartment-specific expression in *Bacillus*.

The relocation of the division site from mid-cell to near the cell pole is a critical event in the process of sporulation. In both binary division and asymmetric division, the process is mediated by the FtsZ protein, a tubulin-like protein, which is assembled into Z-rings (Wang *et al.*, 1993). Asymmetric cell division is mediated by FtsZ rings positioned at sites near both poles (Levin *et al.*, 1996). SpoIIE assembles into E-rings that colocalize with the Z-rings at the septum sites. Using immunofluorescence microscopy, SpoIIE localization was superimposable with the bipolar pattern of the FtsZ (Levin *et al.*, 1997). Further SpoIIE localized to the medial position (with FtsZ) when induced during vegetative growth (Levin *et al.*, 1997). Some *spoIIE* mutants that are unable to activate  $\sigma^F$  can still form asymmetric septa, while null *spoIIE* mutants cannot (Barak *et al.*, 1996). A *spoIIE* mutation was observed that uncoupled asymmetric division, allowing  $\sigma^F$  activation without proper asymmetric septa (Hilbert 2003). Meanwhile, in *ftsZ* mutants, SpoIIE fails to localize to the septum, and  $\sigma^F$  is not activated (Levin *et al.*, 1997). Thus SpoIIE may be the factor that couples proper asymmetric division with the activation of  $\sigma^F$ , ensuring that forespore-specific genes are expressed at the right time.

SpoIIE may also be the protein responsible for compartmentalized gene expression. It has long been question how SpoIIE is regulated to bring about activation of  $\sigma^F$  only in the prespore. It is hypothesized that SpoIIE phosphatase becomes sequestered to the prespore face of the asymmetric septum, ensuring a higher concentration of dephosphorylated SpoIIAA on the



prespore side of the septum (Lewis *et al.*, 1998). Alternatively, the concentrations of SpoIIE could be the same on both faces of the septum, but results in a higher concentration of SpoIIE/SpoIIAB in the forespore. Other evidence suggests that SpoIIE is found in both compartments after septation and must therefore be subject to some sort of regulation (King *et al.*, 1999). This is contrary to the model of sequestering spoIIE to the prespore face. Apparently SpoIIE localizes to the potential asymmetric septum predominantly, but later is found to be associated with the second septum site (Wu *et al.*, 1998). Either way,  $\sigma^F$  appears to be activated only in the prespore compartment through the action of SpoIIE.

### **$\sigma^E$ Activation is Dependent on $\sigma^F$**

*spoIIAC* mutants prevent processing of pro- $\sigma^E$  into mature form (Jonas *et al.*, 1989; Partridge *et al.*, 1991). Like  $\sigma^F$ ,  $\sigma^E$  is produced before septation but not activated until about 2 hrs into sporulation (Haldenwang *et al.*, 1981). Unlike  $\sigma^F$ , however,  $\sigma^E$  is activated only in the mother cell compartment and not the prespore (Losick and Stragier 1992; Stragier and Losick 1996). It is transcribed from *spoIIGB*, with the coexpressed *spoIIGA* being necessary for activation of  $\sigma^E$ . (Jonas *et al.*, 1988; Peters *et al.*, 1994; Stagier *et al.*, 1988). SpoIIGA is a protease responsible for removing 29 amino acids from the N-terminal region of pro- $\sigma^E$ , which is necessary for its activation (Jonas *et al.*, 1988). However, this processing does not occur in-vivo without the signaling protein SpoIIR, transcribed under the control of  $\sigma^F$  (Karow *et al.*, 1995; Londono-Vallejo *et al.*, 1995).

The N-terminal “pro” sequence of pro- $\sigma^E$  is responsible for directing the protein to the sporulation septum, where SpoIIR signaling and SpoIIGA processing are thought to occur. It has been shown that the “pro” sequence of a  $\sigma^E$ -*gfp* fusion is directed to the mother-cell side of the

sporulation septum (Ju *et al.*, 1999). Further, protoplasting and separating the two compartments revealed a GFP signal only in the membrane of the mother cell compartment (Ju *et al.*, 1999). Additional studies found pro- $\sigma$ E associated with the cytoplasmic membrane in the predivisional cell, and later associated with the sporulation septum (Hofmeister *et al.*, 1998). Processed  $\sigma$ E was distributed throughout the mother-cell compartment. The finding that the association between pro- $\sigma$ E and the sporulation septum was SpoIIIGA independent confirms the idea that the “pro” sequence directs the enzyme to the septum.

While signaling from the prespore to mother-cell is important in  $\sigma$ E activation in the mother-cell, there is likely some mechanism that prevents  $\sigma$ E activation in the prespore. Using fluorescence microscopy both pro-E and  $\sigma$ E were found to be absent from the prespore compartment, and their disappearance is dependent on functional *spoIIIE* (Pogliano *et al.*, 1997). Pro- $\sigma$ E can be processed if transcribed with *spoIIIGA* and *spoIIIE* is absent. It has been suggested that a gene for an unknown protease, translocated into the prespore early, is responsible for degradation of SpoIIIGA and pro- $\sigma$ E in the prespore (Ju *et al.*, 1998). Selective degradation of  $\sigma$ E in the prespore compartment has been noted (Ju *et al.*, 1998).

To summarize, a forespore specific signal (SpoIIR) transcribed by  $\sigma$ F is transduced across the septal membrane. This signal, in addition to SpoIIIE, is required for SpoIIIGA to process pro- $\sigma$ E at the septum. Activated  $\sigma$ E in the mother cell compartment is what eventually leads to mother-cell specific transcription. This regulation ensures  $\sigma$ E is not activated until after septation by tying the processing reaction of pro- $\sigma$ E to activated  $\sigma$ F. Over 17 genes have been cloned, sequenced and shown to depend on  $\sigma$ E for expression, which are needed for various aspects of engulfment, cortex and coat formation (Haldenwang *et al.*, 1995).

## **$\sigma$ G Is Also Dependent On $\sigma$ F**

Sigma G is the late prespore  $\sigma$  factor that replaces  $\sigma$ F. Sigma G is the first true compartment specific  $\sigma$  factor, since it is both synthesized and activated in the prespore compartment. It turns out that  $\sigma$ F is very similar to  $\sigma$ G both structurally and functionally. Due to overlapping promoter specificities,  $\sigma$ F may also transcribe genes thought to be  $\sigma$ G dependent such as *gpr*, *gerA* and *sypE* (Sun *et al.*, 1991; Sussman *et al.*, 1991). Both  $\sigma$ F and  $\sigma$ G appear to be active only in the prespore, and both are inhibited in-vitro by SpoIIAB (Rather *et al.*, 1990). Disappearance of SpoIIAB from the forespore compartment also correlates with the activation of  $\sigma$ G in that compartment (Kirchman *et al.*, 1993).

Both  $\sigma$ F and  $\sigma$ G can transcribe *spoIIIG* (encoding  $\sigma$ G) in vitro. Thus  $\sigma$ G is auto-regulated (Sun *et al.*, 1991). However  $\sigma$ F mutants block *spoIIIG* expression (Partridge 1991). This is because  $\sigma$ G synthesis also requires active  $\sigma$ E (Partridge *et al.*, 1993). Sigma G does not become active until the engulfment stage is complete (Stragier 1992). The timing of its activation is thought to correlate with a drop in SpoIIAB after the completion of engulfment, a process dependent on SpoIIIA from the mother cell (Kirchman *et al.*, 1993).

## **Disporic Cells**

The disporic phenotype refers to the production of asymmetric septa at both ends of the cell in certain mutants, including *spoIIA* and *spoIIG* mutants (Setlow *et al.*, 1991; Illing *et al.*, 1991; Fleming *et al.*, 1995). The second septum is formed sequentially after the first septum and at the opposite pole. It is not completely understood why the disporic phenotype occurs in such mutants, but is likely to be dependent on the absence of one or more  $\sigma$ E-transcribed genes. This dependence

means that the second septum is not canceled until the first is completed (active  $\sigma$ E is dependent on active  $\sigma$ F, which is dependent on the sporulation septum).

Three  $\sigma$ E-dependent proteins thought to be responsible for keeping the second septum from forming in wild-type cells have been identified. SpoIIM, SpoIID, and SpoIIP, are partially responsible for the both the degradation of peptidoglycan in the septal membrane (allowing engulfment) and the degradation of complexes at the second polar division site. A *spoIID*, *spoIIM*, *spoIIP* triple mutant mimicked the bipolar phenotype of a  $\sigma$ E mutant, while cells that expressed these proteins prematurely were inhibited in septum formation at both poles (Eichenberger *et al.*, 2001). The disporic phenotype appears both in cells that fail to convert pro- $\sigma$ E to  $\sigma$ E (*spoIIAC* mutant), and in null  $\sigma$ E mutants. Partial double septa may be displayed transiently in a small percentage of wild type cells but are later removed (Pogliano *et al.*, 1999).

The first step in the development of asymmetry seems to be restricting the central division site used in vegetative replication, a process which requires Spo0A. Mutations in *spo0A* which activate the protein in the absence of phosphorylation is sufficient to direct the FtsZ ring from medial to polar (Levin *et al.*, 1996). Then, one of the potential asymmetric division sites is selected, requiring Spo0H. Finally, the second unused division site is permanently blocked, which does not occur in *spoIIA*, *spoIIE*, or *spoIIG* mutants.

At the time of the first asymmetric division, only 30% of the chromosome is located in the forespore compartment; the rest is brought in by SpoIIIE translocase (Wu *et al.*, 1997). This creates not only septal asymmetry, but ‘genetic asymmetry’ as well. This genetic asymmetry is important in the activation of both  $\sigma$ F and  $\sigma$ E. This is because the origin-proximal regions of the chromosome are the first to enter the prespore compartment. This genetic asymmetry is

important in the activation of both  $\sigma^F$  and  $\sigma^E$ . First, the *spoIIA* locus is far from the origin and thus located in the mother-cell at the start of septation. This could allow depletion of SpoIIAB in the forespore, thus allowing  $\sigma^F$  activation (Dworkin *et al.*, 2001). Secondly,  $\sigma^E$  activation is partially ensured by the chromosomal location of *spoIIR*, which is the signaling protein involved in allowing  $\sigma^E$  activation. The *spoIIR* gene is located at 324°, near the origin and thus present in the forespore compartment at septation (Khvorova *et al.*, 2000). It was found that movement of the *spoIIR* to origin-distal regions of the chromosome decreased sporulation efficiency to 20% of the normal rate, while movement to other origin-proximal regions did not negatively effect sporulation (Khorova *et al.*, 2000). Movement of *spoIIR* to origin-distal regions of the chromosome resulted in a delay in pro- $\sigma^E$  processing, leading to the accumulation of disporic cells (Zupancic *et al.*, 2001).

The second septum in  $\sigma^E$  mutants was found to be formed only 6min. after the completion of the first septum and before the completion of chromosome translocation into the first forespore (Pogliano *et al.*, 1999). Abortively disporic cells end up with a chromosome in each of the two polar compartments, leaving the mother-cell portion free of DNA (Setlow *et al.*, 1991; Lewis *et al.*, 1991). Eventually the central compartment lyses, freeing the two prespore compartments into what is termed a 'sporlet' (Magill *et al.*, 1992) The sporlets, unlike the disporic cells, appear to be surrounded by a thick layer of peptidoglycan derived from the mother cell (Magill *et al.*, 1991). Further, sporlets are viable (90% could form colonies) and contained enzymes and ATP levels similar to cells 2 to 3 hours after sporulation (Magill *et al.*, 1991). Sporlets differed from sporulating cells in that macromolecular synthesis was not observed in sporlets, and sporlets were no more heat resistant than vegetative cells (Magill *et al.*, 1991).

## Sporulation and Proteases

It has been established that there is a link between sporulation and exoprotease in *Bacillus* species. Some protease-deficient mutants show decreased intracellular turnover and also lose the ability to form spores (Mandelstam and Waites, 1968). The reason that protease is needed for sporulation is however, not clear. It has been suggested that the protease may be responsible for providing the building blocks needed to form new proteins in the prespore. However, Mandelstam noted that the proteaseless mutant is not deficient in forming new protein (Mandelstam and Waites, 1968). They also noted that the proteaseless mutants retained the protein pattern of vegetative cells, whereas wild-type cells degrade proteins to form a pattern characteristic of sporulation. However, others have reported that a deletion in both the alkaline and neutral proteases had no effect on growth, sporulation, or morphology (Yang *et al.*, 1984).

It is now known that mutations in *spo0* loci lead to protease-deficient cells because many extracellular enzymes are synthesized during stationary phase and early sporulation. However, there is evidence that *spoIIAC* mutations have little effect on proteases in either *B. subtilis* or *B. licheniformis* (Waites *et al.*, 1990; Coote 1992; Fleming *et al.*, 1995).

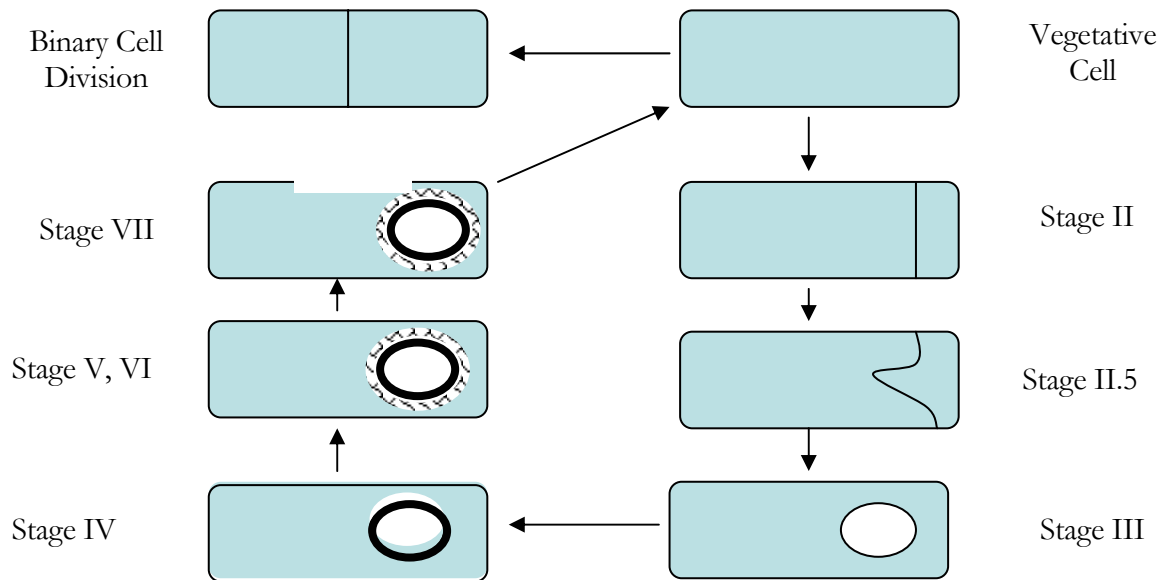
There are several major negative regulators of sporulation including *hpr*, *abrB*, and *sin*. Overexpression of these proteins inhibits sporulation; overexpression of *hpr* also inhibits exoprotease production (Perego and Hock, 1988). *AbrB*, which is a DNA-binding protein, is a negative regulator of several early sporulation genes and also of *aprE*, a major alkaline protease in *Bacillus* species. (Staunch *et al.*, 1989; Zuber *et al.*, 1989).

## Gene Splicing by Overlap Extension (Gene SOEing)

Gene Splicing by Overlap Extension (Gene SOEing) is a PCR based method to create deletions and gene fusions in PCR amplified fragments (Horton *et al.*, 1991; Horton *et al.*, 1993). Gene SOEing allows the creation of large deletions without the use of restriction enzymes or ligase (Horton *et al.*, 1991, Horton *et al.*, 1993). The system is based on the fact that PCR primers can be designed with extra sequences added on to their 5' regions. These 'add on' sequences are complimentary to the primers of a second PCR generated fragment. Thus one strand from the first PCR generated fragment can anneal with one strand from the second PCR generated fragment. The general scheme is depicted in Figure 4.

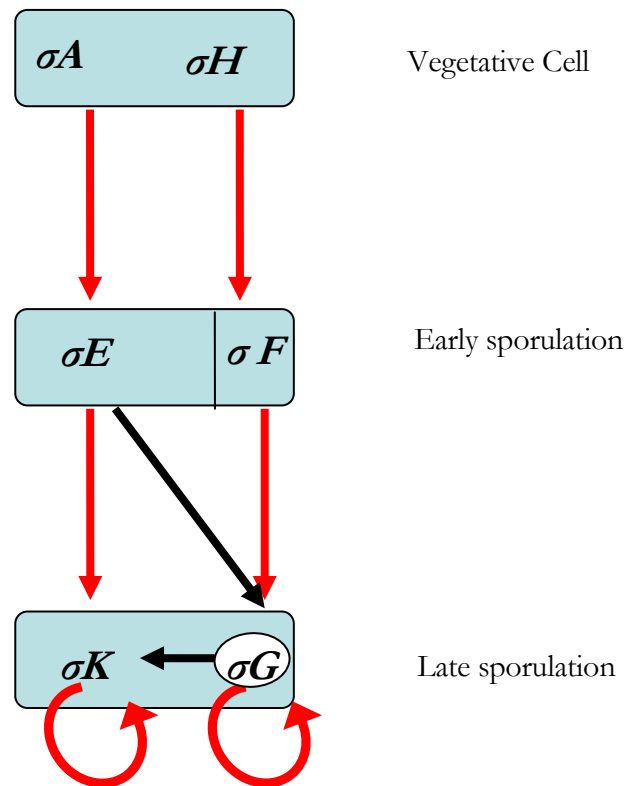
## Selection of *spoIIAC* as the Target for Gene Replacement

After review of the sporulation literature and past research, *spoIIAC* was selected as the target for gene replacement. The ideal sporulation mutant would be blocked at an early enough stage to prevent spores from forming, yet not negatively effect the production of protease. Since proteases and other extracellular enzymes are known to be produced during stationary phase and stage 0 of sporulation, a stage 0 mutant would be less than ideal. Stage II mutants produce normal levels of protease, yet are also blocked in sporulation. However, stage II mutants do not effect the expression of genes produced before asymmetric septation, making them a better gene target. Sigma F (*spoIIAC*) mutants are the logical choice because they also block the activation of  $\sigma E$ ,  $\sigma G$ , and  $\sigma K$ . *B. licheniformis spoIIAC* mutants produced by Gene SOEing have been constructed and shown to be defective in sporulation while able to produce normal levels of protease (Fleming *et al.*, 1995).



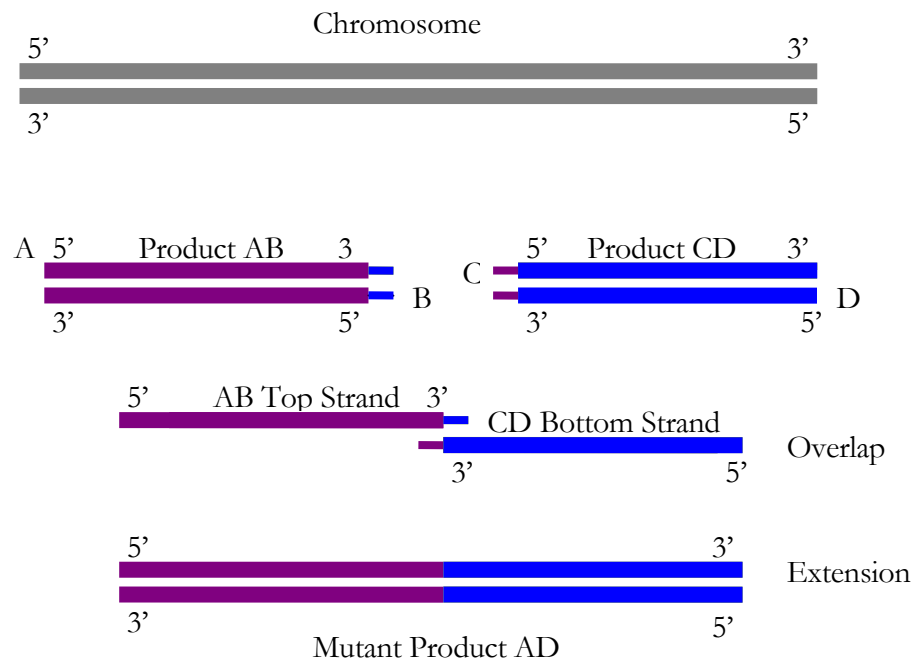
**Fig.1** Morphological Stages of Sporulation. Normally the vegetative cell divides down the middle during binary cell division. Upon entry into sporulation, an asymmetric septum is formed at one end of the cell, marking stage II. During stage III the septum migrates to the pole of the cell, engulfing the forespore compartment and forming a protoplast. Various cortex and coat proteins are deposited during stages IV and V, respectively. The spore has developed its full capacity to resist harsh environmental conditions by stage VI, and is freed from the lysed mother cell during stage VII. The spore is then able to germinate into a normal vegetative cell when conditions are better.





**Fig. 2** Sigma Factor Cascade. This diagram shows the crisscross regulation of  $\sigma$  factors during sporulation. Red arrows indicate that the  $\sigma$  factor is required for the transcription of the next  $\sigma$  factor indicated. Black arrows indicate that one or more products transcribed under the control of that  $\sigma$  factor are needed for the activation of the next  $\sigma$  factor.





**Fig. 4** Splicing by Overlap Extension (Gene SOEing). Four PCR primers (A, B, C, and D) are used in three PCR reactions. Fragment AB is amplified in one tube, fragment CD is amplified in a second tube. Primers B and C contain the 5' 'add on' sequences that allow one strand from AB to overlap with one strand from CD after AB and CD are mixed, denatured and reannealed. In this third PCR reaction, using primers A and D, Taq polymerase extends the 3' OH at the overlap region to create a double stranded molecule with a precise deletion engineered between primers B and C.

## MATERIALS AND METHODS

### Bacterial Strains and Growth Conditions

*B. licheniformis* PWD-1 (ATCC 53757) was used as the wild-type host strain in this study. *B. subtilis* DB104 (Kawamura and Doi, 1984) and *Escherichia coli* DH5 $\alpha$  (Invitrogen, CA) were also used as hosts in subcloning. Most strains were grown overnight, unless otherwise noted, at 37°C. *B. licheniformis* PWD-1 is the only strain of the three used that grows well at 50°C. Specific media used in this study is indicated where appropriate and further described in Appendix B.

### PCR Primers used in the Splicing by Overlap Extension Reaction

The four primers used to construct the reaction are as follows:

**A 5'-(CCATACTGCAGACGATGGATGAACTGACCGA)-3'**

**B 5'-(TCCTGAACGGTCGGAATCCCAGACCAAACAAGACGCA)-3'**

**C 5'-(TGCGTCTTGTTTGGTCTGGGATTCCGACCGTTCAGGA)-3'**

**D 5'-(AGCCGCTGCAGGATTGCGTCTTGTCTTTG)-3'**

The sequences of the primers used were selected from the sequenced *B. licheniformis* *spoIIA* operon (NCBI, GI: 304170, M25260). NetPrimer Software (Premiere Biosoft Int.) was used to deduce T<sub>m</sub>, GC%, primer-dimer, hairpins, and palindromes of potential primers. There were several important considerations in primer design. First, primers B and C would define the deleted region of *spoIIAC*. Therefore primer B was placed just downstream of the start of *spoIIAC*, to ensure that a partially functional  $\sigma^F$  would not be produced. Second, primer C needed to be far enough downstream from primer B to ensure a large deletion. The final consideration in determining the

deletion area was based upon nucleotide BLAST searches which revealed a high level of homology in the region of the gene among related Bacilli. In this study a 256bp deletion was engineered that spanned nucleotides 141 to 397 of the 767 b.p. *spoIIAC* gene. Primers A and D included *PstI* restriction sites for use in cloning. The nucleotide sequence of the desired deletion is indicated in Figure 5.

### **Preparation of Genomic DNA from *B. licheniformis* PWD-1**

In preparation of PCR, genomic DNA from *B. licheniformis* PWD-1 was isolated using the following method modified from Ausubel *et al.*, 1998: 3mL cultures grown in LB were pelleted by centrifugation at maximum speed for one minute. Cells were washed and resuspended in TE buffer and then lysed by 37°C incubation with lysozyme, RNase, SDS, and proteinase K for 1 hour. Following lysis, NaCl and CTAB/NaCl solutions were mixed in, and incubated at 65°C for 10 min. The mixture was phenol extracted, isopropanol precipitated, and the pellet washed with 70% ethanol. The pellet was then resuspended in TE buffer and detected by electrophoresis using a 1% agarose gel.

### **PCR Amplification**

Hotmaster Taq polymerase (Eppendorf, AG), 0.5ul, was used in PCR Reactions. Fifty microliter reactions were set-up using  $Mg^{2+}$  buffer at 2.5mM final concentration. dNTPs were added to a final concentration of 0.25mM each, while the appropriate primers were added to a final concentration of 0.5uM each. PCR was performed as follows: Initial denaturation 94°C, 2 min; denaturation 94°C 20sec; annealing 55°C, 20sec; extension 65°C, 1 min; final extension 65°C, 2 min. The 528bp AB fragment and 296bp CD fragment were PCR amplified in separate tubes.

Thirty cycles each were performed using an Eppendorf Mastercycler thermocycler. PCR products were purified using a PCR Clean-Up kit (Qiagen) as directed by the manufacturer. Products AB and CD were then added as templates for the third PCR reaction to create AD. The 787bp gene SOEing product, AD, was purified by gel extraction kits (Qiagen) as directed by manufacturer. A fourth PCR of the full length AD fragment (1043bp) without the deletion was performed as a control. The Gene SOEing product, AD, is henceforth referred to as *spoIIAC*Δ.

### **Subcloning in *Escherichia coli* DH5α**

The purified 787bp *spoIIAC*Δ fragment was ligated to cloning vector pCR2.1 (Invitrogen, CA). This vector has overhanging “T” nucleotides which keep it linearized in its native state. Ligation can occur because of the intrinsic terminal transferase activity of Taq polymerase, resulting in an “A” nucleotide on the end every molecule amplified. The resulting A-T matchup between insert and vector allows any Taq-amplified PCR fragment to be cloned. A 20ul ligation reaction was set up as follows: 4ul PCR 2.1, 1ul *spoIIAC*Δ, 4ul 5X ligation buffer (Promega), 1ul ligase (BWT), 10ul dH<sub>2</sub>O. The reaction was incubated at 12°C overnight.

*E. coli* DH5α was made competent by the following method: Cells were grown overnight in LB at 37°C; 1mL was transferred to 30mL fresh LB and grown for 2 hrs at 37°C. The cells were pelleted at 6000 RPM at 4°C, resuspended in 15ml ice cold CaCl<sub>2</sub> (100mM), and allowed to sit for 2 hrs. The cells were then centrifuged at 4°C and resuspended in 2ml cold CaCl<sub>2</sub>. The cells were stored at 4°C for 1 hr. A 150ul suspension of competent cells was transferred to a sterile microcentrifuge tube and mixed with the 20ul ligation mixture. The tube was placed on ice for 30 min, heat shocked at 42°C for 2 min., and then returned to ice for 2 min. LB broth, 800ul, was

added and cells were grown at 37°C for 1 hr. The resulting transformants were plated onto LB agar containing 20ug/ul ampicillin.

Putative transformants were first selected by blue/white screening. Since the cloning site in pCR2.1 is in the middle of the *lacZ* gene, insertion of the PCR product destroys *lacZ* function and cells are white. Putative transformants were further analyzed by miniprep (Sambrook and Russell, 2001). The insertion of the PCR product was verified by *HindIII*, *EcoRI*, and *PstI* digestion. The restriction digest consisted of the following: 5ul plasmid DNA, 2ul 10X buffer (A or H as appropriate), 1ul indicated restriction enzyme, and 12ul dH<sub>2</sub>O. The digest was visualized on 1% agarose gel and compared with the expected size fragments derived from the plasmid map.

#### **Sub-Cloning in *B. subtilis* DB104**

Plasmid PCR2.1/*spoIIACA* was digested with *PstI* to release the *spoIIACA* fragment. *PstI*-digested *spoIIACA* was gel extracted and purified using Qiagen kits (Qiagen). Plasmid pE194 (ATTC 37128) was transformed into *B. subtilis* DB104 (Harwood and Cutting, 1990). A single colony was grown in SP1 at 37°C for 3 hr 45 min. One-half milliliter of the SP1 culture was transferred to 4.5ml SP2 medium. The cells were incubated for 90 min. at 37°C. EGTA (100mM, 50uL) was added, and the culture was incubated for 10 min at 37°C. One-half milliliter competent cell culture was mixed with 20ul of the ligation mixture. Cells were incubated for 90 min at 37°C and plated on LB containing 20ug/ul erythromycin.

Plasmid pE194 was miniprepped from *B. subtilis* DB104 using the following method: Transformants were inoculated into 3mL LB containing 20ug/ul and grown for 4 hrs at 37°C. Cells were centrifuged at maximum speed for 1 min, washed with SET buffer, and resuspended

into 80ul SET buffer. Lysozyme (Promega) was added to a final concentration of 4mg/mL. Lytic mix (200ul) was added. The tube was then vortexed and placed on ice for 5 min to allow cell lysis to occur. Potassium acetate (3M, 150ul) was added, and the tube was placed at -20°C for 20 min. The solution was centrifuged for 10 min at 4°C, and the supernatant transferred to a new tube. The mixture was phenol extracted and isopropanol precipitated. The DNA pellet was washed with 70% ethanol and resuspended in dH<sub>2</sub>O.

Miniprep plasmid pE194 was digested with *Pst*I as previously described. *Pst*I-digested pE194, 10 ul, was mixed with 5ul *Pst*I-digested *spoIIACA*, 4ul 5X ligase buffer (Promega), and 1ul ligase. The ligation reaction was allowed to incubate at 12°C overnight. The ligation mixture was transformed into *B. subtilis* DB104 as previously described. Erythromycin-resistant transformants were analyzed by miniprep and *Pst*I digests and gel-electrophoresis. In addition *spoIIACA* was PCR amplified using colony PCR of erythromycin-resistant transformants. The resulting plasmid was termed pSPO. The transformants served as reservoirs of pSPO for use in transformation of *B. licheniformis* PWD-1.

### **Transformation of pSPO in *B. licheniformis* PWD-1**

pSPO was transformed into *B. licheniformis* PWD-1 by protoplast transformation (Harwood and Cutting, 1990). Cells were grown in NBSG-X medium, collected by centrifugation at 6000rpm for 10 min. The cells were resuspended in one-tenth volume 1X SMM media. Lysozyme was added to 2mg/ml and the solution was mixed and incubated for 90 min at 37°C. Protoplasted cells were collected by centrifugation, washed with equal volume SMMP+, and centrifuged again. The pellet was resuspended in one-half volume SMMP+. The protoplasted cell solution (0.5mL), was added to a 40ul mixture containing equal amounts plasmid and 2X SMM. The mixture of cells, plasmid,



and SMM were immediately transferred to 1.5mL PEG. SMMP+ (5mL), was added after 2 min. Cells were then centrifuged, resuspended in 1mL SMMP+, and incubated at 37°C for 90 min.

Transformants were plated on regeneration agar containing 20ug/ul erythromycin. Genetic verification of transformation was accomplished by colony PCR of transformants using primers A and D in the method described above.

### **Gene Integration and Excision**

The method of gene replacement in this experiment takes advantage of the stimulatory effect of rolling-circle replication of thermosensitive plasmids on intramolecular recombination (Hamilton *et al.*, 1989; Biswas *et al.*, 1993). Because thermosensitive plasmids can be maintained in the bacteria before selection is applied, gene replacement in poorly-transformable strains is possible. Since plasmid pE194 is naturally temperature sensitive above 42°C (Noirot *et al.*, 1987), integrants can be selected on the basis of growth at 50°C. Integration of plasmid pE194/*spoIIACA* was accomplished by growing cells at 50°C in LB containing 20ug/mL erythromycin. Those transformants that grew at 50°C were transferred to LB without antibiotics at 37°C. Cells were transferred to fresh LB, grown at 37°C, and transferred to fresh LB several times to select for excision of the plasmid and plasmid cured cells. Erythromycin-sensitive colonies were selected and further analyzed.

### **Verification of Gene Replacement by PCR**

To verify that the deletion was chromosomally based and not plasmid-borne, a fifth primer labeled 'Left' was introduced. The sequence is not found in plasmid pE194/*spoIIACA*, therefore successful amplification must be chromosomally based. The sequence of primer 'Left' is as

follows: 5'-**CCAGGAGGATGAGGATGAGC**-3'. The primer was designed 462bp upstream from primer A and intersects the start codon of *spoIIA4*. The erythromycin-sensitive colonies were subjected to colony PCR using the Left primer and primer D using PCR conditions described above. The results were visualized on a 1% agarose gel. Colonies containing PCR fragments of the desired size were selected as possible mutants. One clone was selected as the final mutant and was named *B. licheniformis* WBG-2.

### **DNA Sequencing**

PCR of *B. licheniformis* WBG-2 and PWD-1 was performed using primer sets A-D and Left-D as described above. PCR samples were purified (Qiagen PCR purification kit), diluted in dH<sub>2</sub>O to 2.5 ng/100 bases/ul. Sequences were analyzed at Iowa State University (Ames, Iowa) using ABI Model 377 Prism DNA Sequencers. The sequences were aligned using Bioedit Software (Tom Hall, North Carolina State University) using a gapped alignment.

### **Heat Treatment Assay**

The heat treatment assay is a standard assay for spores, since spores are very heat resistant and vegetative cells are not. *B. licheniformis* spores are among the most heat-resistant compared to related *Bacilli* (Janštová *et al.*, 1991, Nicholson *et al.*, 2000). To test if the mutant produced spores, a simple heat treatment assay was employed. Cultures of *B. licheniformis* PWD-1 and WBG-2 were grown in Modified Schaeffer's Sporulation medium (Schaeffer *et al.*, 1965, Appendix II) for 24 hr and 37°C to allow sufficient numbers of cells to sporulate. The cultures were boiled for various periods of time, diluted, and survivors plated on LB agar.

## Determination of Proteolytic Activity

Azocasein assays, Azokeratin assays, and milk agar plates were used as measurements of proteolytic activity. The azocasein and azokeratin assays rely on the fact that proteases can cleave the azo group from their substrates, casein and keratin respectively. The azo group is orange colored and release of this molecule can be followed with a spectrophotometer. For both assays, cells were grown in 1% ground feather medium at 37°C or 50°C. Samples were taken from cultures of *B. licheniformis* PWD-1 and WBG-2 periodically during growth.

Azocasein assay: 5ul of sample was added to 800ul reaction mixture (.2% azocasein in .05M Phosphate buffer, pH 7.5). The reaction mixture is mixed and incubated for 10min at 37C. TCA solution (10%, 200ul) is added and the tube is placed on ice for 5 min. to stop the reaction. The tube is centrifuged for 5 min at maximum speed and the supernatant is measured at 450nm.

Azokeratin Assay: 10ul of sample is added to 990ul reaction mixture (5mg azokeratin in .05M Phosphate Buffer, pH 7.5). The reaction mixture is incubated with shaking at 50°C for 30 min. TCA solution (10%, 200ul), is added to stop the reaction. The mixture is centrifuged for 10 min at maximum speed and the supernatant is measured at 450nm.

Milk Agar Assay: Milk agar plates were employed to screen clones for proteolytic activity. The assay was judged visually by judging the diameter of clearing zones due to hydrolysis of milk proteins.

### PCR Amplification of *kerA*

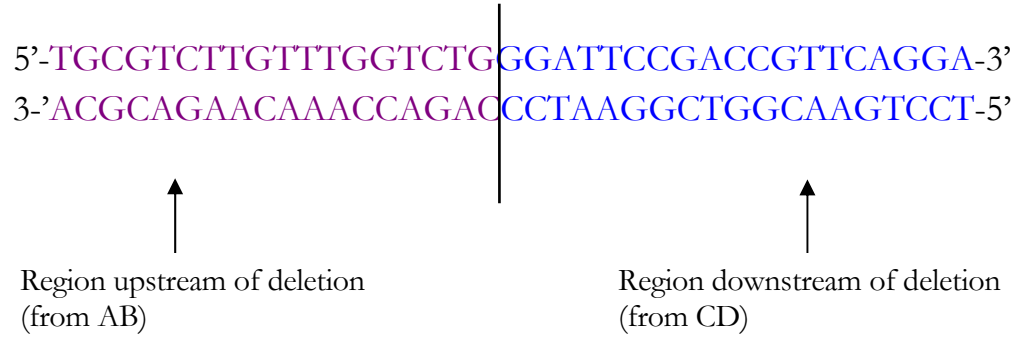
In addition to the determination of enzyme activity, the *kerA* gene was PCR amplified. Overnight cultures of *B. licheniformis* PWD-1 and the WBG-2 were boiled, and the supernatant used as the template in a PCR reaction. The primers used for amplification are as follows:

**KER3: 5'-ATT TAA ATT ATT CTG AAT AAA GAG G-3'**

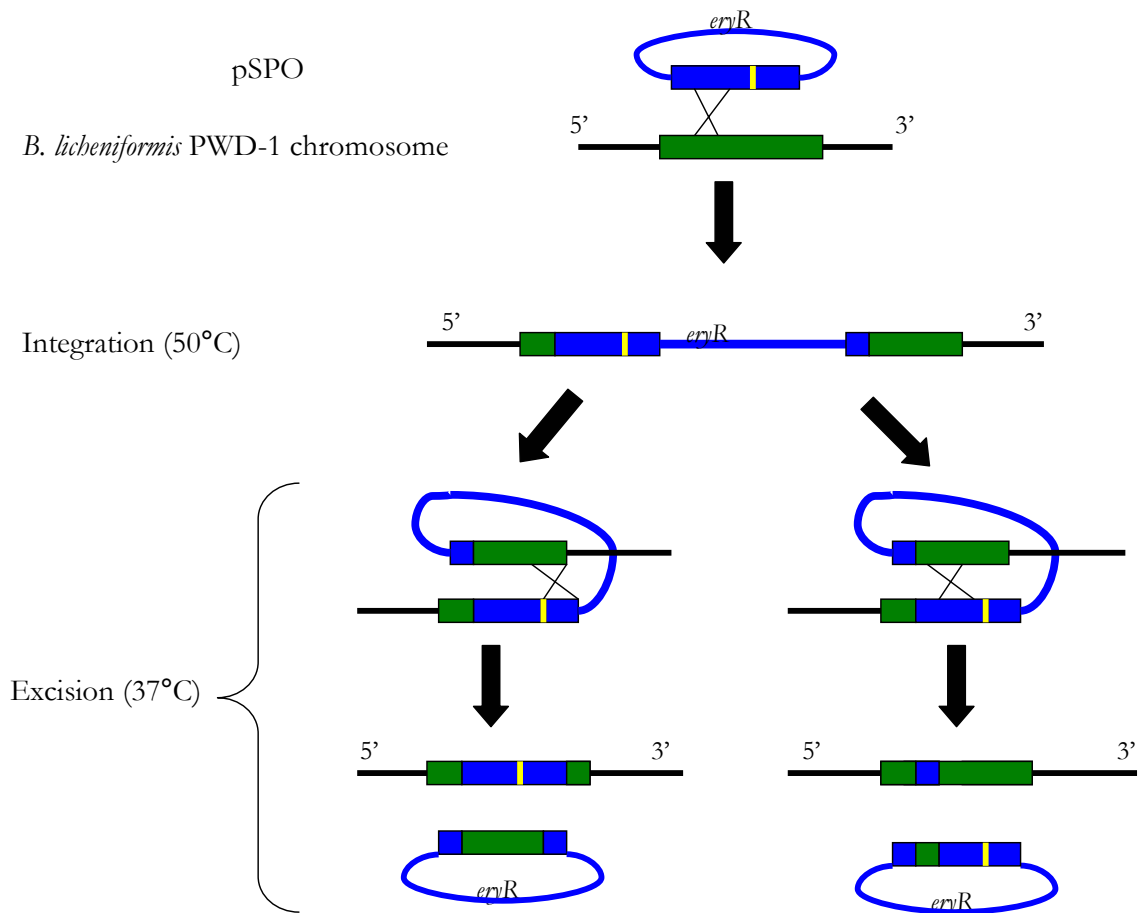
**KER4: 5'-CAC TAG CTT TTT CTA TAT GCT ATT TG-3'**

### Electron Microscopy

*B. licheniformis* PWD-1 and WBG-2 were grown in Modified Schaeffer's Sporulation Medium for 24hr at 37°C to allow for sporulation. Cells were harvested by centrifugation, embedded in Spur's resin, and thin-sectioned. Cells were stained with aqueous uranyl acetate for 1 hr and counterstained with lead citrate for 4 min. Samples were then viewed on a JEOL – 100S Transmission Electron Microscope at 30,000X to 35,000X magnification.



**Fig. 5** Nucleotide Sequence of Predicted Overlap Region



**Figure 6.** Gene Replacement Strategy. The gene replacement strategy (Hamilton *et al.*, 1989; Biswas *et al.*, 1993) results in the 256b.p. deletion (yellow) being located on the chromosome of *B. licheniformis* PWD-1. The first step is integration of plasmid pSPO (blue) into the *B. licheniformis* chromosome (green) by homologous recombination. The temperature-sensitive nature of pSPO allows integrants to be identified by growth at 50°C in the presence of erythromycin. When the temperature is lowered to the permissive temperature of 37°C, a second recombination event occurs, resolving the plasmid. If this second recombination event occurs at a second site (left), the desired gene replacement occurs. However, the second recombination event may also lead to restoration of the original copy of the gene on the chromosome (right).

## **RESULTS**

### **Splicing by Overlap Extension**

The 528bp AB product and the 296bp CD product were amplified separately as shown in Figure 7. Each product was used as the template for the SOEing reaction generating product AD. Three bands were produced, but the band corresponding to the expected 787bp size of AD was gel extracted and purified (Figure 7).

### **Sub-Cloning in *Escherichia coli* DH5 $\alpha$**

Purified AD (*spoIIACA*) was ligated to vector pCR2.1 (Invitrogen, CA) and transformed into *E. coli* DH5 $\alpha$  as described. Transformants were initially selected by blue/white screening, and further examined by miniprep and restriction digests. Figure 9 shows that digestion of plasmid DNA by *HindIII*, *EcoRI*, and *PstI* produced bands of the expected sizes. The 771bp *spoIIACA* insert was digested from the plasmid by *PstI*, gel extracted, and purified.

### **Sub-Cloning in *B. subtilis* DB104**

Plasmid pE194 was linearized with *PstI* and ligated to *PstI*-digested *spoIIACA* (Figure 10, ligation reaction not shown). This plasmid, pSPO (Figure 11), was transformed in *B. subtilis* DB104. Transformants were selected on LB containing 20ug/ml erythromycin. Plasmid pSPO was isolated from transformants and digested with *PstI* (Figure 12). The restriction digest, as well as PCR (not shown), verified the transformation. This *B. subtilis* DB104 transformant was used as a reservoir of plasmid pSPO, for use in transforming *B. licheniformis* PWD-1.

### **Transformation of pSPO in *B. licheniformis* PWD-1**

*B. licheniformis* PWD-1 transformants were selected on Regeneration Agar containing erythromycin. Figure 13 shows a colony-PCR agarose gel using primers A and D to verify the transformants.

### **Gene integration and excision**

A single positive transformant was streaked onto LB plates containing erythromycin and grown at 50°C. Survivors were presumed to be integrants. Integrants were inoculated in LB without antibiotics and grown at 37°C. This was repeated several times to select for excision of the plasmid and for plasmid curing. The organisms were then grown in LB with and without erythromycin, and several erythromycin-sensitive clones were selected for genetic analysis.

### **Verification of gene replacement by PCR**

Clones were tested for integration/excision by P.C.R. using primer sets A-D and Left-D and visualized on a 1% agarose gel (Figure 14). The expected size fragments for the mutation with primers A-D and Left-D were 787bp and 1249bp respectively. Five clones produced the expected size fragments (Clones #2,3,4,5,6), while another produced intermediate products of the wild-type and mutant *spoIIACA* gene (Clone #1). Clone #6 was selected as the final mutant strain and renamed *B. licheniformis* WBG-2. *B. licheniformis* WBG-2 was used in all tests.

### **DNA Sequencing**

Results from sequencing of the four PCR products revealed a precise deletion between primers B and C exactly as expected. The gapped alignment of the sequences is shown in Appendix A



while a graphical interpretation is depicted in Figure 16. The nucleotide sequences showed approximately 99% homology with the Genebank sequence used to originally design the sequence. The use of the Left primer confirmed that the deletion was chromosomally based; thus the *B. licheniformis* WBG-2 contains the desired  $\sigma^F$  deletion.

### **Heat Treatment Assay**

The heat treatment assay is a simple and commonly used method to determine the presence or absence of spores. In this study, *B. licheniformis* was shown to have lost a significant amount of wet-heat resistance compared to the wild-type PWD-1 (Figure 17). No survivors were detected after 5 min. of boiling, even after many repetitions. In contrast, the highly heat-resistance spores of *B. licheniformis* PWD-1 could be detected as long as 20 min. or longer.

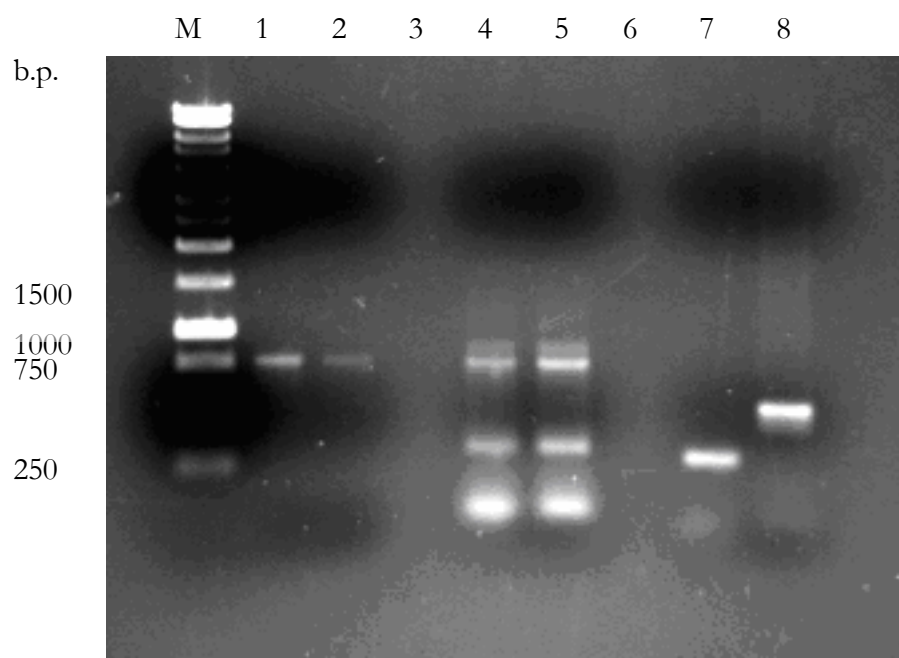
### **Protease Production**

The results of the azokeratin and azocasein assays are shown in Figure 18. *B. licheniformis* WBG-2 was found to secrete slightly higher levels of protease compared with *B. licheniformis* PWD-1. The data suggest that the mutation may be slightly advantageous to keratinase production. However, cell numbers were similar between the two strains. Figure 15 shows that *kerA* was correctly PCR amplified in both the wild-type and mutant strains.

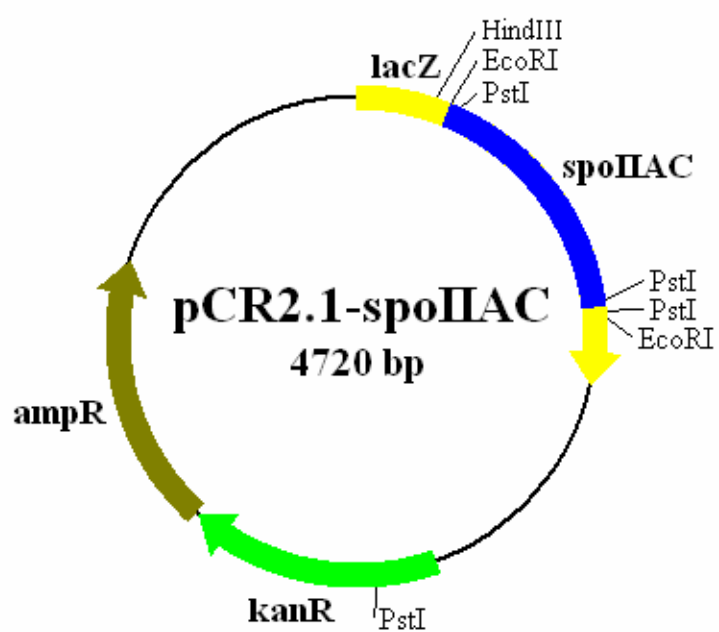
### **Electron Microscopy**

The use of electron microscopy further confirmed the fact that the mutant was totally asporogenic. No spores were detected among many thin-sectioned cells in the mutant. Many of the cells, like the one shown in Fig. 19 B, were clearly disporic, as expected. Other cells of the

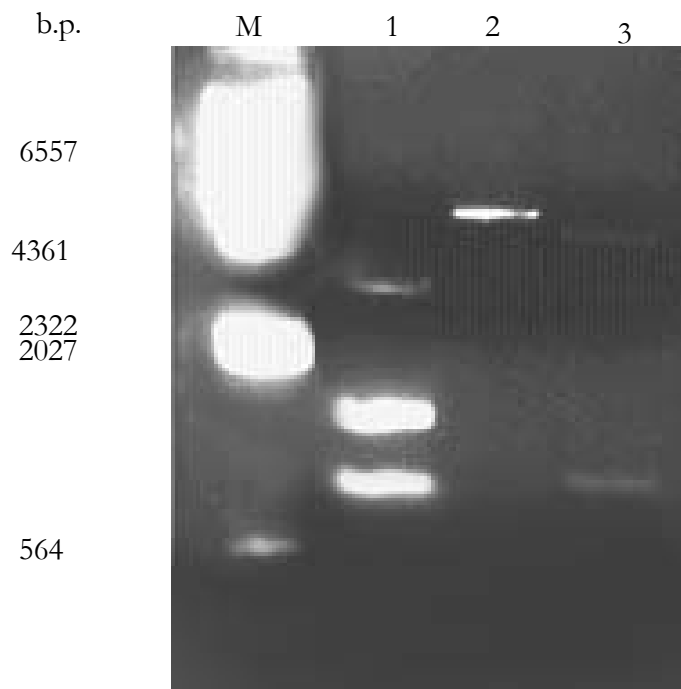
mutant strain were dividing by binary fission or were not in the process of division at all. However, no cells were observed that progressed beyond stage II (septation) of sporulation. When *B. licheniformis* PWD-1 was observed, all of the morphological stages of progression could be observed. The photo from Fig. 19B shows a matured endospore from *B. licheniformis* PWD-1.



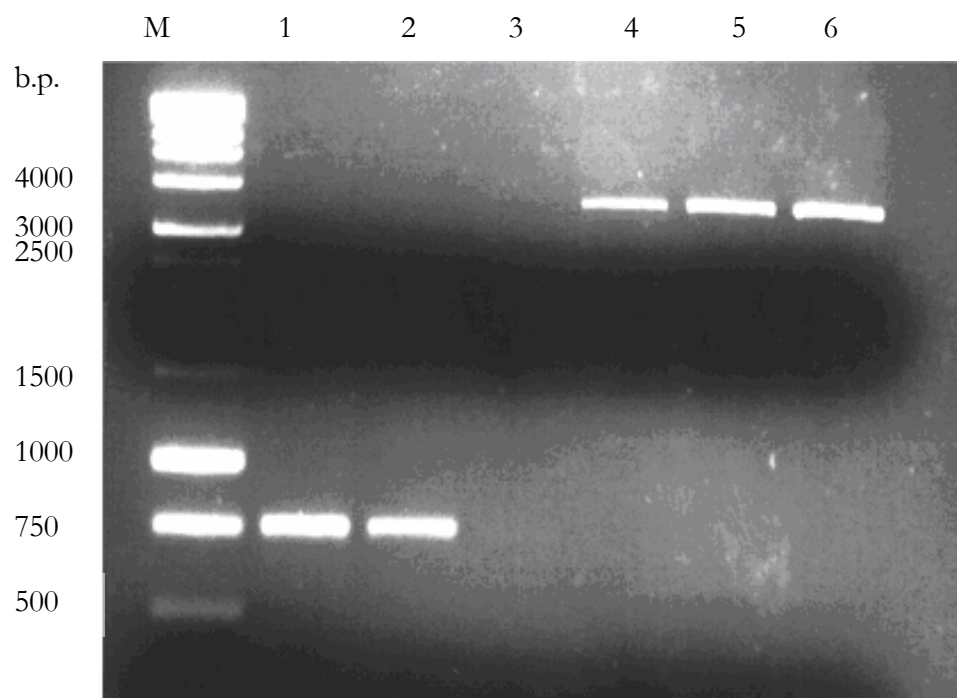
**Fig. 7** Splicing by Overlap Extension PCR. Products AB and CD were separately amplified and added together to create product AD. The 790bp fragment was gel extracted and purified. Lane contents from left: (M) 1 kb DNA Ladder, (1) purified AD (787bp), (2) purified AD (787bp), (3) Blank, (4) AD (787bp), (5) AD (787bp), (6) Blank, (7) CD (296bp), (8) AB (528bp).



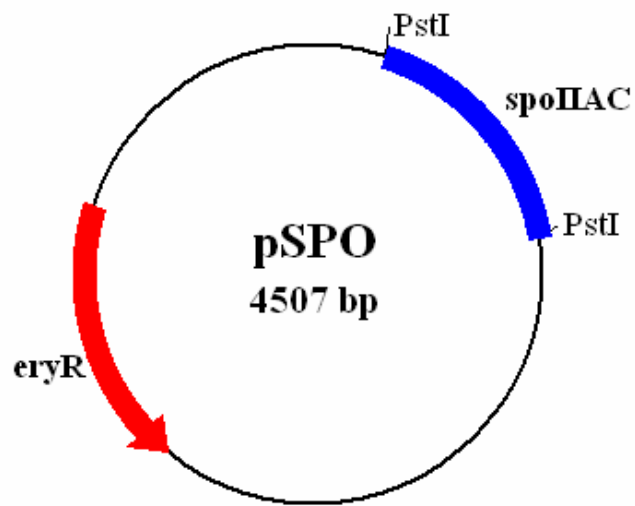
**Fig. 8** Plasmid map of PCR2.1-*spoIIAC*. Product AD (*spoIIAC*) was ligated to pCR2.1, knocking out *lacZ* function.



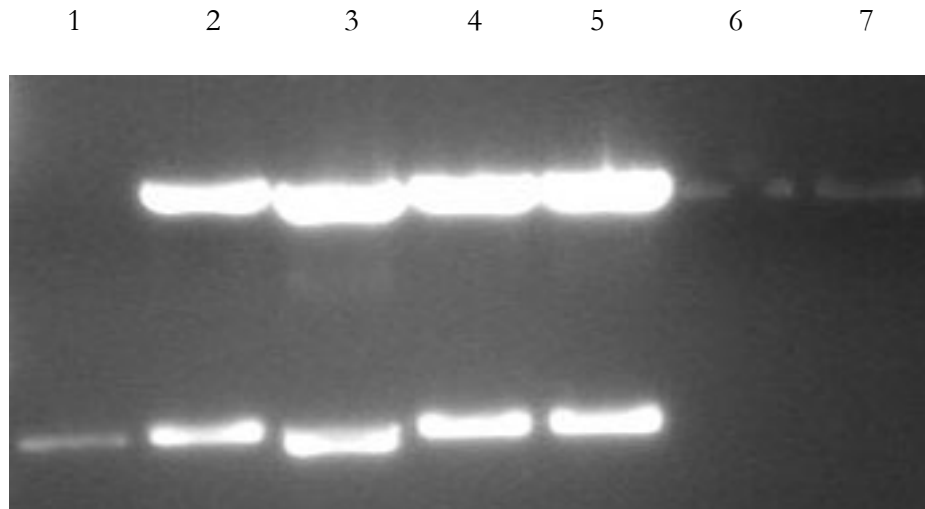
**Fig. 9** Screening of *E. coli* transformant by restriction digest. Lane contents from left: (M): Lambda/*Hind*III Marker, (1) *Pst*I cut (2734bp, 1190bp, 771bp, 22bp), (2) *Hind*III cut ( 4720bp), (3) *Eco*RI cut ( 3913bp, 807bp).



**Fig. 10** *PstI* digests of *spoIIACΔ* and pE194 prior to ligation. Lane contents from left: (M) 1 kb DNA Ladder, (1) *PstI*-cut *spoIIACΔ* (771bp), (2) *PstI*-cut *spoIIACΔ* (771bp), (3) Blank (4) *PstI*-cut pE194 ( 3.9kb), (5) *PstI*-cut pE194 ( 3.9kb), (6) *PstI*-cut pE194 ( 3.9kb)

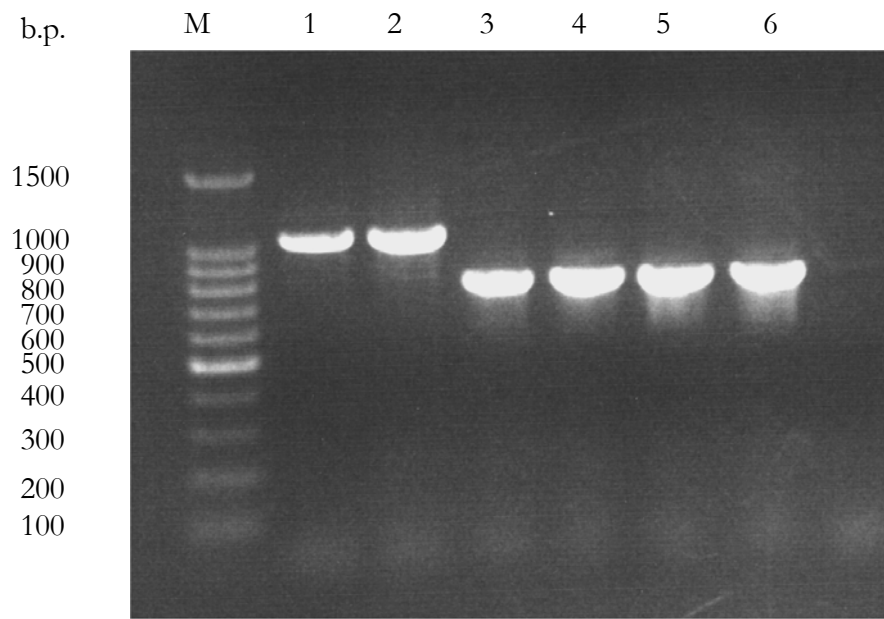


**Fig. 11** Plasmid Map of pSPO. pSPO was created by ligation of *spoIIAC* to pE194 at the *PstI* restriction site. *eryR* includes erythromycin resistance.

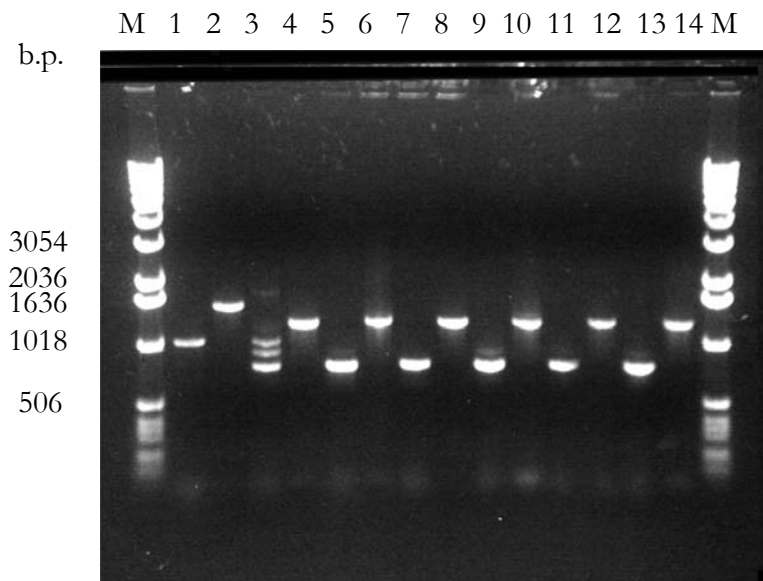


**Fig. 12** Screening of *B. subtilis* DB104 transformants by restriction digest. Lane contents from left: (1) *PstI*-cut *spoIIAC4* ( control, 771bp), (2) *PstI*-digested plasmid from transformant (3.9kb, 771bp), (3) *PstI*-digested plasmid from transformant (3.9kb, 771bp), (4) *PstI*-digested plasmid from transformant (3.9kb, 771bp), (5) *PstI*-digested plasmid from transformant (3.9kb, 771bp), (6) *PstI*-cut pE194 (control, 3.9kb ), (7) *PstI*-cut pE194 (control, 3.9kb)

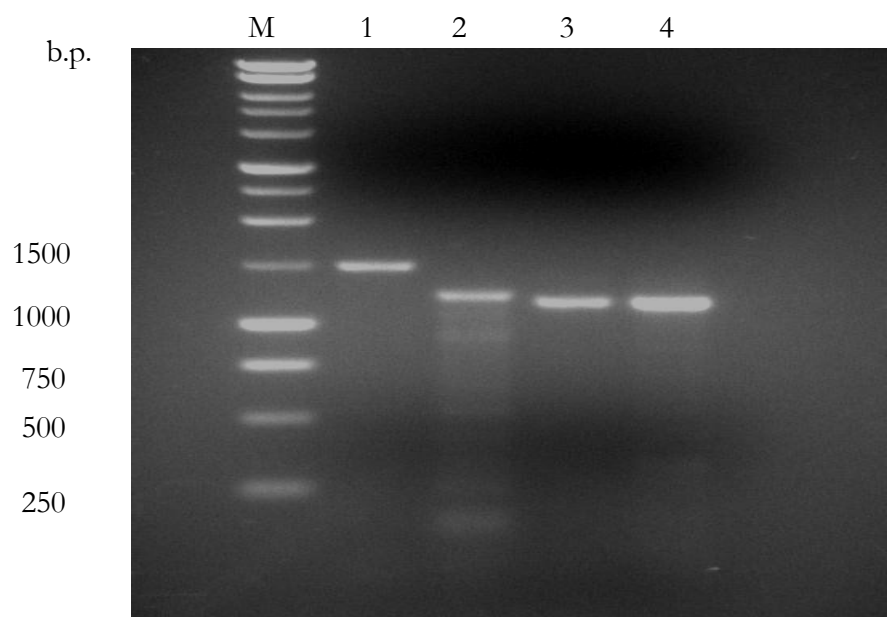




**Fig. 13** Screening of *B. licheniformis* PWD-1 transformants by Colony PCR. Colonies were analyzed using primers A and D and visualized on 1% agarose gel. Lane contents from left: (M) 100bp DNA Ladder, (1) PWD-1 ( 1043bp), (2) PWD-1 ( 1043bp), (3) *B. subtilis* DB104 transformant (787bp), (4) *B. licheniformis* PWD-1 transformant ( 787bp), (5) *B. licheniformis* PWD-1 transformant (787bp), (6) *B. licheniformis* PWD-1 transformant ( 787bp).



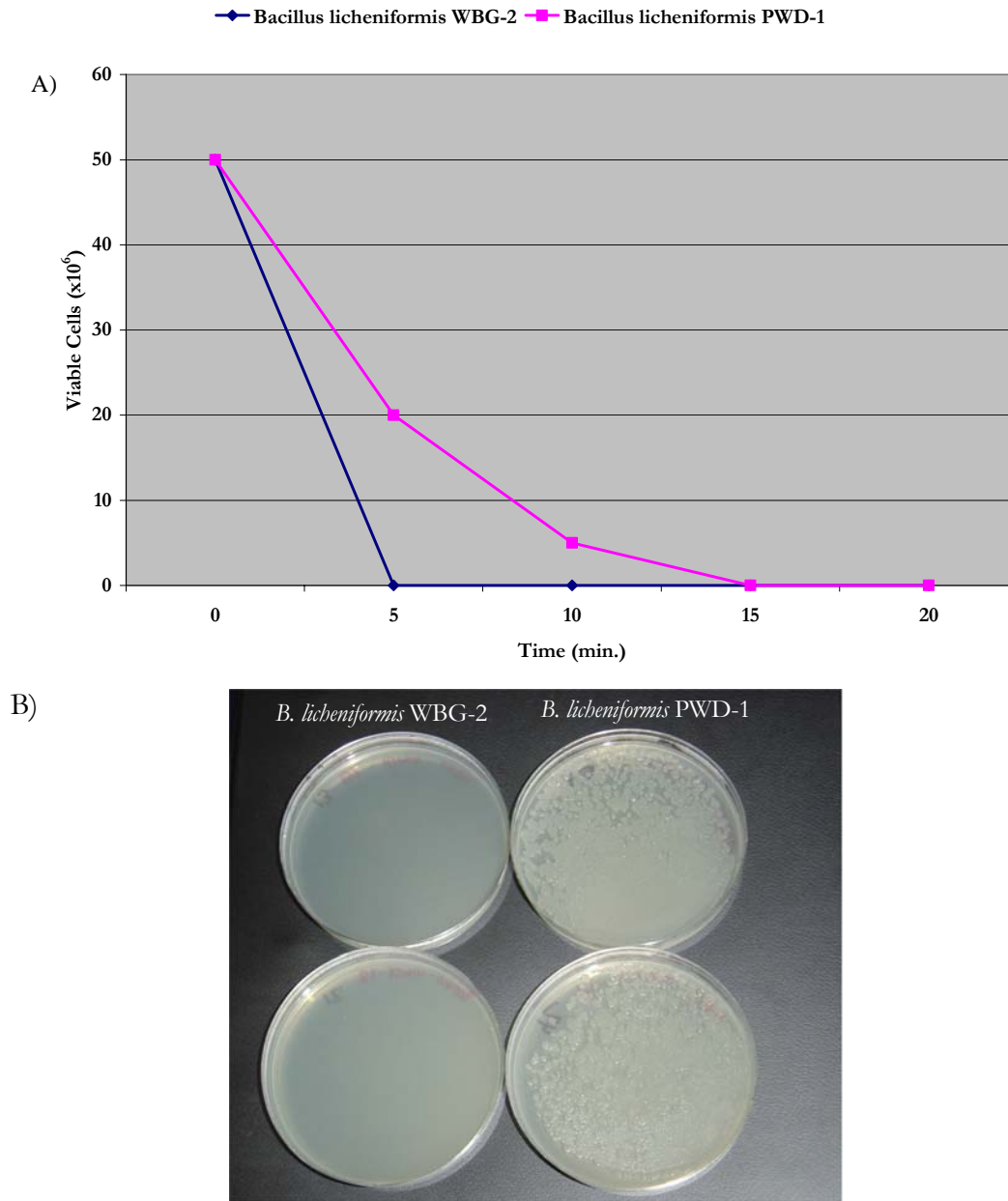
**Fig. 14** Screening of *B. licheniformis* PWD-1 Clones by Colony PCR. Colonies were analyzed using primers A-D and Left-D and visualized on 1% agarose gel. Lane contents from left: (M) 1 kb DNA Ladder, (2) *B. licheniformis* PWD-1 (Primers A-D, 1043bp), (2) *B. licheniformis* PWD-1 (Primers Left-D, 1505bp), (3) *B. licheniformis* PWD-1 Clone #1 (Primers A-D, 787bp), (4) *B. licheniformis* PWD-1 Clone #1 (Primers Left-D, 1249bp), (5) *B. licheniformis* PWD-1 Clone #2 (Primers A-D, 787bp), (6) *B. licheniformis* PWD-1 Clone #2 (Primers Left-D, 1249bp), (7) *B. licheniformis* PWD-1 Clone #3 (Primers A-D, 787bp), (8) *B. licheniformis* PWD-1 Clone #3 (Primers Left-D, 1249bp), (9) *B. licheniformis* PWD-1 Clone #4 (Primers A-D, 787bp), (10) *B. licheniformis* PWD-1 Clone #4 (Primers Left-D, 1249bp), (11) *B. licheniformis* PWD-1 Clone #5 (Primers A-D, 787bp), (12) *B. licheniformis* PWD-1 Clone #5 (Primers Left-D, 1249bp), (13) *B. licheniformis* PWD-1 Clone #6 (Primers A-D, 787bp), (14) *B. licheniformis* PWD-1 Clone #6 (Primers Left-D, 1249bp), (M) 1 kb DNA Ladder.



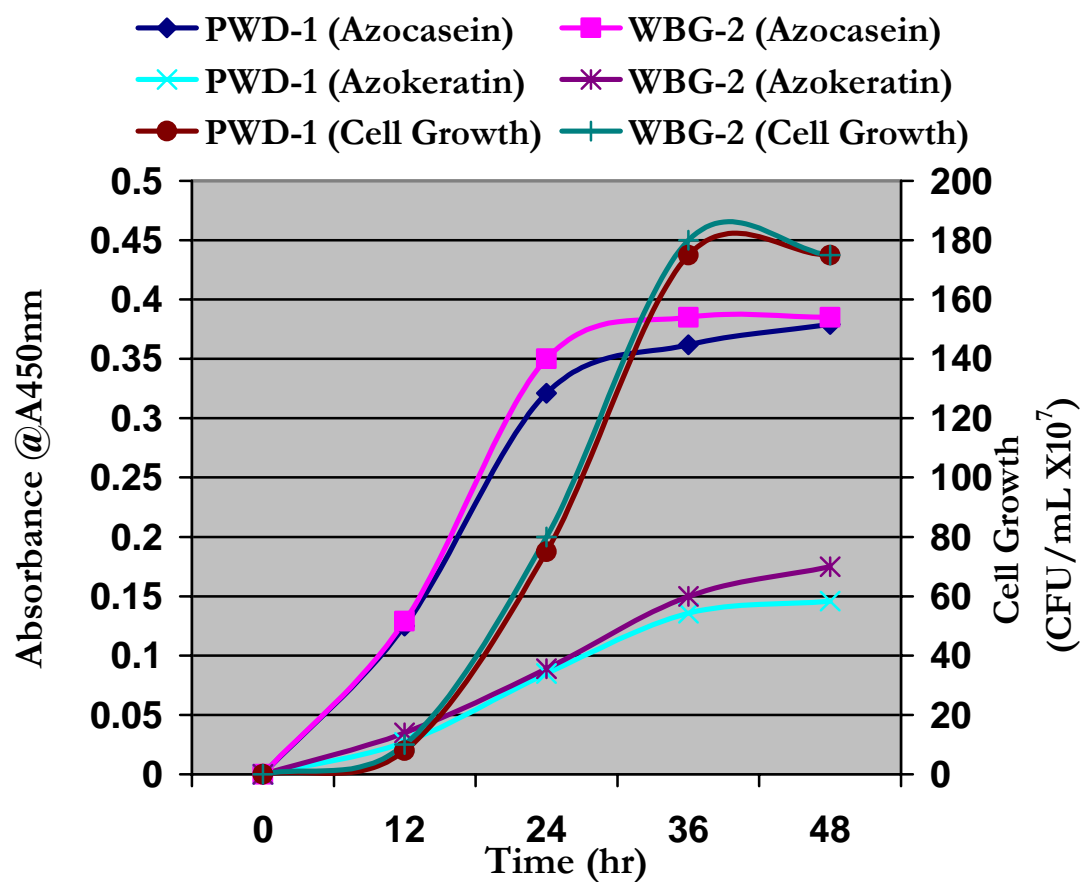
**Fig. 15** Amplification of *kerA* from *B. licheniformis* PWD-1 and *B. licheniformis* WBG-2 using Colony PCR. Lane contents from left: (M) 1 kb DNA Ladder, (1) *B. licheniformis* PWD-1 (Primers Left-D, 1505bp), (2) *B. licheniformis* WBG-2 (Primers Left-D, 1249bp), (3) *B. licheniformis* PWD-1 (Primers KER3-KER4 1211bp), (4) *B. licheniformis* WBG-2 (Primers KER3-KER4, 1211bp)



**Fig. 16** Graphical view of Sequenced PCR Products



**Fig. 17** Heat Treatment Assay. *B. licheniformis* PWD-1 and *B. licheniformis* WBG-2 were grown in Schaeffer's Sporulation medium for 24 hours, and then heated at boiling (100°C). Samples from various time points were plated onto LB agar and survivors counted. A) Killing curve of *B. licheniformis* PWD-1 and *B. licheniformis* WBG-2 at 100°C B) Photograph of 10 min. samples of both strains.



**Fig.18** Proteolytic Activity and Cell Growth

A)



B)



**Fig. 19** Electron Micrograph of *B. licheniformis* PWD-1 and *B. licheniformis* WBG-2. A) A developing spore of *B. licheniformis* PWD-1. B) *B. licheniformis* WBG-2 sporulation mutant showing the disporic phenotype.

## DISCUSSION

The goal of this study was to develop an asporogenic mutant of *B. licheniformis* PWD-1. Such a mutant, *B. licheniformis* WBG-2, was successfully created using the Splicing by Overlap Extension PCR and the use of the thermosensitive vector pE194. This method allowed the creation of a precise 256bp deletion in *spoIIAC*, as verified by PCR and DNA Sequencing. Because the transformation efficiency of *B. licheniformis* is very low, this method proved very useful. In-vivo gene replacement was accomplished with only one *B. licheniformis* PWD-1 transformant. The mutant is not resistant to erythromycin, thus this method is also advantageous because the vector sequences are likely lost.

The results of this study correlate with those of Fleming *et al.*, who first used SOEing to make a deletion in *spoIIAC* of *B. licheniformis*. Fleming reported constructing an 806bp AD fragment containing a 372b.p. deletion, while a 787bp AD fragment containing a 256bp deletion was used in this study. Although the deletion reported here is smaller, it overlaps with the deletion reported by Fleming and should be sufficient to totally disrupt *spoIIAC* transcription. The gene replacement strategy using pE194 was also similar to that of Fleming. Although both studies used DNA sequencing, this study used a PCR primer upstream of the deletion to confirm gene replacement. The use of this upstream primer proved more useful in confirming gene replacement than traditional methods such as Southern-blotting, provided as in this case, the upstream sequence is known.

As predicted, the  $\sigma^F$  mutant is totally asporogenic. The results from the heat treatment assays and electron micrographs confirm this. The disporic phenotype observed is consistent with previous studies of *B. licheniformis* and *B. subtilis* *spoIIA* mutants such as reported by Fleming *et al.*



The mutation does not appear to have any significant detrimental effect on growth or production of keratinase. In fact both azokeratin and azocasein assays reveal that the mutant produces slightly more protease than does wild-type PWD-1. This was an unexpected result, as  $\sigma^F$  mutants of *B. licheniformis* were shown to produce nearly identical amounts of protease by Fleming *et al.* The reason for this is unclear; however it is possible that increased  $\sigma^H$  accumulation in the mutant is responsible for the slight increase in protease. As the amount of protease produced greatly depends on the media and growth conditions used, *B. licheniformis* WBG-2 growth conditions should be optimized.

There is no evidence of suppressor mutations, and the chance of reversion with such a large deletion should be minimal. In summary, the method employed in this study created a sporulation mutant that retains important industrial and commercial capabilities. A similar method could next be used to inactivate all proteases in the sporulation mutant, which could be an advantageous expression host. Further study of this mutant should be conducted to examine fermentation scale-up conditions and processing of keratinase.

## BIBLIOGRAPHY

- Amaya E, Khvorova A, Piggot PJ. "Analysis of promoter recognition in vivo directed by  $\sigma^F$  of *Bacillus subtilis* by using random-sequence oligonucleotides". *J Bacteriol.* 2001 Jun;183(12):3623-30.
- Arabolaza AL, Nakamura A, Pedrido ME, Martelotto L, Orsaria L, Grau RR. "Characterization of a novel inhibitory feedback of the anti-anti-sigma SpoIIAA on Spo0A activation during development in *Bacillus subtilis*". *Mol Microbiol.* 2003 Mar;47(5):1251-63.
- Arigoni, F.L., Duncan. S. Alper, R. Losick, and P. Stragier. 1996, "SpoIIE govern the phosphorylation state of a protein regulating transcription factor  $\sigma^F$  during sporulation in *Bacillus subtilis*". *Proc. Natl. Acad. Sci. USA* 93: 3238-3242.
- Arigoni, F.,K. Posgiano C. Webb, P. Stragier, and R. Losick. 1995, "Localization of protein implicated in establishment of cell type to sites of asymmetric cell division". *Science* 270:637-640
- Arigoni F, Guerout-Fleury AM, Barak I, Stragier P. "The SpoIIE phosphatase, the sporulation septum and the establishment of forespore-specific transcription in *Bacillus subtilis*: a reassessment". *Mol Microbiol.* 1999 Mar;31(5):1407-15.
- Ausubel, Fred M., et. al., editors. *Current Protocols in Molecular Biology*. New York: John Wiley and Sons, 1998.
- Bagyan I, Casillas-Martinez L, Setlow P. "The *katX* gene, which codes for the catalase in spores of *Bacillus subtilis*, is a forespore-specific gene controlled by  $\sigma^F$ , and *katX* is essential for hydrogen peroxide resistance of the germinating spore". *J. Bacteriol.* 1998 Apr;180(8):2057-62.
- Barak I, J Behaari. "Structure and function of the *Bacillus* SpoIIE protein and its localization to sites of sporulation septum assembly". *Mol. Microbiol.* 19 1047-1060
- Barak I, Youngman P. "*SpoIIE* mutants of *Bacillus subtilis* comprise two distinct phenotypic classes consistent with a dual functional role for the SpoIIE protein". *J. Bacteriol.* 1996 Aug;178(16):4984-9.
- Biswas I, Gruss A, Ehrlich SD, Maguin E. "High-efficiency gene inactivation and replacement system for gram-positive bacteria". *J Bacteriol.* 1993 Jun;175(11):3628-35.
- Burbulys D, Trach KA, Hoch JA. "Initiation of sporulation in *B. subtilis* is controlled by a multicomponent phosphorelay". *Cell.* 1991 Feb 8;64(3):545-52.
- Bylund JE, Zhang L, Haines MA, Higgins ML, Piggot PJ. "Analysis by fluorescence microscopy of the development of compartment-specific gene expression during sporulation of *Bacillus subtilis*". *J Bacteriol.* 1994 May;176(10):2898-905.
- Campbell EA, Masuda S, Sun JL, Muzzin O, Olson CA, Wang S, Darst SA. "Crystal structure of the *Bacillus stearothermophilus* anti- $\sigma$  factor SpoIIAB with the sporulation  $\sigma$  factor  $\sigma^F$ ". *Cell.* 2002 Mar 22;108(6):795-807.

- Cheng SW, Hu HM, Shen SW, Takagi H, Asano M, Tsai YC. "Production and characterization of keratinase of a feather-degrading *Bacillus licheniformis* PWD-1". *Biosci Biotechnol Biochem*. 1995 Dec;59(12):2239-43.
- Dancer, B.N., Mandelstam J. "Complementation of Sporulation Mutations in Fused Protoplasts of *Bacillus subtilis*". *J. Gen Microbiology*. 1981 123: 17-26.
- Diederich B, Wilkinson JF, Magnin T, Najafi M, Errington J, Yudkin MD. "Role of interactions between SpoIIAA and SpoIIAB in regulating cell-specific transcription factor sigma F of *Bacillus subtilis*". *Genes Dev*. 1994 Nov 1;8(21):2653-63.
- Driks A, Losick R. "Compartmentalized expression of a gene under the control of sporulation transcription factor sigma E in *Bacillus subtilis*". *Proc Natl Acad Sci U S A*. 1991 Nov 15;88(22):9934-8.
- Duncan L, Losick R. "SpoIIAB is an anti- $\sigma$  factor that binds to and inhibits transcription by regulatory protein  $\sigma$  F from *Bacillus subtilis*". *Proc Natl Acad Sci U S A*. 1993 Mar 15;90(6):2325-9.
- Duncan L, Alper S, Arigoni F, Losick R, Stragier P. "Activation of cell-specific transcription by a serine phosphatase at the site of asymmetric division". *Science*. 1995 Oct 27;270(5236):641-4.
- Duncan L, Alper S, Losick R. "SpoIIAA governs the release of the cell-type specific transcription factor sigma F from its anti-sigma factor SpoIIAB". *J Mol Biol*. 1996 Jul 12;260(2):147-64.
- Duncan L, Losick R. "SpoIIAB is an anti-sigma factor that binds to and inhibits transcription by regulatory protein sigma F from *Bacillus subtilis*". *Proc Natl Acad Sci U S A*. 1993 Mar 15;90(6):2325-9.
- Eichenberger P, Fawcett P, Losick R. "A three-protein inhibitor of polar septation during sporulation in *Bacillus subtilis*". *Mol Microbiol*. 2001 Dec;42(5):1147-62.
- Errington J. "Determination of cell fate in *Bacillus subtilis*". *Trends Genet*. 1996 Jan;12(1):31-4.
- Errington J, Illing N. "Establishment of cell-specific transcription during sporulation in *Bacillus subtilis*". *Mol Microbiol*. 1992 Mar;6(6):689-95.
- Errington J., Fort P., and Mandelstam J. "Duplicated sporulation genes in bacteria: Implications for simple developmental systems". *FEBS* 188:2. 1985.
- Errington J, Mandelstam J. "Use of a *lacZ* gene fusion to determine the dependence pattern of sporulation operon *spoIIA* in spo mutants of *Bacillus subtilis*". *J Gen Microbiol*. 1986 Nov;132 ( Pt 11):2967-76.
- Fawcett P, Eichenberger P, Losick R, Youngman P. "The transcriptional profile of early to middle sporulation in *Bacillus subtilis*". *Proc Natl Acad Sci U S A*. 2000 Jul 5;97(14):8063-8.
- Feucht A, Magnin T, Yudkin MD, Errington J. "Bifunctional protein required for asymmetric cell division and cell-specific transcription in *Bacillus subtilis*". *Genes Dev*. 1996 Apr 1;10(7):794-803.
- Fleming AB, Tangney M, Jorgensen PL, Diderichsen B, Priest FG. "Extracellular enzyme synthesis in a sporulation-deficient strain of *Bacillus licheniformis*". *Appl Environ Microbiol*. 1995 Nov;61(11):3775-80.

- Frandsen N, Barak I, Karmazyn-Campelli C, Stragier P. "Transient gene asymmetry during sporulation and establishment of cell specificity in *Bacillus subtilis*". *Genes Dev.* 1999 Feb 15;13(4):394-9.
- Haldenwang WG. "The  $\sigma$  factors of *Bacillus subtilis*". *Microbiol Rev.* 1995 Mar;59(1):1-30
- Hamilton CM, Aldea M, Washburn BK, Babitzke P, Kushner SR. "New method for generating deletions and gene replacements in *Escherichia coli*". *J Bacteriol.* 1989 Sep;171(9):4617-22.
- Harwood, C. R. & Cutting, S. M. *Molecular Biological Methods for Bacillus*. 1990 (Wiley, New York)
- Hilbert DW, Piggot PJ. "Novel *spoIIIE* mutation that causes uncompartimentalized  $\sigma^F$  activation in *Bacillus subtilis*". *J Bacteriol.* 2003 Mar;185(5):1590-8.
- Ho MS, Carniol K, Losick R. "Evidence in support of a docking model for the release of the transcription factor sigma F from the antisigma factor SpoIIAB in *Bacillus subtilis*". *J Biol Chem.* 2003 Jun 6;278(23):20898-905. Epub 2003 Apr 03.
- Ho SN, Hunt HD, Horton RM, Pullen JK, Pease LR. "Site-directed mutagenesis by overlap extension using the polymerase chain reaction". *Gene.* 1989 Apr 15;77(1):51-9.
- Hoch JA. "Regulation of the phosphorelay and the initiation of sporulation in *Bacillus subtilis*". *Annu Rev Microbiol.* 1993;47:441-65.
- Hofmeister A. "Activation of the proprotein transcription factor pro- $\sigma^E$  is associated with its progression through three patterns of subcellular localization during sporulation in *Bacillus subtilis*". *J Bacteriol.* 1998 May;180(9):2426-33.
- Horton RM, Hunt HD, Ho SN, Pullen JK, Pease LR. "Engineering hybrid genes without the use of restriction enzymes: gene splicing by overlap extension". *Gene.* 1989 Apr 15;77(1):61-8.
- Horton RM, Ho SN, Pullen JK, Hunt HD, Cai Z, Pease LR. "Gene splicing by overlap extension". *Methods Enzymol.* 1993;217:270-9.
- Iglesias A, Bensi G, Canosi U, Trautner TA. "Plasmid transformation in *Bacillus subtilis*. Alterations introduced into the recipient-homologous DNA of hybrid plasmids can be corrected in transformation". *Mol Gen Genet.* 1981;184(3):405-9.
- Illing N, Errington J. "Genetic regulation of morphogenesis in *Bacillus subtilis*: roles of sigma E and sigma F in prespore engulfment". *J Bacteriol.* 1991 May;173(10):3159-69.
- Janštová B., J. Lukášová: "Heat Resistance of *Bacillus* spp. Spores Isolated from Cow's Milk and Farm Environment". *Acta Vet. Brno* 70, 2001: 179-184
- Jonas RM, Peters HK 3rd, Haldenwang WG. "Phenotypes of *Bacillus subtilis* mutants altered in the precursor-specific region of sigma E". *J Bacteriol.* 1990 Aug;172(8):4178-86..
- Jonas RM, Haldenwang WG. "Influence of spo mutations on sigma E synthesis in *Bacillus subtilis*". *J Bacteriol.* 1989 Sep;171(9):5226-8.
- Ju J, Haldenwang WG. "The "pro" sequence of the sporulation-specific  $\sigma$  transcription factor  $\sigma^E$  directs it to the mother cell side of the sporulation septum". *J Bacteriol.* 1999 Oct;181(19):6171-5.

- Ju J, Luo T, Haldenwang WG. "Forespore expression and processing of the SigE transcription factor in wild-type and mutant *Bacillus subtilis*". *J Bacteriol.* 1998 Apr;180(7):1673-81.
- Ju J, Mitchell T, Peters H 3rd, Haldenwang WG. "Sigma factor displacement from RNA polymerase during *Bacillus subtilis* sporulation". *J Bacteriol.* 1999 Aug;181(16):4969-77.
- Juan Wu L, Errington J. "Identification and characterization of a new prespore-specific regulatory gene, *rsfA*, of *Bacillus subtilis*". *J Bacteriol.* 2000 Jan;182(2):418-24.
- Karow, L "Identification of a gene, *spoIIR* which links the activation of  $\sigma$ E to the transcriptional activity of  $\sigma$  F during sporulation in *Bacillus subtilis*". *Proc Natl Acad Sci* 1995 92:2012-2016.
- Kawamura F, Doi RH. "Construction of a *Bacillus subtilis* double mutant deficient in extracellular alkaline and neutral proteases". *J Bacteriol.* 1984 Oct;160(1):442-4.
- Kenney TJ, Moran CP Jr. "Organization and regulation of an operon that encodes a sporulation-essential  $\sigma$  factor in *Bacillus subtilis*". *J Bacteriol.* 1987 Jul;169(7):3329-39
- Khvorova A, Chary VK, Hilbert DW, Piggot PJ. "The chromosomal location of the *Bacillus subtilis* sporulation gene *spoIIR* is important for its function". *J Bacteriol.* 2000 Aug;182(16):4425-9.
- Khvorova A, Zhang L, Higgins ML, Piggot PJ. "The *spoIIE* locus is involved in the Spo0A-dependent switch in the location of FtsZ rings in *Bacillus subtilis*". *J Bacteriol.* 1998 Mar;180(5):1256-60.
- King N, Dreesen O, Stragier P, Pogliano K, Losick R. "Septation, dephosphorylation, and the activation of sigma F during sporulation in *Bacillus subtilis*". *Genes Dev.* 1999 May 1;13(9):1156-67.
- Kirchman PA, DeGrazia H, Kellner EM, Moran CP Jr. "Forespore-specific disappearance of the  $\sigma$ -factor antagonist SpoIIAB: implications for its role in determination of cell fate in *Bacillus subtilis*". *Mol Microbiol.* 1993 May;8(4):663-71.
- Kroos L, Zhang B, Ichikawa H, Yu Y.T. "Control of sigma factor activity during *Bacillus subtilis* sporulation". *Mol Microbiol.* 1999 Mar;31(5):1285-94.
- Kuwana R, Kasahara Y, Fujibayashi M, Takamatsu H, Ogasawara N, Watabe K. "Proteomics characterization of novel spore proteins of *Bacillus subtilis*". *Microbiology.* 2002 Dec;148(Pt 12):3971-82.
- Lee CS, Clarkson J, Shu JC, Campbell ID, Yudkin MD. "*Bacillus subtilis* mutations that alter the pathway of phosphorylation of the anti-anti-sigmaF factor SpoIIAA lead to a Spo- phenotype". *Mol Microbiol.* 2001 Apr;40(1):9-19.
- Levin PA, Losick R. "Transcription factor Spo0A switches the localization of the cell division protein FtsZ from a medial to a bipolar pattern in *Bacillus subtilis*". *Genes Dev.* 1996 Feb 15;10(4):478-88.
- Levin PA, Losick R, Stragier P, Arigoni F. "Localization of the sporulation protein SpoIIE in *Bacillus subtilis* is dependent upon the cell division protein FtsZ". *Mol Microbiol.* 1997 Sep;25(5):839-46.
- Lewis PJ, Magnin T, Errington J. "Compartmentalized distribution of the proteins controlling the prespore-specific transcription factor  $\sigma$  F of *Bacillus subtilis*". *Genes Cells.* 1996 Oct;1(10):881-94

- Lewis PJ, Wu LJ, Errington J. "Establishment of prespore-specific gene expression in *Bacillus subtilis*: localization of SpoIIE phosphatase and initiation of compartment-specific proteolysis". *J Bacteriol.* 1998 Jul;180(13):3276-84.
- Lin X, Kelemen DW, Miller ES, Shih JC. "Nucleotide sequence and expression of *kerA*, the gene encoding a keratinolytic protease of *Bacillus licheniformis* PWD-1". *Appl Environ Microbiol.* 1995 Apr;61(4):1469-74.
- Londono-Vallejo JA, Stragier P. "Cell-cell signaling pathway activating a developmental transcription factor in *Bacillus subtilis*". *Genes Dev.* 1995 Feb 15;9(4):503-8.
- Lord M, Barilla D, Yudkin MD. "Replacement of vegetative  $\sigma$ A by sporulation-specific  $\sigma$ F as a component of the RNA polymerase holoenzyme in sporulating *Bacillus subtilis*". *J Bacteriol.* 1999 Apr;181(8):2346-50.
- Losick R, Pero J. "Cascades of Sigma factors". *Cell.* 1981 Sep;25(3):582-4.
- Losick R, Stragier P. "Crisscross regulation of cell-type-specific gene expression during development in *B. subtilis*". *Nature.* 1992 Feb 13;355(6361):601-4.
- Louie P., Lee A., Stansmore. K. "Roles of *rpoD*, *spoIIF*, *spoIIG*, *spoIIN*, and *sin* in Regulation of *Bacillus subtilis* Stage II Sporulation-Specific Transcription". *J Bacteriology*, June 1992 p3570-3576
- Magill NG, Setlow P. "Properties of purified sporlets produced by *spoII* mutants of *Bacillus subtilis*". *J Bacteriol.* 1992 Dec;174(24):8148-51.
- Mandelstam J, Waites WM. "Sporulation in *Bacillus subtilis*. The role of exoprotease". *Biochem J.* 1968 Oct;109(5):793-801.
- Margolis P, Driks A, Losick R. "Establishment of cell type by compartmentalized activation of a transcription factor". *Science.* 1991 Oct 25;254(5031):562-5.
- Min KT, Hilditch CM, Diederich B, Errington J, Yudkin MD. "Sigma F, the first compartment-specific transcription factor of *B. subtilis*, is regulated by an anti- $\sigma$  factor that is also a protein kinase" *Cell.* 1993 Aug 27;74(4):735-42.
- Nicholson WL, Munakata N, Horneck G, Melosh HJ, Setlow P. "Resistance of *Bacillus* endospores to extreme terrestrial and extraterrestrial environments". *Microbiol Mol Biol Rev.* 2000 Sep;64(3):548-72.
- Noirot P, Petit MA, Ehrlich SD. "Plasmid replication stimulates DNA recombination in *Bacillus subtilis*". *J Mol Biol.* 1987 Jul 5;196(1):39-48.
- Partridge SR, Foulger D, Errington J. "The role of  $\sigma$ F in prespore-specific transcription in *Bacillus subtilis*." *Mol Microbiol.* 1991 Mar;5(3):757-67.
- Partridge SR, Errington J. "The importance of morphological events and intercellular interactions in the regulation of prespore-specific gene expression during sporulation in *Bacillus subtilis*". *Mol Microbiol.* 1993 May;8(5):945-55.

- Perez AR, Abanes-De Mello A, Pogliano K. "SpoIIB localizes to active sites of septal biogenesis and spatially regulates septal thinning during engulfment in *Bacillus subtilis*". *J Bacteriol.* 2000 Feb;182(4):1096-108.
- Piggot PJ, Coote JG. "Genetic aspects of bacterial endospore formation". *Bacteriol Rev.* 1976 Dec;40(4):908-62.
- Pogliano J, Osborne N, Sharp MD, Abanes-De Mello A, Perez A, Sun YL, Pogliano K. "A vital stain for studying membrane dynamics in bacteria: a novel mechanism controlling septation during *Bacillus subtilis* sporulation". *Mol Microbiol.* 1999 Feb;31(4):1149-59.
- Rather PN, Coppolecchia R, DeGrazia H, Moran CP Jr. "Negative regulator of  $\sigma$  G-controlled gene expression in stationary-phase *Bacillus subtilis*". *J Bacteriol.* 1990 Feb;172(2):709-15.
- Molecular Cloning. A Laboratory Manual (volumes 1-3). J. Sambrook and D.W. Russell. Cold Spring Harbor Laboratory Press, New York, 2001
- Schaeffer, P., J. Millet, and J.-P. Aubert. 1965. "Catabolite repression of bacterial sporulation". *Proc. Natl. Acad. Sci. USA* 54:704-711
- Schmidt R, Margolis P, Duncan L, Coppolecchia R, Moran CP Jr, Losick R. "Control of developmental transcription factor  $\sigma$  F by sporulation regulatory proteins SpoIIAA and SpoIIAB in *Bacillus subtilis*". *Proc Natl Acad Sci U S A.* 1990 Dec;87(23):9221-5.
- Serrano M, Hovel S, Moran CP Jr, Henriques AO, Volker U. "Forespore-specific transcription of the *lonB* gene during sporulation in *Bacillus subtilis*". *J Bacteriol.* 2001 May;183(10):2995-3003.
- Serrano M, Corte L, Opdyke J, Moran CP Jr, Henriques AO. "Expression of *spoIIIJ* in the prespore is sufficient for activation of  $\sigma$  G and for sporulation in *Bacillus subtilis*". *J Bacteriol.* 2003 Jul;185(13):3905-17.
- Setlow B, Magill N, Febbroriello P, Nakhimovsky L, Koppel DE, Setlow P. "Condensation of the forespore nucleoid early in sporulation of *Bacillus* species". *J Bacteriol.* 1991 Oct;173(19):6270-8.
- Sharp MD, Pogliano K. "MinCD-dependent regulation of the polarity of SpoIIIE assembly and DNA transfer". *EMBO J.* 2002 Nov 15;21(22):6267-74.
- Sonenshein AL. "Control of sporulation initiation in *Bacillus subtilis*". *Curr Opin Microbiol.* 2000 Dec;3(6):561-6.
- Stragier P, Bonamy C, Karmazyn-Campelli C. "Processing of a sporulation  $\sigma$  factor in *Bacillus subtilis*: how morphological structure could control gene expression". *Cell.* 1988 Mar 11;52(5):697-704.
- Sun DX, Cabrera-Martinez RM, Setlow P. "Control of transcription of the *Bacillus subtilis* *spoIIIG* gene, which codes for the forespore-specific transcription factor  $\sigma$  G". *J Bacteriol.* 1991 May;173(9):2977-84.
- Sun D, Fajardo-Cavazos P, Sussman MD, Tovar-Rojo F, Cabrera-Martinez RM, Setlow P. "Effect of chromosome location of *Bacillus subtilis* forespore genes on their *spo* gene dependence and transcription by  $\sigma$  F: identification of features of good  $\sigma$  F-dependent promoters". *J Bacteriol.* 1991 Dec;173(24):7867-74.

- Sun DX, Stragier P, Setlow P. "Identification of a new  $\sigma$ -factor involved in compartmentalized gene expression during sporulation of *Bacillus subtilis*". *Genes Dev.* 1989 Feb;3(2):141-9.
- Sun YL, Sharp MD, Pogliano K. "A dispensable role for forespore-specific gene expression in engulfment of the forespore during sporulation of *Bacillus subtilis*". *J Bacteriol.* 2000 May;182(10):2919-27.
- Sussman MD, Setlow P. "Cloning, nucleotide sequence, and regulation of the *Bacillus subtilis* *gpr* gene, which codes for the protease that initiates degradation of small, acid-soluble proteins during spore germination". *J Bacteriol.* 1991 Jan;173(1):291-300.
- Wang JJ, Shih J. "Fermentation production of keratinase from *Bacillus licheniformis* PWD-1 and a recombinant *B. subtilis* FDB-29". *J Ind Microbiol Biotechnol.* 1999 Jun;22(6):608-616.
- Wu LJ, Errington J. "Septal localization of the SpoIIIE chromosome partitioning protein in *Bacillus subtilis*". *EMBO J.* 1997 Apr 15;16(8):2161-9.
- Wu LJ, Feucht A, Errington J. "Prespore-specific gene expression in *Bacillus subtilis* is driven by sequestration of SpoIIIE phosphatase to the prespore side of the asymmetric septum". *Genes Dev.* 1998 May 1;12(9):1371-80.
- Wu JJ, Howard MG, Piggot PJ. "Regulation of transcription of the *Bacillus subtilis* *spoIIA* locus". *J Bacteriol.* 1989 Feb;171(2):692-8.
- Yang MY, Ferrari E, Henner DJ. "Cloning of the neutral protease gene of *Bacillus subtilis* and the use of the cloned gene to create an in vitro-derived deletion mutation". *J Bacteriol.* 1984 Oct;160(1):15-21.
- Yudkin MD, Appleby L, Smith AJ. "Nucleotide sequence of the *Bacillus licheniformis* homologue of the sporulation locus *spoIIA* of *Bacillus subtilis*". *J Gen Microbiol.* 1989 Apr;135 ( Pt 4):767-75.
- Zhang L, Higgins ML, Piggot PJ, Karow ML. "Analysis of the role of prespore gene expression in the compartmentalization of mother cell-specific gene expression during sporulation of *Bacillus subtilis*". *J Bacteriol.* 1996 May;178(10):2813-7.
- Zupancic ML, Tran H, Hofmeister AE. "Chromosomal organization governs the timing of cell type-specific gene expression required for spore formation in *Bacillus subtilis*". *Mol Microbiol.* 2001 Mar;39(6):1471-81.



APPENDIX A. DNA SEQUENCE ALIGNMENT OF PCR PRODUCTS WITH  
GENEBANK SEQUENCE OF *B. LICHENIFORMIS SPOILA* OPERON (M25260).

M25260	1	TCCAGGCCCCCTGTCAAAAAAAGGCACAGAGCTTGGGACGCTCGTTTTGAA
PWD-1 (Left-D	1	~~~~~
PWD-1 (A-D)	1	~~~~~
WBG-2(Left-D)	1	~~~~~
WBG-2(A-D)	1	--~~~~~
M25260	51	AAAGGATGGAAAGGTGCTCGCTGAAAGTCCTCTTGTCGCTGAAAAAGATA
PWD-1 (Left-D	1	~~~~~
PWD-1 (A-D)	1	~~~~~
WBG-2(Left-D)	1	~~~~~
WBG-2(A-D)	1	~~~~~
M25260	101	TGGACAAAAGCGGGGATGTGGACGATGTTCAAGCGGACGATGACCCACTGG
PWD-1 (Left-D	1	~~~~~
PWD-1 (A-D)	1	~~~~~
WBG-2(Left-D)	1	~~~~~
WBG-2(A-D)	1	~~~~~
M25260	151	ACGAAGTGGAGTGAATAATGCCGAACGGTCACTAGTTTTGTACCGGTGAA
PWD-1 (Left-D	1	~~~~~
PWD-1 (A-D)	1	~~~~~
WBG-2(Left-D)	1	~~~~~
WBG-2(A-D)	1	~~~~~
M25260	201	GGAATTTATAAAGTCTGAAGCGAAACACTCATTATCCGATTTTAAACCAAG
PWD-1 (Left-D	1	~~~~~
PWD-1 (A-D)	1	~~~~~
WBG-2(Left-D)	1	~~~~~
WBG-2(A-D)	1	~~~~~
M25260	251	GAGGAATGAGGATGAGCCTCGGAATCGATATTACAGTCAAAGAATCCGTA
PWD-1 (Left-D	1	~~~~~
PWD-1 (A-D)	1	~~~~~
WBG-2(Left-D)	1	~~~~~
WBG-2(A-D)	1	~~~~~
M25260	301	TTATGCATTTCGGTTGACAGGTGAACTCGATCACCATACAGCTGAAACCT
PWD-1 (Left-D	33	TTATGCATTTCGGTTGACAGGTGAACTCGATCACCATACAGCAGAAACCT
PWD-1 (A-D)	1	~~~~~
WBG-2(Left-D)	33	NTATGCATTTCGGTTGACAGGTGAACTCGATCACCATACAGCAGAAACCT
WBG-2(A-D)	1	~~~~~
M25260	350	TGAG-AAAACAAGT-CAGTGGCCATCTGGAACAAACCG-ACATTGCCAT



PWD-1 (A-D)	22	G-AAGCGGTACAAACGCG-ATCATTACGGTTATGAAAACTCAGGGCAG
WBG-2 (Left-D)	480	G-AAGCGGTACAAACGCG-ATCATTACGGTTATGAAAACTCAGGGCAG
WBG-2 (A-D)	20	GTAAGCGGTACAAACGCGTATCATTACGGTTATGAAAACTCAGGGCAG
M25260	795	GGAAACGTATATATTTCCGTCACCTCTCGAGGACCATAT--GTCTATTTTAA
PWD-1 (Left-D)	531	GGAAACGTATATATTTCCGTCACCTCTCGAGGACCATATTTGTCTATTTTAA
PWD-1 (A-D)	70	GGAAACGTATATATTTCCGTCACCTCTCGAGGACCATAT-TGTCTATTTTAA
WBG-2 (Left-D)	528	GGAAACGTATATATTTCCGTCACCTCTCGAGGACCATAT-TGTCTATTTTAA
WBG-2 (A-D)	70	GGAAACGTATATATTTCCGTCACCTCTCGAGGACCATAT-TGTCTATTTTAA
M25260	843	CGATCCGCGACG-AAGGAGTCGGCATCCCTGATCTTGAAGAAGCGCGCCA
PWD-1 (Left-D)	581	CGATCCGCGACGAAAGGAGTCGGCATCCCTGATCTTGAAGAAGCGCGCCA
PWD-1 (A-D)	119	CGATCCGCGACG-AAGGAGTCGGCATCCCTGATCTTGAAGAAGCGCGCCA
WBG-2 (Left-D)	577	CGATCCGCGACG-AAGGAGTCGGCATCCCTGATCTTGAAGAAGCGCGCCA
WBG-2 (A-D)	119	CGATCCGCGACG-AAGGAGTCGGCATCCCTGATCTTGAAGAAGCGCGCCA
M25260	892	GCCCCGTGTTACGACAAAGCCTGAACTCGAGCGGTCGGGAATGGGCCTTTA
PWD-1 (Left-D)	631	GCCCCGTGTTACGACAAAGNNCTGAACTCGAGCGGTCGGGAATGGGCCTTT-
PWD-1 (A-D)	168	GCCCCGTGTTACGACAAAGCCTGAACTCGAGCGGTCGGGAATGGGCCTTTA
WBG-2 (Left-D)	626	GCCCCGTGTTACGACAAAGCCTGAACTCGAGCGGTCGGGAATGGGCCTTTA
WBG-2 (A-D)	168	GCCCCGTGTTACGACAAAGCCTGAACTCGAGCGGTCGGGAATGGGCCTTTA
M25260	942	CGATCATGGAAAAATTCATGGATGATATTTTCGATCGACTCCTCACCTGAG
PWD-1 (Left-D)	680	CNATCATGGNAAAATT-C-TGGATGATNTTTN-ATCGACTCCTCAC-TGAG
PWD-1 (A-D)	218	CGATCATGGAAAAATTCATGGATGATATTTTCGATCGACTCCTCACCTGAG
WBG-2 (Left-D)	675	CGATCATGGAAAAATT-C-NGGATGATATTTTCGATCGACTCCTCACCTGAG
WBG-2 (A-D)	218	CGATCATGGAAAAATTCATGGATGATATTTTCGATCGACTCCTCACCTGAG
M25260	992	ATGGGAACCAACAATACACTTAACAAAGCACTTATCAAAAAGCAAAGCGCT
PWD-1 (Left-D)	726	AGGGGAACCAAC-ATACANTTA-CAA-GCACTAA-CAAAAAGCAAAGCGCT
PWD-1 (A-D)	268	ATGGGAACCAACAATACACTTAACAAAGCACTTATCAAAAAGCAAAGCGCT
WBG-2 (Left-D)	723	AGGGAAACC-CA-TACACTTA-CNAAAGCACTTNTCAAAA-GCAA-GCGCT
WBG-2 (A-D)	268	ATGGGAACCAACAATACACTTAACAAAGCACTTATCAAAAAGCAAAGCGCT
M25260	1042	TTGCAATTAAGGGAGATTTGTTATGGATGTGGAGGTTAAAAAGAAAA-C
PWD-1 (Left-D)	772	TGGCAATTAAGGGAGATTTGTTAAGGAGTGGGAGGTAAAAAGAAAAAC
PWD-1 (A-D)	318	TTGCAATTAAGGGAGATTTGTTATGGATGTGGAGGTTAAAAAGAAAA-C
WBG-2 (Left-D)	768	TTGCNATTAAGG-AGATT-GTNTGGAGNNGGNTNAAAAAAACCGAA-C
WBG-2 (A-D)	318	TTGCAATTAAGGGAGATTTGTTATGGATGTGGAGGTTAAAAAGAAAA-C
M25260	1091	CAGAACTCTCAGCTTAAAGACCATGAA-GTGAAAGAACTGATTAAAAACA
PWD-1 (Left-D)	822	CGAACTCTCAGCTTAAAGACCATGAAAGTGAAAGAACTGATTAAANAACA
PWD-1 (A-D)	367	CAGAACTCTCAGCTTAAAGACCATGAA-GTGAAAGAACTGATTAAAAACA
WBG-2 (Left-D)	815	CAGAACTCTCAGCTTAAAGACCATGAA-GTGAAAGAACTGATTAAAAACA
WBG-2 (A-D)	367	CAGAACTCTCAGCTTAAAGACCATGAA-GTGAAAGAACTGATTAAAAACA
M25260	1140	GCCAGAACGGCGATCAAAAAGCAAGGGACCTCCTCATAGAAAAAACATG
PWD-1 (Left-D)	872	GCCAGAACGGCGATCAAAAAGCAAGGGACCTCCTCATAGANAAAAACATG
PWD-1 (A-D)	416	GCCAGAACGGCGATCAAAAAGCAAGGGACCTCCTCATAGAAAAAACATG

WBG-2 (Left-D) 864	GCCAGAACGGCGATCAAAAAGCAAGGGACCTCCTCATAGAAAAAACATG
WBG-2 (A-D) 416	GCCAGAACGGCGATCAAAAAGCAAGGGACCTCCTCATAGAAAAAACATG
M25260 1190	CGTCTTGTTTGGTCTGTCGTTTCAGCGTTTTTTTGAACAGAGGCTATGAGCC
PWD-1 (Left-D) 922	CGTCTTGTTTGGTCTGTCGTTTCAGCGTTTTTTTGAACAGAGGCTATGAGCC
PWD-1 (A-D) 466	CGTCTTGTTTGGTCTGTCGTTTCAGCGTTTTTTTGAACAGAGGCTATGAGCC
WBG-2 (Left-D) 914	CGTCTTGTTTGGTCT-----
WBG-2 (A-D) 466	CGTCTTGTTTGGTCT-----
M25260 1240	TGACGACCTCTTTCAAATCGGCTGCATCGGCCTCTTGAAGTCGGTGGACA
PWD-1 (Left-D) 972	TGACGACCTCTTTCAAATCGGCTGCATCGGCCTCTTGAAGTCGGTGGACA
PWD-1 (A-D) 516	TGACGACCTCTTTCAAATCGGCTGC-----CTCTTGAAGTCGGTGGACA
WBG-2 (Left-D) 928	-----
WBG-2 (A-D) 481	-----
M25260 1290	AATT~CGATCTTTCTTATG~ACGTTTCGGTTTTCCACCTACGCCGTTCCGA
PWD-1 (Left-D) 1022	AATTACGATCTTTCTTATG~ACGTTTCGGTTTTCCACCTACGCCGTTCCGA
PWD-1 (A-D) 560	AATT~CGATCTTTCTTATG~ACGTTTCGGTTTTCCACD~TACGCCGTTCCGA
WBG-2 (Left-D) 928	-----
WBG-2 (A-D) 481	-----
M25260 1338	TGATTATCGGCGAGATTTCAGCGGTTTATTCAGAGATGACGGAACCGTCAAA
PWD-1 (Left-D) 1072	TGATTATCGGCGAGATTTCAGCGGTTTATTCAGAGATGACGGAACCGTCAAA
PWD-1 (A-D) 608	TGATTATCGGCGAGATTTCAGCGGTTTATTCAGAGATGACGGAACCGTCAAA
WBG-2 (Left-D) 928	-----
WBG-2 (A-D) 481	-----
M25260 1388	GTGAGCCGCTCGCTGAAAGAACTCGGCAACAAAATCCGGCGGGCGAAAGA
PWD-1 (Left-D) 1122	GTGAGCCGCTCGCTGAAAGAACTCGGCAACAAAATCCGGCGGGCGAAAGA
PWD-1 (A-D) 658	GTGAGCCGCTCGCTGAAAGAACTCGGCAACAAAATCCGGCGGGCGAAAGA
WBG-2 (Left-D) 928	-----
WBG-2 (A-D) 481	-----
M25260 1438	GGAGCTTTCCAAGTCAAACGGCCGGATTCCGACC~GTTTCAGGAAATCGCC
PWD-1 (Left-D) 1172	GGAGCTTTCCAAGTCAAACGGCCGGATTCCGACC~GTTTCAGGAAATCGCC
PWD-1 (A-D) 708	GGAGCTTTCCAAGTCAAACGGCCGGATTCCGACC~GTTTCAGGAAATCGCC
WBG-2 (Left-D) 928	-----GGGATTCCGACC~GTTTCAGGAAATCGCC
WBG-2 (A-D) 481	-----GGATTCCGACC~GTTTCAGGAAATCGCC
M25260 1487	GATTATCTCGAAATCAGTTCAGAAGAGGTTCGTGATGGCCCAGGAAGCGGT
PWD-1 (Left-D) 1222	GATTATCTCGAAATCAGTTCAGAAGAGGTTCGTGATGGCCCAGGAAGCGGT
PWD-1 (A-D) 757	GATTATCTCGAAATCAGTTCAGAAGAGGTTCGTGATGGCCCAGGAAGCGGT
WBG-2 (Left-D) 956	GATTATCTCGAAATCAGTTCAGAAGAGGTTCGTGATGGCCCAGGAAGCGGT
WBG-2 (A-D) 508	GATTATCTCGAAATCAGTTCAGAAGAGGTTCGTGATGGCCCAGGAAGCGGT
M25260 1537	CCGCTCTCCCTCGTCCATTATGAAACGGTTTACGAAAACGACGGGGACC
PWD-1 (Left-D) 1272	CCGCTCTCCCTCGTCCATTATGAAACGGTTTACGAAAACGACGGGGACC
PWD-1 (A-D) 807	CCGCTCTCCCTCGTCCATTATGAAACGGTTTACGAAAACGACGGGGACC
WBG-2 (Left-D) 1006	CCGCTCTCCCTCGTCCATTATGAAACGGTTTACGAAAACGACGGGGACC

WBG-2 (A-D) 558 CCGCTCTCCCTCGTCCATTTCATGAAACGGTTTACGAAACGACGGGGACC  
 M25260 1587 CGATTACACTTCTGGACCAAAATCGCCGATCAGTCGGAAGAGAAGTGGT  
 PWD-1 (Left-D 1322 CGATTACACTTCTGGACCAAAATNCGCCGATCAGTCGGAAGAGAAGTGGT  
 PWD-1 (A-D) 857 CGATTACACTTCTGGACCAAAATCGCCGATCAGTCGGAAGAGAAGTGGT  
 WBG-2(Left-D)1056 CGATTACACTTCTGGACCAAAATCGCCGATCAGTCGGAAGAGAAGTGGT  
 WBG-2 (A-D) 608 CGATTACACTTCTGGACCAAAATCGCCGATCAGTCGGAAGAGAAGTGGT  
 M25260 1635 TCGATAAAATCGCTTTGAAGGAAGCCATCAAGGATTTGGATGAACGGGA  
 PWD-1 (Left-D 1371 TCGATAAAATCGCTTTGAAGGAAGCCATCAAGGATTTGGATGAACGGGA  
 PWD-1 (A-D) 906 TCGATAAAATCGCTTTGAAGGAAGCCATCAAGGATTTGGATGAACGGGA  
 WBG-2(Left-D)1104 TCGATAAAATCGCTTTGAAGGAAGCCATCAAGGATTTGGATGAACGGGA  
 WBG-2 (A-D) 656 TCGATAAAATCGCTTTGAAGGAAGCCATCAAGGATTTGGATGAACGGGA  
 M25260 1684 AAAGCTGATTGTTTATTTAAGGTATTACAAAGACAAGACGCAATCTGAAG  
 PWD-1 (Left-D 1420 AAAGCTGATTGTTTATTTAAGGTATTA~  
 PWD-1 (A-D) 956 AAAGCTGATTGTTTATTTAAGGTATTA~  
 WBG-2(Left-)1153 ANNCGCGATNGTTTATTTANGGTATTNN~  
 WBG-2 (A-D) 706 AAAGCTGATTGTTTATTTAAGGTATTA~  
 M25260 1734 TTGCCGACCGCCTCGGCATATCCCAGGTGCAAGTCTCGAGGCTTGAGAAA  
 PWD-1(Left-D 1446 ~~~~~~  
 PWD-1 (A-D) 982 ~~~~~~  
 WBG-2(Left-D)1180 ~~~~~~  
 WBG-2 (A-D) 732 ~~~~~~  
 M25260 1784 AAGATTTTAAAGCAAAATCAAAAACCAGATGGACCATTTTGAAAGCTGAGG  
 PWD-1 (Left-D 1446 ~~~~~~  
 PWD-1 (A-D) 982 ~~~~~~  
 WBG-2 (Left-D) 1180 ~~~~~~  
 WBG-2 (A-D) 732 ~~~~~~  
 M25260 1834 CTGCTCATGTTGCAGGCAGCCTCGGATGTCCGATGAAAAACCGGACGCTC  
 PWD-1 (Left-D 1446 ~~~~~~  
 PWD-1 (A-D) 982 ~~~~~~  
 WBG-2 (Left-D)1180 ~~~~~~  
 WBG-2 (A-D) 732 ~~~~~~  
 M25260 1884 TTGGGAGCGTTCCGTTTTTTTGT~  
 PWD-1 (Left-D 1446 ~~~~~~  
 PWD-1 (A-D) 982 ~~~~~~  
 WBG-2 (Left-D)1180 ~~~~~~  
 WBG-2 (A-D) 732 ~~~~~~

## APPENDIX B. MEDIA FORMULATIONS (HARWOOD AND CUTTING, 1990)

### ***B. licheniformis* protoplast transformation reagents and media:**

PEG – 10g dissolved in 25ml 1X SMM

SMMP – equal volumes of 2X SMM and 4X PAB

2X SMM – per liter: 342g sucrose (1M); 4.72g sodium maleate (0.04M); 8.12g  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$  (0.04M); pH 6.5 with NaOH.

4X PAB – 7g Difco antibiotic medium no. 3 per 100mL

SMMP+ - 100ml SMMP; 0.2ml 20% BSA (filter sterilized)

NBSG-X – 500mL of Solution A mixed 500ml Solution B. Shortly before use, add: 0.5ml  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$  (stock 8mg/ml); 0.1ml  $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$  (stock 2.5 mg/ml); 2.0ml glycerol (stock 250mg/ml).

Solution A – per 500ml: 56.0g  $\text{K}_2\text{HPO}_4$ ; 24.0g  $\text{KH}_2\text{PO}_4$ ; 2.0g  $(\text{NH}_4)\text{SO}_4$ ; pH 8.0.

Solution B – per 500ml: 3.0g Difco beef extract; 5.0g Difco bacto peptone; 1.2g  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ ; 6.0g trisodium citrate  $2\text{H}_2\text{O}$ .

Regeneration Agar – mix equal volumes of Solutions 1 to 4 cool to 50°C. Add 1.0ml of VB-spore element solution, 2.0ml of 50% glucose, 0.5ml of 20% BSA (filter sterilized), and 20ug/ml erythromycin.

Solution 1 – per 250ml: 7.0g  $\text{K}_2\text{HPO}_4$ ; 3.0g  $\text{KH}_2\text{PO}_4$

Solution 2 – per 250ml: 1.25g  $(\text{NH}_4)\text{SO}_4$ ; 0.35g  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$

Solution 3 – per 250ml: 37.5g KCl

Solution 4 – 15g Difco agar

VB Spore element – per 100ml: KI 10ug;  $(\text{NH}_4)_2\text{Fe}(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$  250mg;  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$  50ug;  $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$  30ug;  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  15ug;  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$  15ug;  $\text{H}_3\text{BO}_3$  5ug;  $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$  5.5ug; pH 3.0 with 4M HCl.

***B. subtilis* transformation reagents and media:**

SP1 medium – 97mL SP1 salts, 1mL 50% glucose, 1mL Casamino yeast extract, 1mL 100X tryptophan

Casamino acid – Yeast extract – 1g casamino acids, 5g yeast extract in 50ml dH<sub>2</sub>O

100X tryptophan – 0.25g tryptophan in 50ml dH<sub>2</sub>O

SP1 salts – per 500ml: (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> 1.0g; K<sub>2</sub>HPO<sub>4</sub> 7.0g; KH<sub>2</sub>PO<sub>4</sub> 3.0g; Na citrate 2H<sub>2</sub>O 0.5g; MgSO<sub>4</sub> 7H<sub>2</sub>O 0.1g.

SP2 medium – 98ml SP1 medium, 1ml 250mM MgCl<sub>2</sub>, 1ml 50mM CaCl<sub>2</sub>

***B. subtilis* and *B. licheniformis* miniprep reagents**

SET buffer – 20% sucrose, 50mM EDTA, 50mM Tris pH 7.6

Lytic Mix – 1% SDS, 0.2N NaOH

**Heat Treatment Assay and Electron Microscopy Growth Media (Schaeffer *et al.*, 1965)**

Modified Schaeffer's Sporulation Medium – per liter: Difco nutrient broth 16.0g; KCl 2.0g; MgSO<sub>4</sub> 7H<sub>2</sub>O 0.5g; agar (if desired) 17.0g. Adjust pH to 7.0, autoclave, and cool to 50C. Add 1.0ml 1M Ca(NO<sub>3</sub>)<sub>2</sub>; 1.0ml 0.1M MnCl<sub>2</sub> 4H<sub>2</sub>O; 1ml 1mM FeSO<sub>4</sub>;

**Proteolytic Activity Media**

1% Feather Medium – per liter: 10g ground feather, 2.1g K<sub>2</sub>HPO<sub>4</sub>, 0.1g MgCl<sub>2</sub>, 0.5g NaCl