REQUIREMENTS FOR COMPARTMENTALIZATION OF PENICILLIN BINDING PROTEINS DURING SPORULATION IN *BACILLUS SUBTILIS*

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Requirements for compartmentalization of penicillin-binding proteins during sporulation in *Bacillus subtilis*

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(ABSTRACT)

Penicillin-binding proteins (PBP's) are membrane-associated enzymes involved in the polymerization of peptidoglycan. PBP's are divided into three classes based upon their molecular weights and functional domains. Gene expression is regulated in the two differentiated cells in *Bacillus subtilis*, the mother cell and the forespore, by coordinated expression of different sigma factors that recognize specific promoters in each compartment. The functional and compartmental specificity of individual penicillin-binding proteins from the different classes of PBP's were examined during sporulation in *B. subtilis.* Analyses of three class A high molecular weight PBP's indicated that *pbpF* and *pbpG* must be expressed in the forespore to carry out their specific role during spore peptidoglycan synthesis. Expressing *pbpD* in either the forespore or the mother cell could not complement for the loss of *pbpF* and *pbpG*, suggesting that there must be additional sequence information in PBP2c and PBP2d that allows them to carry out their specific role during germ cell wall synthesis. Analyses of a low molecular weight PBP, PBP5*, suggested that expressing *dacB* in either the mother cell or in the forespore could regulate the level of spore peptidoglycan cross-linking to what is typical of wild type spore peptidoglycan.

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CHAPTER ONE

Introduction and Review of the Literature

Stages of Sporulation

Some Gram-positive bacteria, such as *Bacillus* and *Clostridium*, form spores in response to nutrient limitation. Spores are dormant, metabolically inactive cells that are resistant to high temperatures, UV radiation and penetration of many chemicals (4).

When *B. subtilis* cells become starved for carbon, phosphorus and nitrogen, sporulation can be induced. The first step in sporulation involves the replication of DNA until the cell contains two complete sets of the genome (Fig. 1, 3,4). Through invagination of the cytoplasmic membrane, the cell forms a spore septum containing a thin layer of peptidoglycan. This asymmetric septation divides the vegetative cell into two parts: the mother cell and the forespore. The mother cell is the larger remainder of the vegetative cell that will break down after it contributes to the development of the endospore. The smaller division of the vegetative cell is known as the forespore, which eventually will become the mature spore. Both the mother cell and the forespore contain complete copies of the genome (3,4).

The mother cell engulfs the forespore, producing a double membrane that encloses the cytoplasmic contents of the forespore (3,4). The two membranes are known as the inner forespore membrane (IFM) and the outer forespore membrane (OFM). Spore peptidoglycan is produced between the IFM and the OFM. A proteinaceous spore coat is formed around the outer forespore membrane and finally the mother cell lyses, releasing the mature, dormant endospore (3,4).

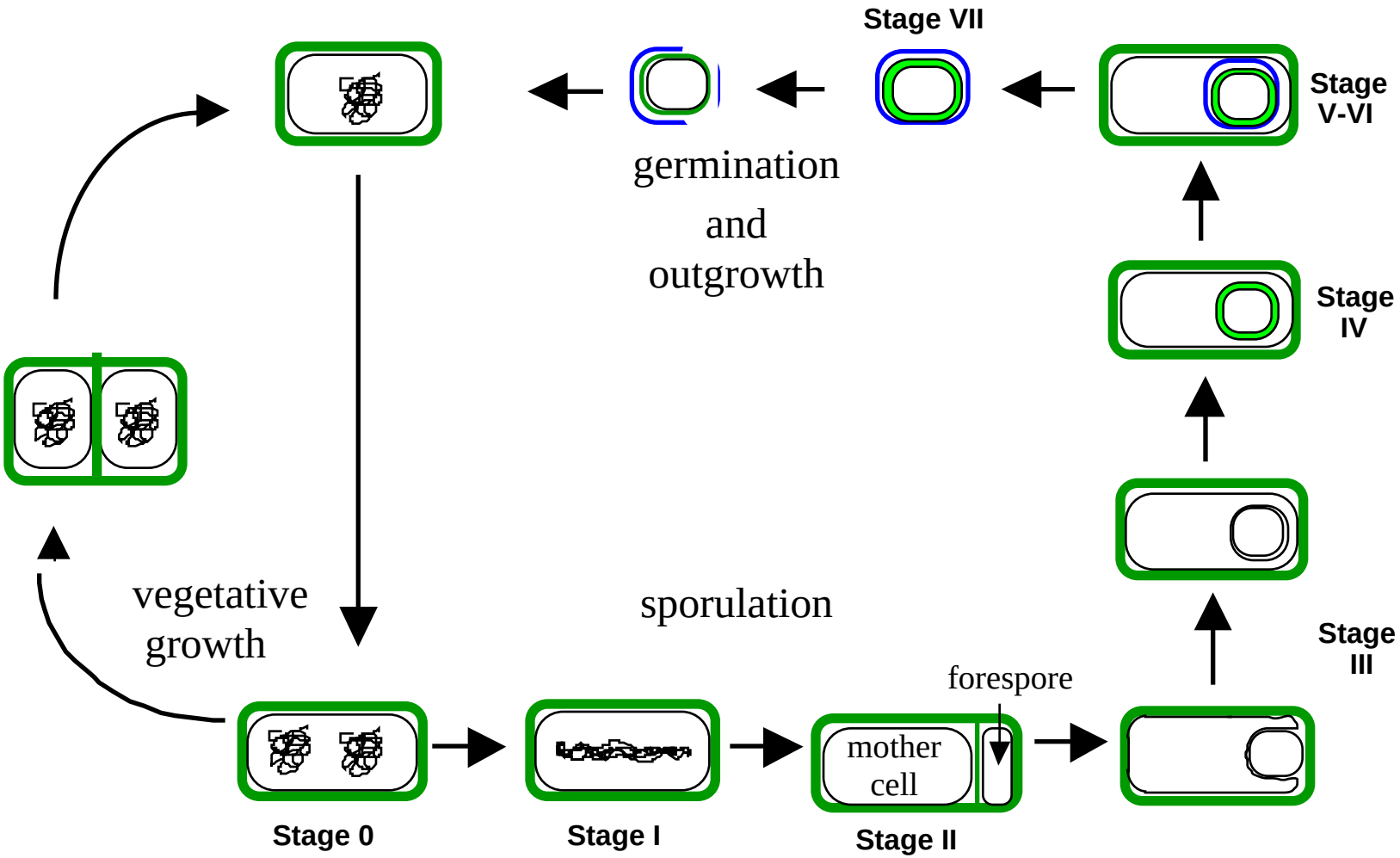

Fig. 1. Stages of the *Bacillus subtilis* life cycle. Stage 0 is a vegetative cell that is nutrient deprived. In stage I, the cell has decided to enter sporulation and there is gathering of the two nucleoids, forming an axial filament. In stage II, the cell undergoes an asymmetric septation producing a larger cell, the mother cell and a smaller cell known as the forespore. The mother cell engulfs the forespore during stage III. The peptidoglycan wall called the spore cortex is synthesized between two membranes in stage IV. During stage V, spore coat proteins are deposited around the forespore. Stage VI is loosely defined as spore maturation and finally in stage VII, the mother cell lyses and releases the dormant endospore.

Sequential activation of sigma factors during sporulation

Gene expression is regulated in the two differentiated cells, the mother cell and forespore, by coordinated expression of different sigma factors that recognize specific promoters in each compartment (8). When *B.subtilis* becomes nutrient starved, sigma A, the major sigma factor in growing cells, and sigma H direct transcription of genes needed for the spore septum to form and the genes encoding sigma F and sigma E. Sigma F is made before the polar septum appears, but becomes activated only in the forespore after the septum forms. With the activation of sigma F, a cascade of regulated gene expression follows in the forespore and also the first mother cell-specific sigma factor, sigma E, is activated. Like sigma F, sigma E is made before the polar septum forms, but remains in its inactive form, pro-σE, until proteolytic processing, which is linked to septation (8).

Both sigma E and sigma F products are required for the engulfment of the forespore by the mother cell (8). Once the forespore in engulfed, sigma G becomes activated in the forespore. After the activation of sigma G in the forespore, sigma K is activated in the mother cell. Sigma K drives the expression of the genes needed for synthesis of coat proteins and spore maturation functions. It is not clear yet if sigma G or sigma K is required for cortex synthesis or simply to produce stable spores that do not degrade the cortex (8).

Differential gene expression

The progression of the sporulation process can be monitored using biochemical tests for temporally correlated sporulation products (21). Dipicolinic acid (DPA) and coat proteins are synthesized in the mother cell, while small-acid soluble proteins (SASPS) and glucose dehydrogenase (GDH) are synthesized in the forespore. DPA is actively pumped into the forespore where it is involved in mineralization of the spore. DPA is able to chelate calcium and is required for the accumulation of calcium in the forespore, which is believed to assist in stabilizing the protein structure of the dormant endospore (21).

Spore peptidoglycan

Peptidoglycan, the structural component of the cell wall, is an important substance in vegetative cells and endospores. Peptidoglycan is a polymer consisting of glycan strands cross-linked by peptide side chains (1, Fig. 2). The function of peptidoglycan is to provide the cell with shape and strength against internal turgor pressure. The structure of vegetative peptidoglycan consists of repeating N-acetylglucosamine (NAG) units linked to N-acetylmuramic acid (NAM) units. The pentapeptides attached to every NAM unit enable the cells to form numerous cross-links with other side chains, providing a rigid cell wall. The polymerization of vegetative and endospore peptidoglycan is similar, however, endospore peptidoglycan differs from vegetative peptidoglycan in the degree of cross-linking and the presence of modified side chains. Endospore peptidoglycan enables the spore to maintain dehydration creating heat

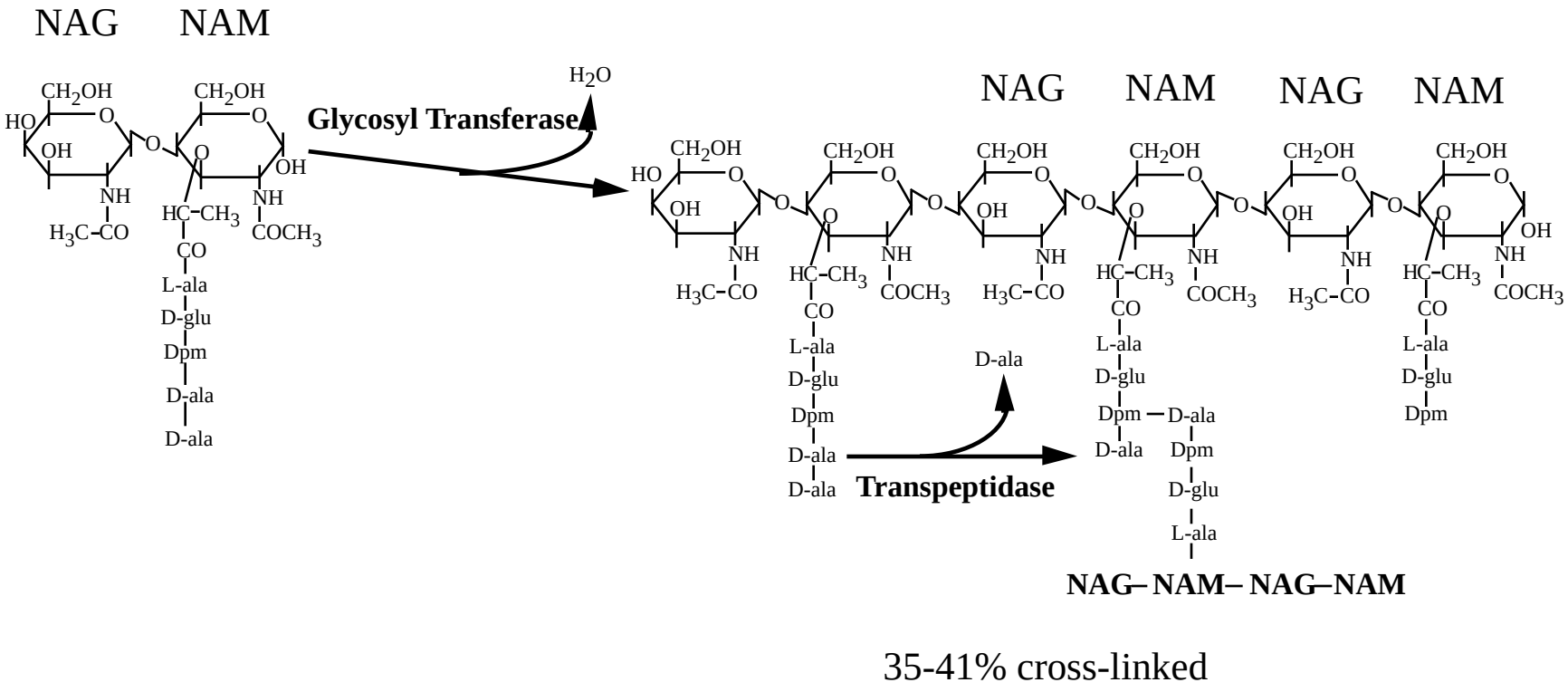

Fig. 2. Synthesis of Vegetative PG. The structure of vegetative PG consists of repeating N-acetylglucosamine (NAG) units linked to N-acetylmuramic acid (NAM) units. The pentapeptide attached to every NAM unit enable the cells to form numerous cross-links with other side chains. The glycosyl transferase activity polymerizes the disaccharide units into glycan strands. The transpeptidase activity cross-links the peptide side chains to one another.

resistant properties (1, 11, 15).

The first, thin layer of peptidoglycan in the endospore that develops next to the inner forespore membrane is known as the germ cell wall (Fig. 3) (11). Its structure is similar to vegetative peptidoglycan and it is believed to be the initial cell wall during germination and outgrowth, unlike the cortex peptidoglycan, which is completely degraded at germination. The cortex comprises 70-90% of the spore peptidoglycan. It is located outside and adjacent to the germ cell wall and next to the outer forespore membrane (11, 15).

The main difference between germ cell wall and cortex peptidoglycan is that 50% of NAM units in the cortex are converted to muramic-δ-lactam. Muramic-δ-lactam is produced when pentapeptide side chains are completely removed. The removal or partial cleavage of the side chains makes fewer sites available for cross-linking. Researchers believe that the low degree of cross-linking adds flexibility and desiccation abilities to the sporulating cell (22). In the germ cell wall, there is little or no muramic-δ-lactam present and the amount of cross-linking is greater. (11, 15)

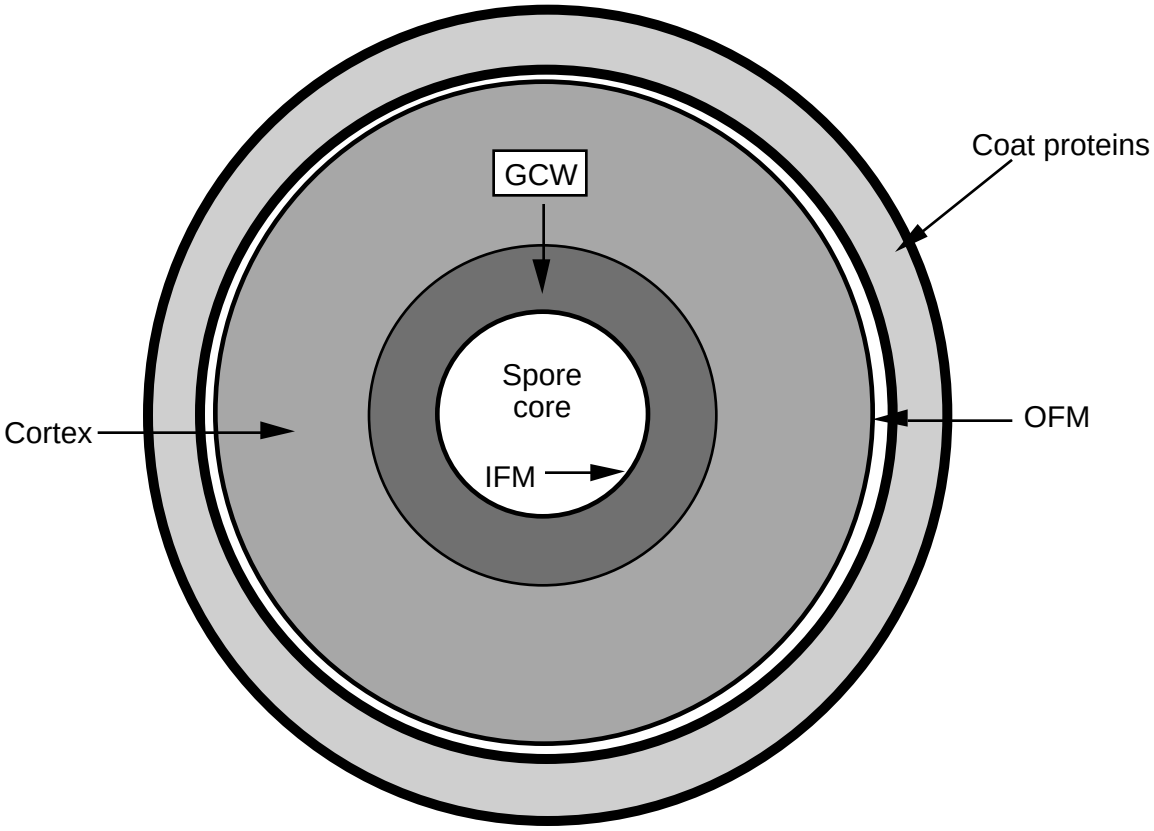

Figure 3. Model of a mature spore. The thin layer of peptidoglycan in the endospore that develops next to the inner forespore membrane (IFM) is known as the germ cell wall (GCW). Its structure is similar to vegetative peptidoglycan. The cortex comprises 70-90% of the spore peptidoglycan. It is located outside and adjacent to the GCW and next to the outer forespore membrane (OFM). The cortex has less cross-linking than vegetative peptidoglycan and contains muramic-δ-lactam.

Classes of Penicillin-binding Proteins (PBP's)

First identified by their ability to bind penicillin, penicillin-binding proteins (PBP's) are membrane-associated enzymes involved in the polymerization of peptidoglycan. PBP's are divided into three classes based upon their molecular weights and functional domains. The three classes include: low molecular weight PBP's, Class A high molecular weight PBP's and Class B high molecular weight PBP's (6).

Low molecular weight PBP's generally possess monofunctional D,D-carboxypeptidase activity. This activity can regulate the number of peptide cross-links by removal of the terminal D-alanine of a peptide side chain (3, 11, 15, 20). With the removal of the D-alanine, the side chain can no longer cross-link with another side chain.

Class A PBP's possess both glycosyl transferase and transpeptidase activity. These two activities are involved in the polymerization of the disaccharide units into glycan strands and in cross-linking the peptide side chains to one another, respectively. Class B high molecular weight PBP's differ from Class A in that they contain only transpeptidase activity (6). Class B PBP's cannot fully synthesize peptidoglycan, but they are essential for the regulation of cell shape and septation (6).

Low molecular weight PBP's

PBP5* (*dacB*)

Low molecular weight PBP's, such as PBP5* and DacF, possess D,D-carboxypeptidase activity. The protein product of *dacB*, PBP5*, is synthesized in the third stage of sporulation. PBP5* is expressed in the mother cell and is dependent on the activation of sigma E (2,4, Figure 4). The amino acid sequence of PBP5* contains a C-terminal amphipathic alpha helix and a cleavable signal peptide in the N-terminal portion of the protein. The amphipathic alpha helix is believed to anchor the PBP5* protein in the outer forespore membrane because PBP5* copurifies with the membrane (3). A *dacB* mutant produces heat sensitive spores, which is most likely due to the fact that *dacB* cortex peptidoglycan is known to be more highly cross-linked because of the loss of D,D-carboxypeptidase activity (11, 15). Studies show that with improper cross-linking in the cortex peptidoglycan, the spore cannot maintain its core dehydration during heating, allowing water to be taken up into the cell and heat resistance is lost (15).

DacF (*dacF*)

Another low molecular weight PBP-encoding gene is *dacF*. This gene produces a protein, DacF, in the forespore and its expression is dependent on sigma F (4, 20, Fig. 4). The amino acid sequence of *dacF* shows an apparent C-terminal amphipathic alpha helix (23) and is presumed to possess a signal peptide suggesting that DacF should be associated with the inner forespore membrane. However, DacF has never been

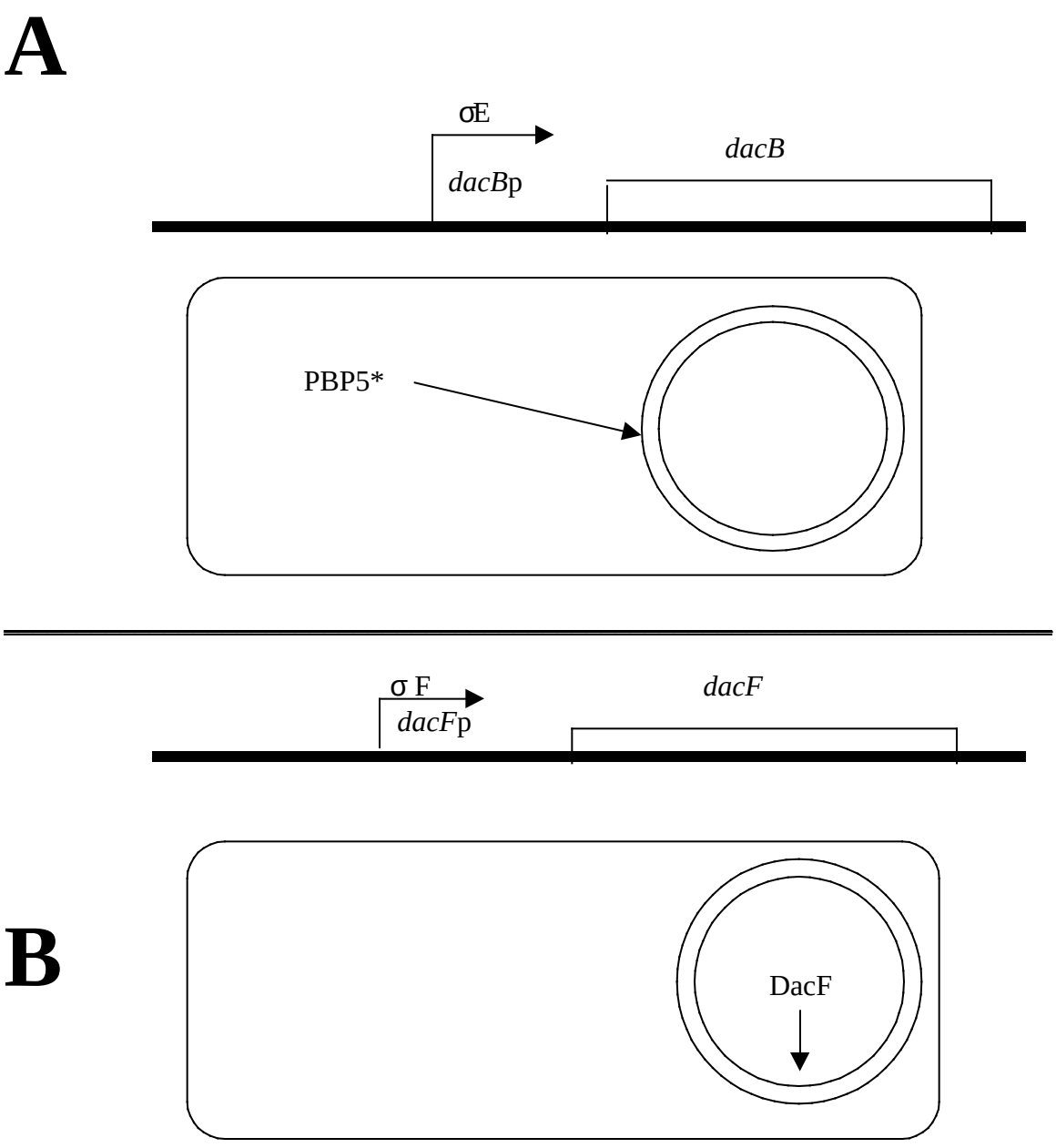

Figure 4. Low molecular weight PBP's, PBP5* and DacF. The *dacB* gene encodes the protein PBP5* and the *dacF* gene encodes the protein DacF. A) Wild type expression of the *dacB* gene with sigma E occurs in the mother cell. B) Wild type expression of the *dacF* gene with sigma F occurs in the forespore.

visualized in a membrane preparation using labeled penicillin. This suggests that DacF is either not membrane associated or it does not bind penicillin well. A *dacF* mutant produces wild type germ cell wall and cortex peptidoglycan (15).

The *spoIIA* operon

The *spoIIA* operon lies immediately downstream of *dacF* and is expressed early in sporulation under the control of sigma H (Fig. 5). The *spoIIA* operon consists of three genes: *spoIIAA, spoIIAB* and *spoIIAC* (20). The first two genes of this operon control the activity of sigma F, which is encoded by the third gene *spoIIAC*. Sigma F becomes active in the forespore and one of the genes expressed under its control is *dacF*. Transcription through *dacF* leads to an increased expression of *spoIIA*, therefore, it is possible that a polar *dacF* mutation could affect sporulation through an effect on *spoIIA* expression (20).

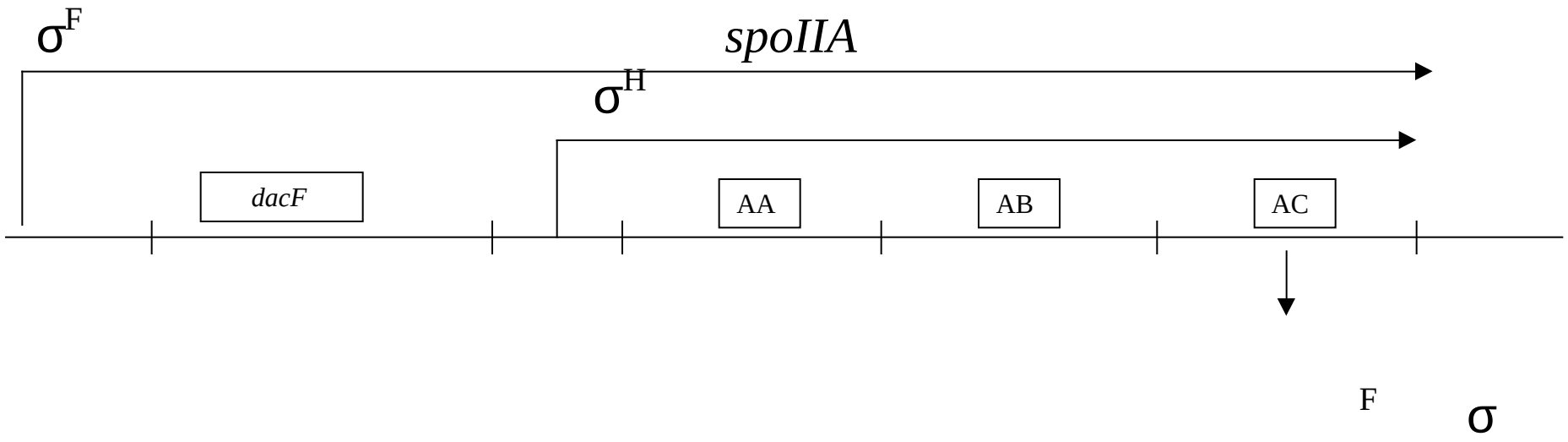

Figure 5: Wild type *spoIIA* operon. The *spoIIA* operon lies downstream of *dacF* and is expressed early in sporulation under the control of sigma H. The *spoIIA* operon consists on three genes, *AA, AB* and *AC*. *SpoIIAA* and *AB* control the activity of sigma F, which is encoded by *spoIIAC*. Sigma F becomes activated in the forespore and expresses *dacF*. Transcription through *dacF* leads to an increased expression of *spoIIA*.

Phenotypic characteristics of a *dacB dacF* double mutant

When a *dacB dacF* double mutant is examined, the spores have a greater amount of cross-linking in the cortex than the single *dacB* mutant (15). These data indicate that DacF must be able to affect the cortex, an effect that can be masked by PBP5* activity. It is amazing that DacF can affect the structure of the cortex when DacF is made in the forespore and only germ cell wall is made adjacent to the inner forespore membrane. All of the cortex peptidoglycan is made adjacent to the outer forespore membrane. One of the two possible theories to explain the results of *dacB dacF* double mutants is that in the absence of DacF in the inner forespore membrane, the germ cell wall is made incorrectly, and this in turn causes abnormal cortex synthesis. The other possible explanation is that DacF does not remain associated with the inner forespore membrane, and affects the cortex by entering the intermembrane space.

Goal of the low molecular weight PBP research

The aim of the low molecular weight PBP research presented here was to construct a set of strains that have compartmentalized expression of *dacB* and *dacF* to determine the functional and compartmental specificity of PBP5* and DacF, respectively. The experiments with *dacB* and *dacF* constructs were performed in a *dacB dacF* double mutant. First, *dacF* was expressed in the correct compartment, the forespore, as a control to be sure it is functional. A phenotype similar to a *dacB* single mutant was expected. Then *dacF* was expressed in the mother cell, where it may have entered the outer forespore membrane. If the first theory was correct and in the absence of DacF the germ cell wall is made incorrectly and thus the cortex is made incorrectly, then DacF expressed in the mother cell should not have solved the cortex synthesis problem. However, if the second theory was correct and DacF does not remain associated with the inner forespore membrane, but enters the intermembrane space to affect the cortex, then when DacF is expressed in the mother cell, the problem of cortex synthesis should have been solved.

Second, DacB was correctly expressed in the mother cell and a phenotype of a *dacF* single mutant was expected, which displays wild type germ cell wall and cortex peptidoglycan. DacB was then expressed in the forespore. If the first theory was correct, then DacB expressed in the forespore should have solved the cortex synthesis problem, but only if DacB was functionally equivalent to DacF. However, if theory two was correct, then DacB expressed in the forespore would not fix the problem with cortex synthesis because DacB would have been embedded in the inner forespore membrane.

Class A high molecular weight PBP's

PBP2d (*pbpG*)

Class A PBP's, such as the products of *pbpF*, *pbpG* and *pbpD*, possess both transpeptidase and glycosyl transferase activity. The protein product of *pbpG* is PBP2d. *pbpG* is expressed under the control of forespore-specific sigma F and its expression is sporulation specific (14, Figure 6). PBP2d possesses a signal peptide and is membrane-associated with the inner forespore membrane. A *pbpG* mutant produces normal heat resistant spores and spore cortex structure (10,14).

PBP2c (*pbpF*)

The gene that encodes PBP2c, *pbpF*, is expressed both in the vegetative state and during sporulation (17, Figure 6). In vegetative cells, PBP2c is weakly expressed. In the fourth stage of sporulation, PBP2c is expressed under the control of forespore specific sigma G (17). PBP2c is potentially present in all membranes, but probably its presence is strongest in the inner forespore membrane. PBP2c has been identified and is membrane-associated (19). The spore peptidoglycan structure of a *pbpF* mutant is similar to that of the wild type (10).

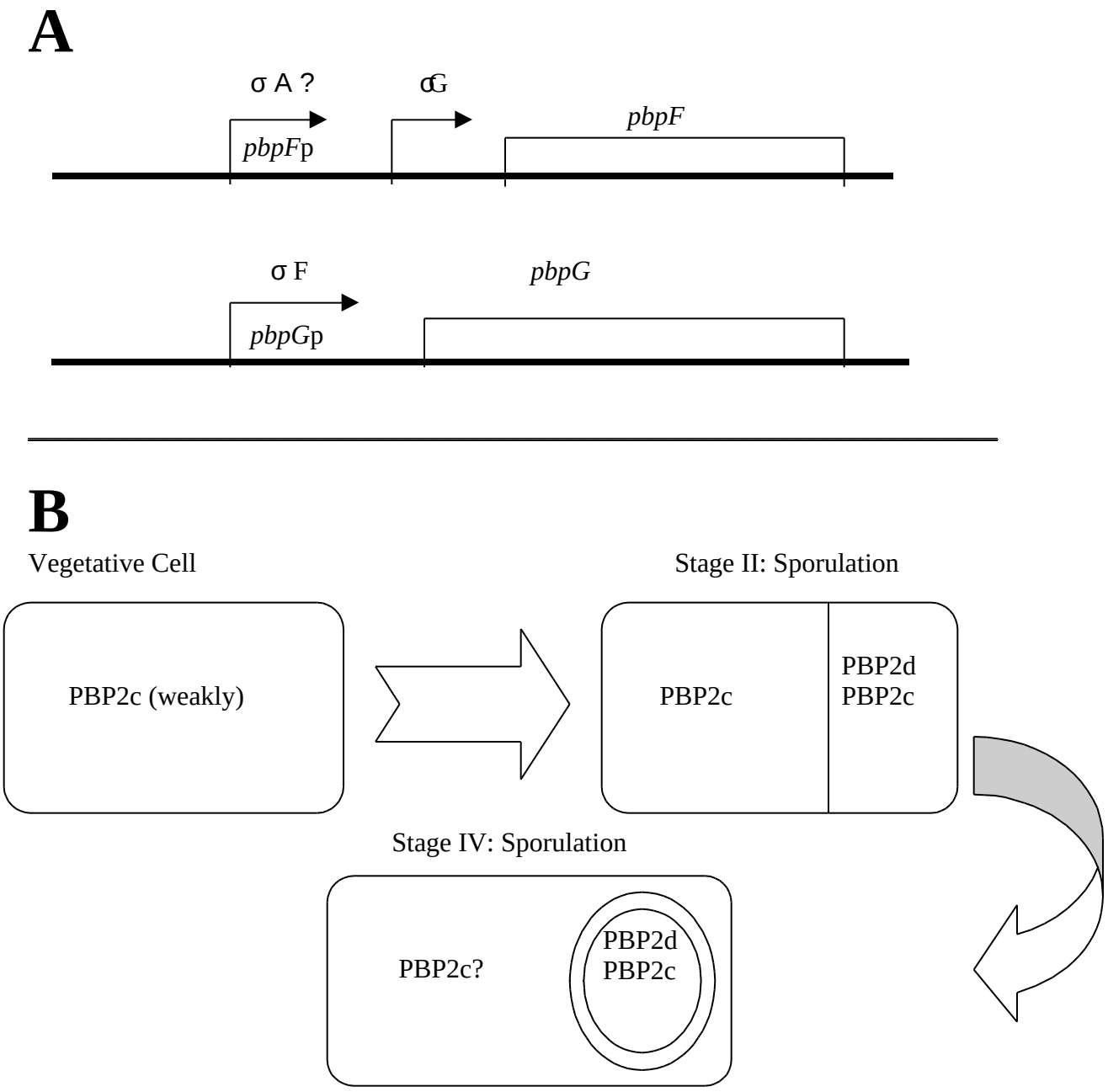

Figure 6. Class A HMW PBP's, PBP2c and PBP2d. A) The two pictures show the promoter configurations for the *pbpG* and *pbpF* genes in a wild type cell. B) *pbpG* is expressed in the forespore, which is demonstrated in the last two cells of the cascade. *pbpF* encodes PBP2c, and it is weakly expressed in the vegetative cell with sigma A factor. During the fourth stage of sporulation, PBP2c can be expressed in the forespore and possibly some residual of the PBP2c expressed vegetatively lingers in the mother cell in the outer forespore membrane.

Phenotypic characteristics of a *pbpF pbpG* double mutant

When the *pbpF pbpG* double mutant was examined previously, the cells were unable to carry out sporulation correctly. The double mutant makes a normal amount of spore peptidoglycan but the cortex structure is altered (10). The cortex appears to be made in large masses at the poles of the forespore, instead of in a sphere around the forespore.

Based on these results, it was proposed that PBP2d and PBP2c are functionally redundant in germ cell wall synthesis. Only when both proteins are absent, is the germ cell wall made incorrectly (10). The germ cell wall may function as a template for cortex deposition. This first theory indicates that when the germ cell wall is not made properly, then cortex synthesis will be impaired. An alternative explanation for the data on PBP2c and PBP2d is that the requirement for PBP2c or PBP2d is actually in the outer forespore membrane for cortex synthesis. PBP2c could be in the outer forespore membrane following vegetative expression (10). PBP2d could also play this role if it does not remain associated with the inner forespore membrane or if *pbpG* is very weakly expressed during vegetative growth.

PBP4 (*pbpD)*

PBP4 is a Class A high molecular weight PBP, encoded by *pbpD*, and plays a minor role in vegetative peptidoglycan synthesis (18, 19). PBP4 is the smallest and simplest Class A PBP. Its protein extension sizes outside of the highly conserved N and C terminal domains are very small (6). PBP4 is also a membrane-associated protein. A

pbpD mutant strain shows no obvious change in growth, spore germination, cell division, sporulation, spore peptidoglycan structure or spore heat resistance (18, 19).

Goals of Class A high molecular weight PBP research

The aim of the Class A high molecular weight PBP research presented here was to construct a set of strains that have compartmentalized expression of *pbpF, pbpG* and *pbpD* to determine the functional and compartmental specificity of PBP2c and PBP2d. The experiments with *pbpF, pbpG* and *pbpD* constructs were performed in a *pbpF pbpG* double mutant. In the first experiment PBP2c or PBP2d were expressed in the forespore. If the proposal that PBP2c and PBP2d play a redundant role in germ cell wall synthesis was correct, then either PBP2c or PBP2d should have corrected the double mutant defect. The alternative explanation is that PBP2c is required to be in the outer forespore membrane for cortex synthesis and PBP2d can act in the place of PBP2c if it does not remain associated with the inner forespore membrane. If this explanation was correct then we should have observed that forespore-specific expression of PBP2d corrects cortex synthesis but PBP2c does not.

The second experiment had either PBP2c or PBP2d expressed in the mother cell. If the first possibility was correct that PBP2c or PBP2d must function from the inner forespore membrane, then neither PBP2c nor PBP2d expressed in the mother cell would have been able to correct the double mutant defect. However, if the alternative hypothesis was correct, then either PBP2c or PBP2d would have been able to correct the defect.

The final experiment involved expressing *pbpD* in the mother cell and forespore. If *pbpD* could complement for the loss of *pbpF* or *pbpG*, expressing *pbpD* in the forespore should have corrected the cortex defect. If *pbpD* could not complement for the loss of *pbpF* or *pbpG*, than the cortex defect would not have been corrected and it can be

assumed that not just any Class A high molecular weight PBP is required in the forespore. This further means that PBP2c and PBP2d must possess an activity, lacking in PBP4, that allows them to carry out a specific role in spore peptidoglycan synthesis.

CHAPTER TWO

Materials and Methods

Construction of mother cell-specific and forespore-specific vectors

Plasmid pDG364 (7) (Table 1), which contains chlorophenicol resistance and *amyE* sequences for recombination into the *B. subtilis* chromosome at the nonessential *amyE* locus, was digested with the restriction enzymes *Spe*I and *Sac*II for one hour at 37°C. The digest was treated with the Klenow fragment of DNA polymerase and 2.5 mM of each nucleotide (ATP, GTP, CTP, TTP) for 15 minutes at room temperature. The enzymes were heat inactivated at 70°C for 15 minutes and then the vector was ligated back together to create pDPV92 (Table 1), which would be used to construct the mother cell-specific and forespore-specific vectors. With the removal of *Spe*I and *Sac*II sites from pDG364, it was possible to insert new *Spe*I and *Sac*II restriction sites that were advantageous to cloning in the coding sequences of the desired genes.

Primer pairs BsdacF1 + BsdacFp and BsdacBP2 + BsdacBP3 (Table 2) were used to amplify the *dacF* and *dacB* promoter regions, respectively, from *B. subtilis* chromosomal DNA. These PCR products were cloned into the pGEM-T vector (Promega) at the *EcoR*V site, in the opposite orientation of *lacZ* to produce pDPV86 and pDPV101 (Table 1). Next, a *Pst*I-*Sac*II-*Hind*III linker composed of primers PSHL3 and PSHL4 was added to a microfuge tube that contained pDPV92 digested with *EcoR*I and *Hind*III, and either pDPV86 or pPDV101 digested with *EcoR*1 and *Pst*I. After a ligation of all the above elements, the mother cell-specific and forespore-specific vectors were created (pDPV116 and pDPV115 (Table 1), respectively), and the new *Spe*I and *Sac*II sites were available downstream of each promoter for insertion of the desired genes.

Table 1. Plasmids used in constructing new *B.subtilis* mutant strains.

Plasmid	Vector	Vector Sites	Insert	Insert Sites	Description/Use	Resistance
pDG364					*amyE* sites for recombination into *B.subtilis* chromosome	Ap, Cm
pDPV86	pGEM-T	EcoRV	250bp PCR w/ BsdacF1 and BsdacFp		*dacFp* in opposite orientation to lacZ	Ap
pDPV92	pDG364	SpeI-SacII			Deletion of SpeI, XbaI and SacII sites	Ap, Cm
pDPV101	pGEM-T	EcoRV	258 bp PCR with BsdacBP2 and BP3		*dacB* promoter in opposite orientation to lacZ	Ap
pDPV105	pGEM-T	EcoRV	1170 bp PCR with BsdacB4 and BsdacB5		*dacB* in same orientation as lacZ	Ap
pDPV134	pUC19	HincII	1200 bp PCR with dacF5SpeI and dacF6SacII		*dacF* Pfu polymerase PCR product in pUC19	Ap
pDPV115	pDPV92	EcoRI-HindIII	250 bp dacFp from pDPV86	EcoRI-PstI	*dacFp* into *amyE* replacement vector along with PstI-SacII-HindIII linker	Ap, Cm

Plasmid	Vector	Vector Sites	Insert	Insert Sites	Description/Use	Resistance
pDPV116	pDPV92	EcoRI-HindIII	258 bp *dacBp* from pDPV101	EcoRI-PstI	*dacBp* into amyE replacement vector along with PstI-SacII-HindIII linker	Ap, Cm
pDPV117	pDPV115	SpeI-SacII	1200 bp *dacB* from pDPV105	SpeI-SacII	*dacFp-dacB* in pDPV92	Ap, Cm
pDPV118	pDPV115	SpeI-SacII	1200 bp *dacF* from pDPV134	SpeI-SacII	*dacFp-dacF* in pDPV92	Ap, Cm
pDPV119	pDPV116	SpeI-SacII	1200 bp *dacB* from pDPV105	SpeI-SacII	*dacBp-dacB* in pDPV92	Ap, Cm
pDPV120	pDPV116	SpeI-SacII	1200 bp *dacF* from pDPV134	SpeI-SacII	*dacBp-dacF* in pDPV92	Ap, Cm
pDPV135	pGEM-T	EcoRV	1875 bp PCR with pbpD1SpeI and pbpD2SacII		*pbpD* in orientation opposite lacZ	Ap
pDPV136	pGEM-T	EcoRV	2142 bp PCR with pbpF1SpeI and pbpF2SacII		*pbpF* in orientation opposite lacZ	Ap
pDPV137	pGEM-T	EcoRV	2170 bp PCR with pbpG1SpeI and pbpG2SacII		*pbpG* in orientation opposite lacZ	Ap
pDPV140	pDPV115	SpeI-SacII	1875 bp *pbpD* from pDPV135	SpeI-SacII	*dacFp-pbpD*	Ap, Cm
pDPV141	pDPV116	SpeI-SacII	1875 bp *pbpD* from pDPV135	SpeI-SacII	*dacBp-pbpD*	Ap, Cm
pDPV142	pDPV116	SpeI-SacII	2142 bp *pbpF* from pDPV136	SpeI-SacII	*dacBp-pbpF*	Ap, Cm

Plasmid	Vector	Vector Sites	Insert	Insert Sites	Description/Use	Resistance
pDPV143	pDPV115	SpeI-SacII	2170 bp *pbpG* from pDPV137	SpeI-SacII	*dacFp-pbpG*	Ap, Cm
pDPV144	pDPV116	SpeI-SacII	2170 bp *pbpG* from pDPV137	SpeI-SacII	*dacBp-pbpG*	Ap, Cm
pDPV145	pDPV115	SpeI-SacII	2142 bp *pbpF* from pDPV136	SpeI-SacII	*dacFp-pbpF*	Ap, Cm
PJF751					Translational vector EcoRI and BamHI *lacZ* sites	Ap, Cm
pDPV166	pJF751	EcoRI-BamHI	250 bp PCR product of spoIIAAEcoRI and spoIIAABamHI	EcoRI-BamHI	*spoIIAA-lacZ*	Ap

Table 2. Primer sequences used to construct mother cell-specific and forespore-specific vectors.

Primer Name	Length	Sequence 5'-3'	Function
BsdacBP2	21	GGTTTGTACAAGTTTATGCGC	*dacB* promoter - downstream
BsdacBP3	23	GGAATTCTTATACCGGGGTCAGC	*dacB* promoter-upstream with *EcoR*I site
BsdacF1	20	GCCGGAATTCTGGATCAGCC	*dacF* promoter-upstream
BsdacFp	31	GGTCTAGAATCCTTTTTATTTTTTCCAAGCG	*dacF* promoter-downstream
PSHL3	11	GAGCCGCGGAA	*Pst*I-*SacII*-*Hind*III linker
PSHL4	19	AGCTTTCCGCGGCTCTGCA	*Pst*I-*Sac*II-*Hind*III linker

Construction of Class A high molecular weight PBP plasmids

Plasmids pDPV135, pDPV136 and pDPV137 (Table 1) were constructed with the PCR products produced from *B.subtilis* chromosomal DNA using primer pairs BspbpD1SpeI and BspbpD2SacII, BspbpF1SpeI and BspbpF2SacII, BspbpG1SpeI and ywhE2SacII, respectively (Table 3). These PCR products contained *Spe*I and *Sac*II restriction sites because the primer sequences were designed with these sites. The PCR products were *Spe*I and *Sac*II-digested and directly cloned into the pDPV115 (forespore-specific) and pDPV116 (mother cell-specific) vectors. The correct gene sequences in all the plasmid constructs were confirmed through DNA sequencing (Virginia Bioinformatics Institute core laboratory).

Construction of mutant Class A high molecular weight PBP *Bacillus subtilis* strains

DPVB208, DPVB209, DPVB211, DPVB212, DPVB225 and DPVB226 (Table 4, Fig. 7) were made by using plasmids pDPV135, pDPV136 and pDPV137 (Table 1). The plasmids were linearized with *Sac*I for *pbpG* strains and *Sca*I for *pbpF* strains and transformed into DPVB56 (*pbpF*$^-$*pbpG*$^-$*)* with selection for chlorophenicol resistance. The linear DNA was expected to recombine into the nonessential *amyE* locus in the *B. subtilis* chromosome. A starch hydrolysis test was performed to verify the double crossover event into the *amyE* locus of DPVB56.

Table 3. Primer sequences used to construct strains with *pbpF*, *pbpG* and *pbpD*.

Primer Name	Length	Sequence 5'-3'	Function
BspbpF1SpeI	28	CCACTAGTTAGAAAGGCGAGGTGAGTTC	*pbpF* coding sequence-upstream
BspbpF2SacII	29	TCCCCGCGGCCGAATTCCATTAAGAGGAA	*pbpF* coding sequence-downstream
BspbpG1SpeI	29	CCACTAGTAAAAAAAGGGGGAACCCGTTG	*pbpG* coding sequence-upstream
YwhE2SacII	29	TCCCCGCGGAGCTCGATTGTCTAACATTC	*pbpG* coding sequence-downstream
BspbpD1SpeI	28	CCACTAGTAGAATTTAGGAGAAAAGAGA	*pbpD* coding sequence - upstream
BspbpD2SacII	31	TCCCCGCGGGGATCCTTTAATAAGCCGCTTG	*pbpD* coding sequence - downstream

Table 4. *Bacillus subtilis* strains used for analysis.

Strain[a]	Recipient Strain	Genotype[b]	Compartmentalization
VB208	VB56 (*pbpG::Kn pbpF:: MLS*)	*pbpG::Kn pbpF:: MLS dacBp-pbpF*	*dacBp-pbpF*
VB209	VB56	*pbpG::Kn pbpF:: MLS dacBp-pbpG*	*dacBp-pbpG*
VB211	VB56	*pbpG::Kn pbpF:: MLS dacFp-pbpF*	*dacFp-pbpF*
VB212	VB56	*pbpG::Kn pbpF: :MLS dacFp-pbpG*	*dacFp-pbpG*
VB225	VB56	*pbpG::Kn pbpF:: MLS dacFp-pbpD*	*dacFp-pbpD*
VB226	VB56	*pbpG::Kn pbpF:: MLS dacBp-pbpD*	*dacBp-pbpD*

[a]Laboratory stock strain numbers. [b]Abbreviations: Kn, kanamycin; MLS, resistance to erthromycin and lincomycin

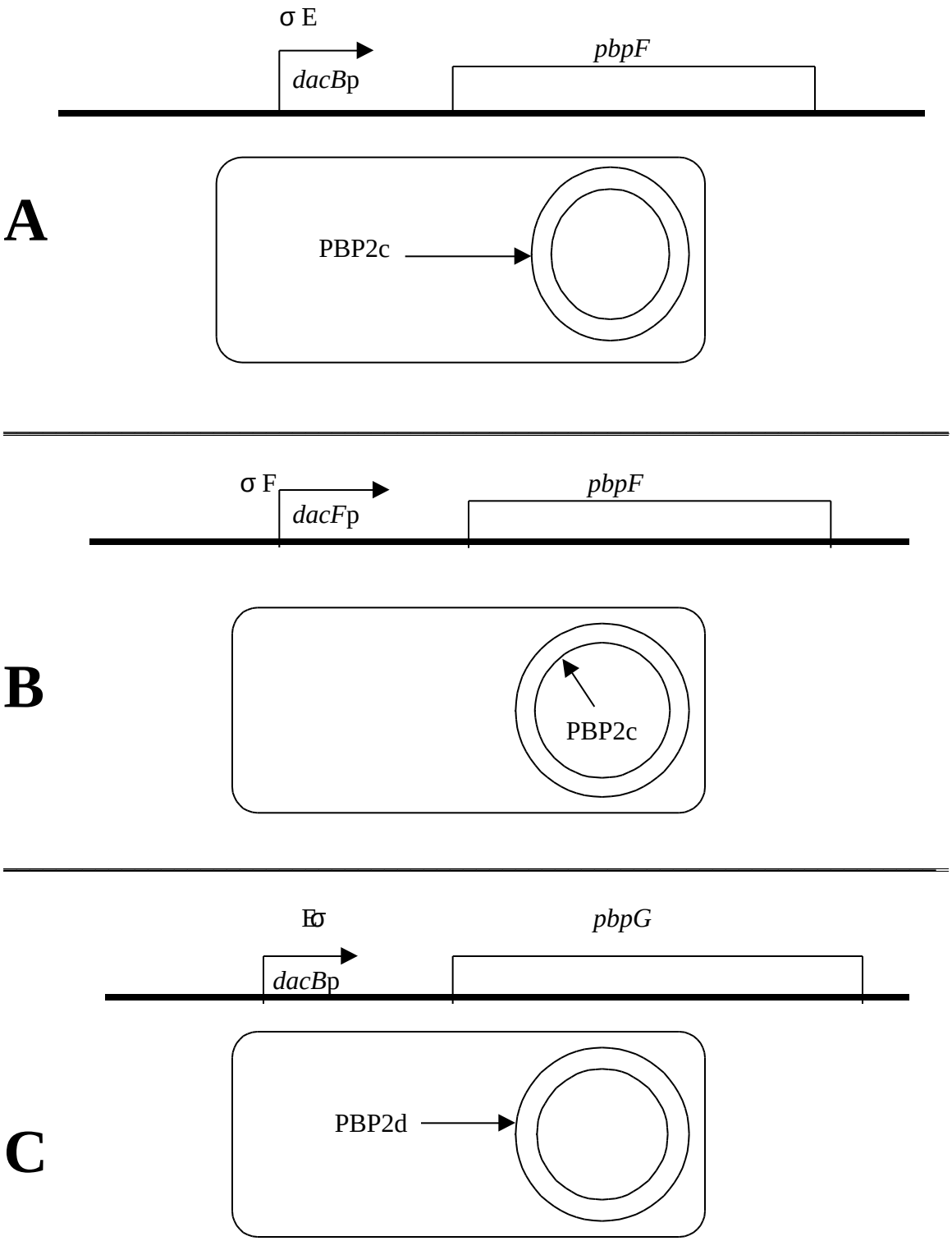

Fig. 7. Genetic constructs for incorrect expression of *pbpF* and *pbpG*. The above figures demonstrate the constructs that were recombined into the *amyE* locus and the resulting compartmentalized PBP expression. A) Strain DPVB208 B) Strain DPVB211 C) DPVB209.

Construction of low molecular weight PBP plasmids

Plasmids pDPV96 and pDPV105 (Table 1) were constructed with the PCR products produced using primer pairs BsdacF5 + BsdacF6 and BsdacB4 + BsdacB5 (Table 5). These PCR products were cloned into pGEM-T at the *EcoR*V site in the same orientation as *lacZ.* The *dacF* and *dacB* coding sequences were cut out of pDPV96 and pDPV105 using *Spe*I and *Sac*II. These were inserted into the mother cell and forespore-specific vectors digested with *Spe*I and *Sac*II to create plasmids pDPV117, pDPV118, pDPV119 and pDPV120 (Table 1). The correct gene sequences in all the plasmid constructs were confirmed through DNA sequencing (Virginia Bioinformatics Institute core laboratory).

Construction of mutant low molecular weight PBP *Bacillus subtilis* strains

DPVB161, DPVB162, DPVB163 and DPVB164 (Table 6, Fig. 8) were made using plasmids pDPV117, pDPV118, pDPV119 and pDPV120 (Table 1). The plasmids were linearized by digesting each plasmid with *Sac*I for 2 hours at 37°C. The linearized plasmids were transformed into DPVB98 *(*Δ*dacB dacF*::Kn) and DPVB219 (Δ*dacB* Δ*dacF*) with selection for chlorophenicol resistance. The linear DNA was expected to recombine into the nonessential *amyE* locus via a double crossover in the *B. subtilis* chromosome. To confirm a double crossover event occurred at the *amyE* locus, a starch hydrolysis test was performed. The strains were verified through PCR to be sure that Δ*dacB* was still present at its normal locus.

Table 5. Primer sequences used to construct strains with *dacB* and *dacF*.

Primer Name	Length	Sequence 5'-3'	Function
BsdacB4	22	GGGATCCCAAATTATATTGACC	*dacB* coding sequence - downstream
BsdacB5	16	CCACAAGGACGTGAGC	*dacB* coding sequence -upstream
BsdacF5	19	GAAATGGAGGGCTTTTGAG	*dacF* coding sequence -upstream
BsdacF6	17	GTGGTCATTCGGCATAA	*dacF* coding sequence- downstream
BsdacF5SpeI	27	CCACTAGTGAAATGGAGGGCTTTTGAG	*dacF* coding sequence -upstream with *Spe*I site
BsdacF6SacII	26	TCCCCGCGGGTGGTCATTCGGCATAA	*dacF* coding sequence- downstream with *Sac*II site

Table 6. *Bacillus subtilis* strains used for analysis.

Strain[a]	Recipient Strain	Genotype[b]	Compartmentalization
VB161	VB98 (Δ *dacB dacF::Kn*)	Δ *dacB dacF::Kn dacFp-dacB*	*dacFp-dacB*
VB162	VB98	Δ*dacB dacF::Kn dacFp-dacF*	*dacFp-dacF*
VB163	VB98	Δ*dacB dacF::Kn dacBp-dacB*	*dacBp-dacB*
VB164	VB98	Δ*dacB dacF::Kn dacBp-dacF*	*dacBp-dacF*
VB201	VB219 *(*Δ *dacB* Δ*dacF*)	Δ*dacB* Δ*dacF dacFp-dacF*	*dacFp-dacF*
VB204	VB219	Δ*dacB* Δ*dacF dacBp-dacF*	*dacBp-dacF*

[a]Laboratory stock strain numbers. [b]Abbreviations: Kn, kanamycin

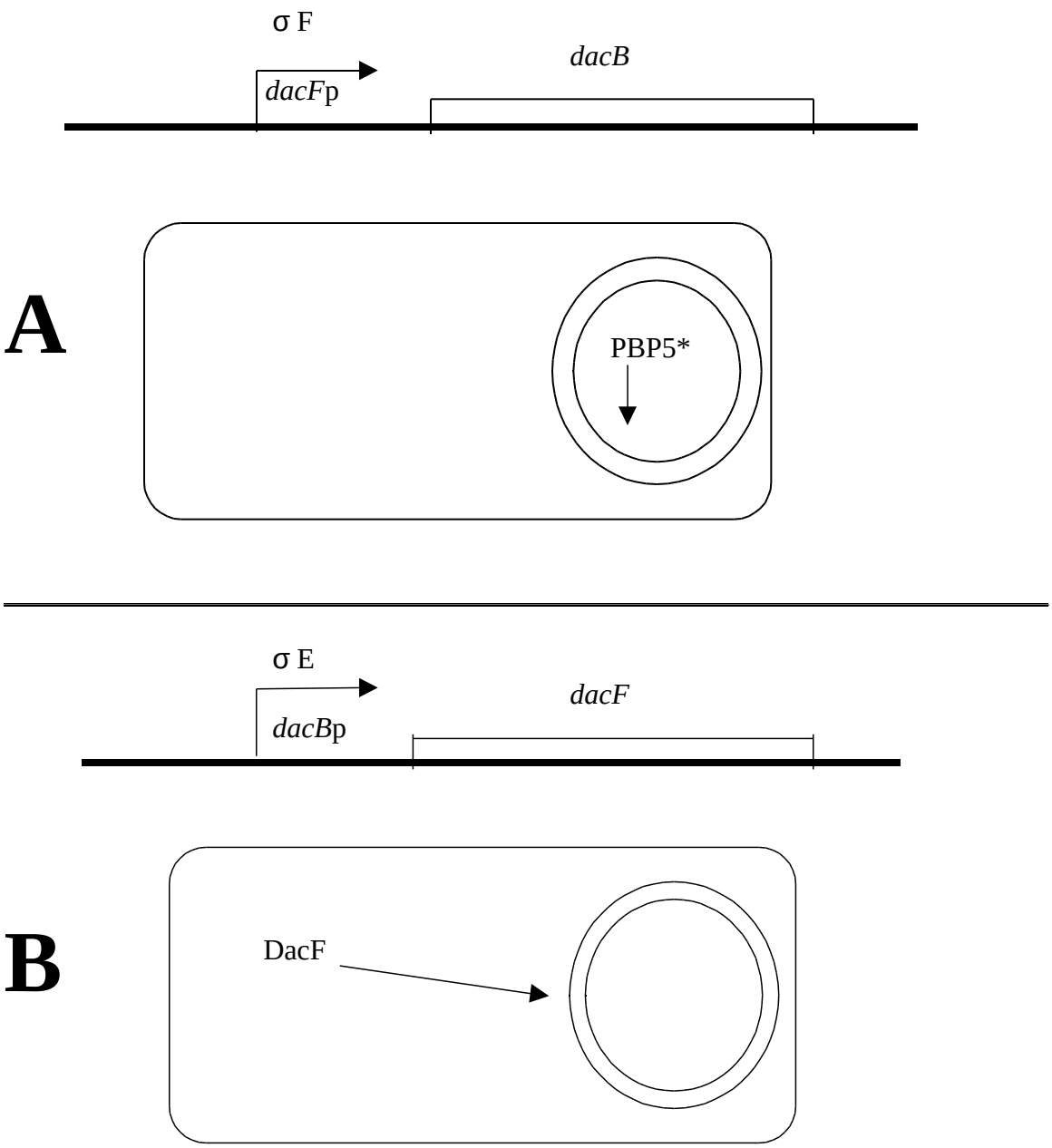

Figure 8. Genetic constructs for incorrect expression of *dacB* and *dacF*. A) The *dacB* gene was expressed in the forespore by being placed downstream of the forespore specific sigma factor F. B) The *dacF* gene was expressed in the mother cell under the control of mother cell specific sigma E.

Construction of sigma F-dependent gene transcriptional fusions

Chromosomal DNA from *B. subtilis* strains, DPVB169 (*pbpI-lacZ*) and DPVB237 (*pbpG-lacZ*), was transformed into two different *dacB dacF* double mutant strains (DPVB98 and DPVB219) with selection for chlorophenicol resistance. After purification of the new strains, they were named DPVB256, DPVB257, DPVB258 and DPVB259 (Table 8).

Construction of *spoIIAA-lacZ* translational fusion

Plasmid pDPV166 (Table 1) was constructed using the PCR product produced from *B.subtilis* chromosomal DNA using the primer pair, spoIIAAEcoRI and spoIIAABamHI (Table 7). This 250bp PCR product and translational fusion vector pJF751 (5) (Table 1) were digested with *Eco*RI and *BamH*I and ligated together. Once the construct was verified by DNA sequencing to have the *spoIIAA* insert upstream of *lacZ*, the plasmid was transformed into three different *B. subtilis* strains where it recombined into the chromosome via a Campbell-type insertion. The three *B. subtilis* strains used for the transformation were PS832 (wild type), DPVB98 (Δ*dacB dacF::Kn*) and DPVB219 (Δ*dacB* Δ*dacF*). After verifying the cross over event occurred by selecting for chlorophenicol resistance, the strains were named DPVB263, DPVB264 and DPVB265 (Table 8).

Table 7. Primer sequences used to construct *spoIIAA-lacZ* translational fusion.

Primer Name	Length	Sequence 5'-3'	Function
SpoIIAAEcoRI	27	CGGAATTCTATGCCGAATGACCACTAG	Upstream primer for *spoIIAA*
SpoIIAABamHI	23	CGGGATCCATAAAGGAAAGGTCC	Downstream primer for *spoIIAA*

Table 8. *Bacillus subtilis* strains used for β-galactosidase activity analyses.

Strain[a]	Genotype[b]
DPVB256	Δ *dacB* Δ*dacF pbpI-lacZ*
DPVB257	Δ *dacB* Δ*dacF pbpG-lacZ*
DPVB258	Δ*dacB dacF::Kn pbpI-lacZ*
DPVB259	Δ*dacB dacF::Kn pbpG-lacZ*
DPVB169	*pbpI-lacZ*
DPVB237	*pbpG-lacZ*
DPVB263	*spoIIAA-lacZ*
DPVB264	Δ*dacB dacF::Kn spoIIAA-lacZ*
DPVB265	Δ *dacB* Δ*dacF spoIIAA-lacZ*

[a]Laboratory stock strain numbers. [b]Abbreviations: Kn, kanamycin

Molecular Biology Methods

Restriction enzymes were purchased from Promega and digestions were incubated at 37°C for one hour. DNA was purified from low melt agarose gel slices with Wizard PCR Preps DNA purification system (Promega). Ligations were carried out at 4°C with T4 DNA ligase (Promega). Plasmid DNA was transformed into competent *E.coli* cells, strain JM109, through heat shock at 42°C and gentle shaking in rich medium for 1 hour at 37°C (2).

Plasmid DNA was extracted through the plasmid miniprep procedure from 3 mls of saturated culture. After the cells were pelleted, 100 µl of GET buffer (50 mM glucose, 10 mM EDTA, 25 mM Tris HCl pH 8.0) and 200 µl of lysis solution (0.2 N NaOH, 1% sodium dodecyl sulfate (SDS) (Sigma) were added and the pelleted cells were resuspended. Next, 150 µl of 3 M cold potassium acetate, 2 N glacial acetic acid was added and the suspension was put on ice for 5 minutes. Samples were centrifuged at 13,000 rpm for 10 minutes at 4°C. The supernatant was transferred to a new microfuge tube and 5 µl of 10 mg/ml RNase A was added. The samples were incubated at 37°C for 30 minutes. An equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) was added to the samples and vortexed for 45 seconds. The samples were then centrifuged at 13,000 rpm for 3 minutes and the top layer was transferred to a new microfuge tube. One milliliter of cold 95% ethanol was added and incubated for 10 minutes at room temperature. The samples were centrifuged at 13,000 rpm for 10 minutes and the supernatant was removed. The pellets were washed with cold 70% ethanol. Finally the samples were vacuum dried and resuspended in water (2).

Preparation and analysis of forespore peptidoglycan

B. subtilis cultures were induced to sporulate by the nutrient exhaustion method in 2xSG medium (9).

Samples for peptidoglycan analysis and biochemical assays were taken from sporulating cultures at 15 and 30-minute intervals. Samples of 30 mls were harvested and centrifuged at 8,000 rpm for 5 minutes at 20°C. The supernatant was discarded and the pellet was resuspended in 5 mls of SMM protoplast solution (2) by vortexing. Next, 25 mg of lysozyme was added and incubated at 37°C for 15 minutes to degrade the mother cell wall. The protoplasted cells were then added into 45 mls of boiling and stirring 4% SDS, 50 mM DTT and incubated for 20 minutes. The samples were allowed to cool, then transferred to a 50 ml centrifuge tube and centrifuged at 13,000 rpm for 30 minutes at 20°C. The supernatant was poured off and the pellet was resuspended in 1 ml of warm (65° -70°C) sterile MilliQ water. Finally the suspension was transferred to a 1.5 ml fresh microfuge tube. The tubes were boiled for 5 minutes and then centrifuged immediately at 21,000 g for 20 minutes. The supernatant was aspirated and the pellet was resuspended and washed repeatedly in 1 ml of warm, sterile MilliQ water until SDS could not be detected in the supernatant. The samples were then digested for 2 hours at 37°C with 1 ml of 100 mM Tris HCl pH 7, 20 mM $MgSO_4$, 15 µg DnaseI and 75 µg Rnase A. 150 µg of Trypsin and $CaCl_2$ (10 mM) were added and incubation continued for 16 hours at 37°C. The samples were centrifuged at 15,000 rpm for 20 minutes and the supernatant was aspirated. The pellet was resuspended in 1 ml of 1% SDS and boiled for 20 minutes. Samples were washed with 1 ml of warm sterile MilliQ water until SDS was not detected. The isolated spore PG was digested with 125 units of Mutanolysin

(Sigma) in a total volume of 250 µl 12.5 mM $NaPO_4$ (pH 5.5) for 16 hours at 37°C. The solubilized muropeptides were prepared for HPLC analysis (16).

Biochemical assays

GDH and DPA were assayed as previously described (13). Spore heat resistance was measured as described (13). To measure the hexosamine content, 0.5 mL samples were taken at each time point during sporulation and centrifuged at 13,000 rpm for 45 seconds. After the supernatant was carefully aspirated, the cells were resuspended in 0.5 ml of cold 1 mM $MgCl_2$. The samples were centrifuged again at 13,000 rpm for 45 seconds and the supernatant aspirated. The cells were finally resuspended in 0.5 ml of cold 6 N HCl and 20 µl was transferred to a new microfuge tube. The 20 µl samples were hydrolyzed at 95°C for 4 hours and the amino sugars were analyzed (11).

β-galactosidase Assay

The strains were grown in 2xSG media and sporulation was induced by the nutrient exhaustion method. Samples of 1 ml were taken every 30 minutes during sporulation (t_0-t_5) and centrifuged at 13,000 rpm for 30 seconds. The supernatant was aspirated and the pellets were stored at –80°C until they were ready to be assayed.

To prepare for the β-galactosidase assay, the pellets were resuspended in 1 ml of Z buffer (12) containing 0.5 mg lysozyme and incubated at 37°C for 10 minutes. Then, 10 µl of 10% Triton was added to each sample with brief vortexing. To begin the assay, 400 µl of the cell extract and 400 µl of Z Buffer were combined in a fresh microfuge tube and placed at 30°C for 5 minutes before starting the reaction. To start each reaction, 160

µl of o-nitrophenyl-β-D-galactopyranoside (ONPG, 4 mg/ml) in Z buffer was added, the tubes were vortexed and placed back in the 30°C water bath. Once the solution in the tube was clearly becoming yellow, 0.4 ml of 1 M Na_2CO_3 was added to stop the reaction. The time at which the reaction began and stopped was recorded. After 60 minutes, all remaining reactions were stopped, regardless of their color. All samples were spun at 13,000 rpm for 5 minutes to remove insoluble material and the supernatant was poured into a cuvette. The absorbance of each sample was read at 420 nm. The amount of β-galactosidase activity was calculated in terms of "Miller Units" (12).

CHAPTER THREE

Results from analyses of Class A high molecular weight PBP's

The goal of the Class A high molecular weight PBP research was to determine the functional and compartmental specificity of PBP2c and PBP2d. A set of strains were constructed that had compartmentalized expression of the Class A high molecular weight PBP-encoding genes, *pbpF, pbpG* and *pbpD*. The aim of creating these strains was to answer the following questions. Do PBP2c and PBP2d play a redundant role in germ cell wall synthesis or is PBP2c required to be in the outer forespore membrane for cortex synthesis? Can PBP2d act in the place of PBP2c if it does not remain associated with the inner forespore membrane? Do PBP2c and PBP2d function only from the inner forespore membrane, presumably creating a template of germ cell wall for proper cortex synthesis? Can PBP4 carry out the role played by PBP2c or PBP2d in spore peptidoglycan synthesis?

All the constructed plasmids containing *pbpF, pbpG* and *pbpD* were inserted into a *pbpF pbpG* double mutant. Each gene was placed downstream of a mother cell-specific promoter, *dacBp,* and a forespore-specific promoter, *dacFp* and these vectors allowed expression specifically in the mother cell or the forespore. Each gene contained its native ribosome-binding site for expression of the gene in the designated compartment. The vectors containing a specific promoter and gene were recombined into the nonessential *amyE* locus in the *pbpF pbpG* double mutant chromosome.

Timing of biochemical and phenotypic sporulation markers

During the period of spore peptidoglycan synthesis, samples were harvested from sporulating cultures and the forespore peptidoglycan was extracted. Experiments were performed in duplicate for the Class A high molecular weight PBP's. Only one data set from each strain is presented but the other results looked similar.

Cultures were induced to sporulate by the nutrient exhaustion method in 2xSG medium (9). GDH was the first sporulation marker examined. It is expressed during stage III of sporulation (21). GDH increases and reaches its maximum during the seventh hour of sporulation (Fig. 9). GDH activity in the strains that expressed *pbpF* and *pbpG* in the mother cell (DPVB208 and DPVB209) began to increase around the fourth hour of sporulation, similar to the control strains that expressed *pbpF* and *pbpG* in the forespore (DPVB211 and DPVB212). However, by the sixth hour of sporulation, DPVB208 and DPVB209 had less GDH activity than did DPVB211 and DPVB212. By the twenty-fourth hour, all four strains expressing *pbpF* and *pbpG* in the mother cell and in the forespore dropped near zero. GDH activity dropped near zero at the end of sporulation because the spores became resistant to lysozyme. Any GDH in the spores was therefore not released during the assay. GDH activity produced in the strains expressing *pbpD* (DPVB225 and DPVB226) in the mother cell and in the forespore was similar to a *pbpF pbpG* double mutant strain, with lower activity around the sixth hour of sporulation (Fig. 10). GDH activity reached the maximum at the eighth hour of sporulation and then dropped near zero at t_{24} for both *pbpD* strains.

DPA began to increase around the fifth hour of sporulation, following GDH activity, and continued to increase throughout the remainder of sporulation in DPVB211

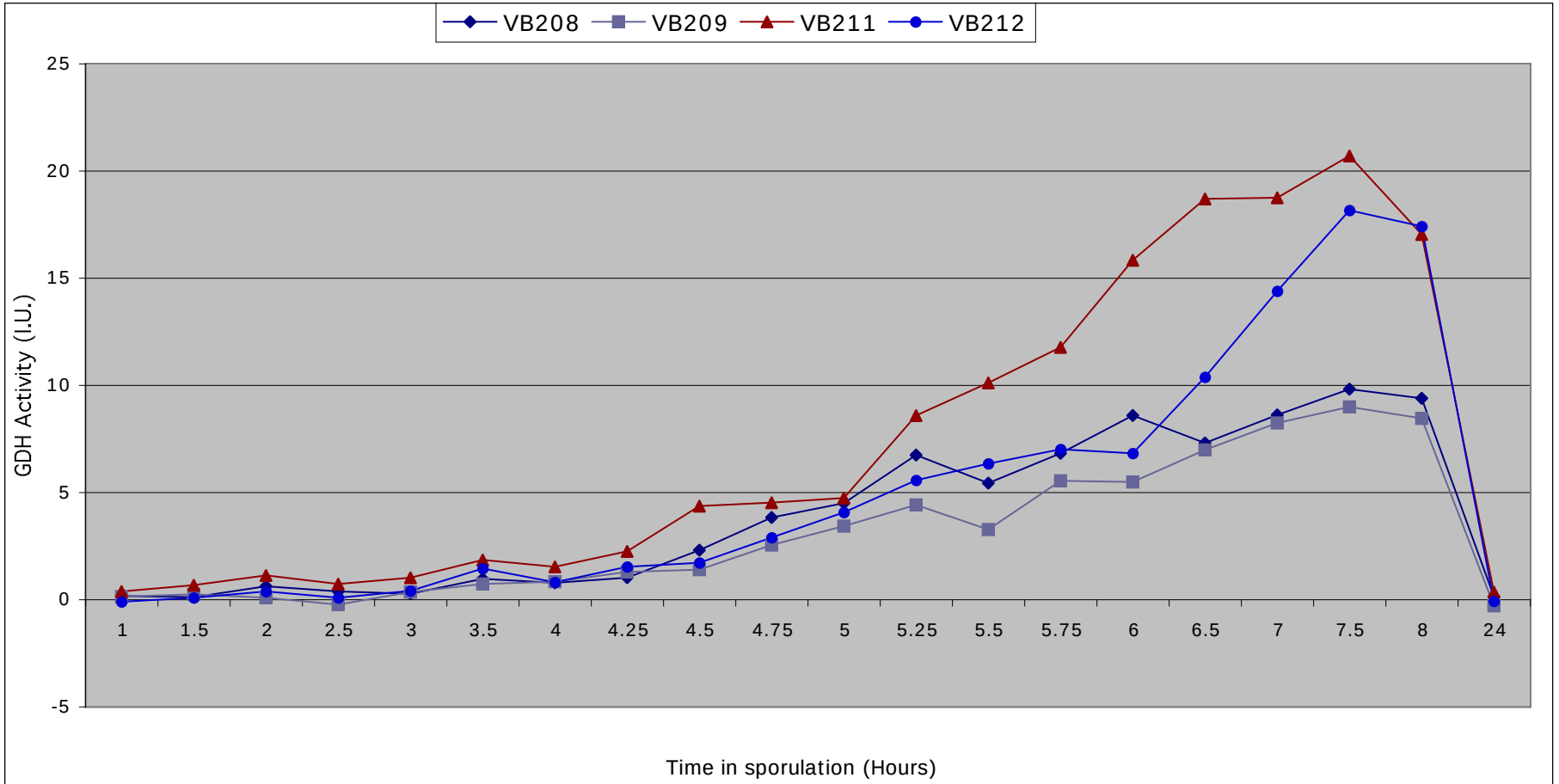

Fig. 9 - GDH activity produced in Class A PBP strains expressing PBP2c and PBP2d. Strains expressing pbpF and pbpG in the mother cell had less GDH activity at the 6th hr of sporulation compared to the strains expressing pbpF and pbpG in the forespore.

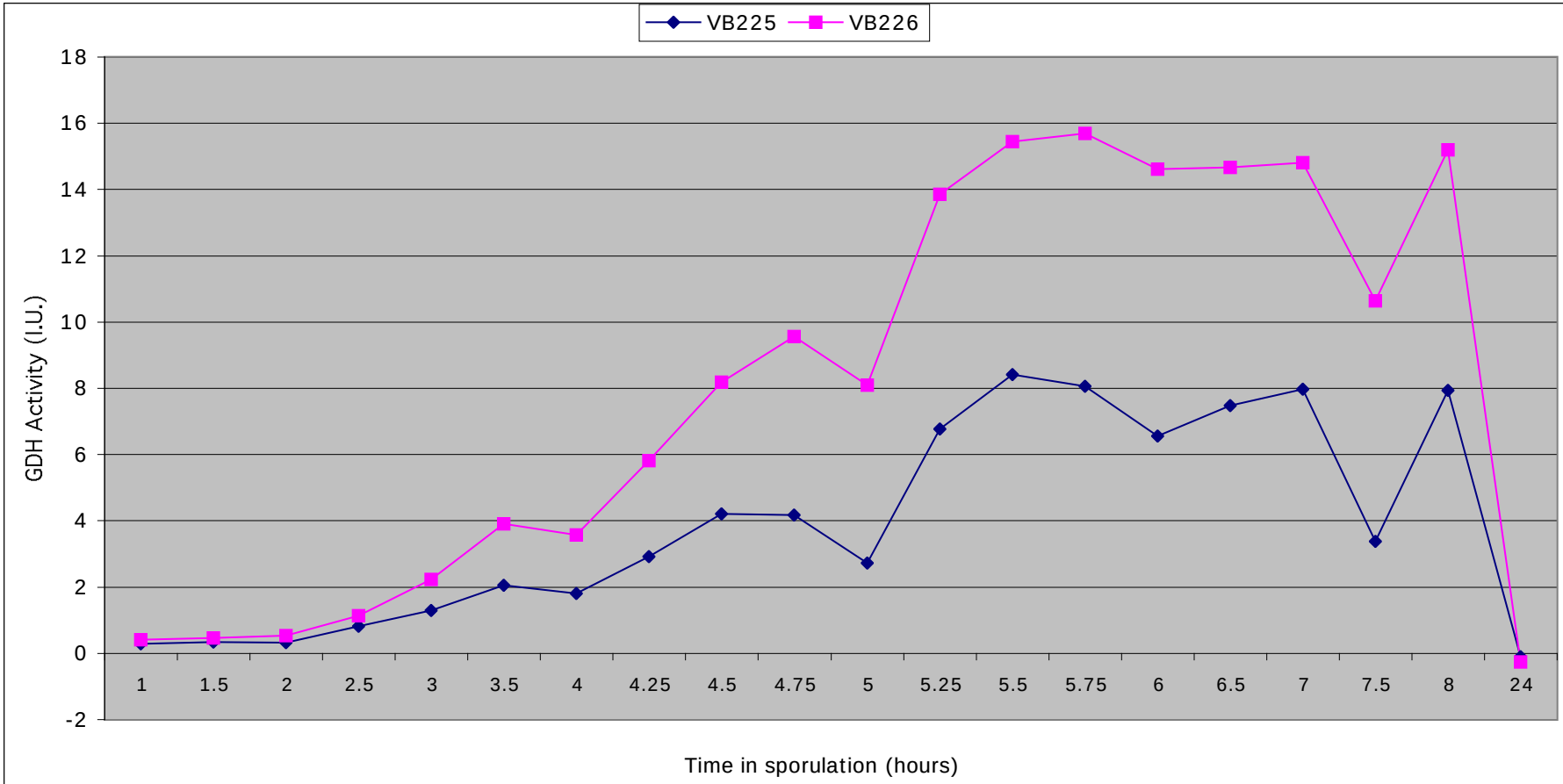

Fig 10. GDH activity produced in Class A PBP strain expressing PBP4. GDH activity produced in the strains expressing pbpD in the MC and FS was similar to a pbpFpbpG double mutant, with lower activity around the 6th hour of sporulation.

and DPVB212 (Fig. 11). However, in strains DPVB208 and DPVB209, DPA never accumulated to similar levels. In both *pbpD* strains (DPVB225 and DPVB226), DPA accumulation was lower compared to wild type strains (Fig. 12).

The third sporulation marker, heat resistance, is measured by viable cell counts and follows the appearance of DPA and GDH. In strains DPVB211 and DPVB212, where *pbpF* and *pbpG* were expressed in their correct compartment, the forespore, there are a greater amount of heat resistance spores formed than compared to strains DPVB208 and DPVB209 (where *pbpF* and *pbpG* were expressed in the mother cell, Fig.13). These results indicated that expressing *pbpF* and *pbpG* in their correct compartment allowed for the complementation of *pbpF* and *pbpG* in the double mutant. However, when *pbpF* and *pbpG* were expressed in the mother cell, the forespores were unstable and could not produce normal resistant spores. A low amount of heat resistant spores were also formed in the *pbpD* expressing strains compared to strains DPVB211 and DPVB212. This would indicate that expressing *pbpD* in either the mother cell or the forespore cannot change the phenotype of the *pbpF pbpG* double mutant (Fig.13).

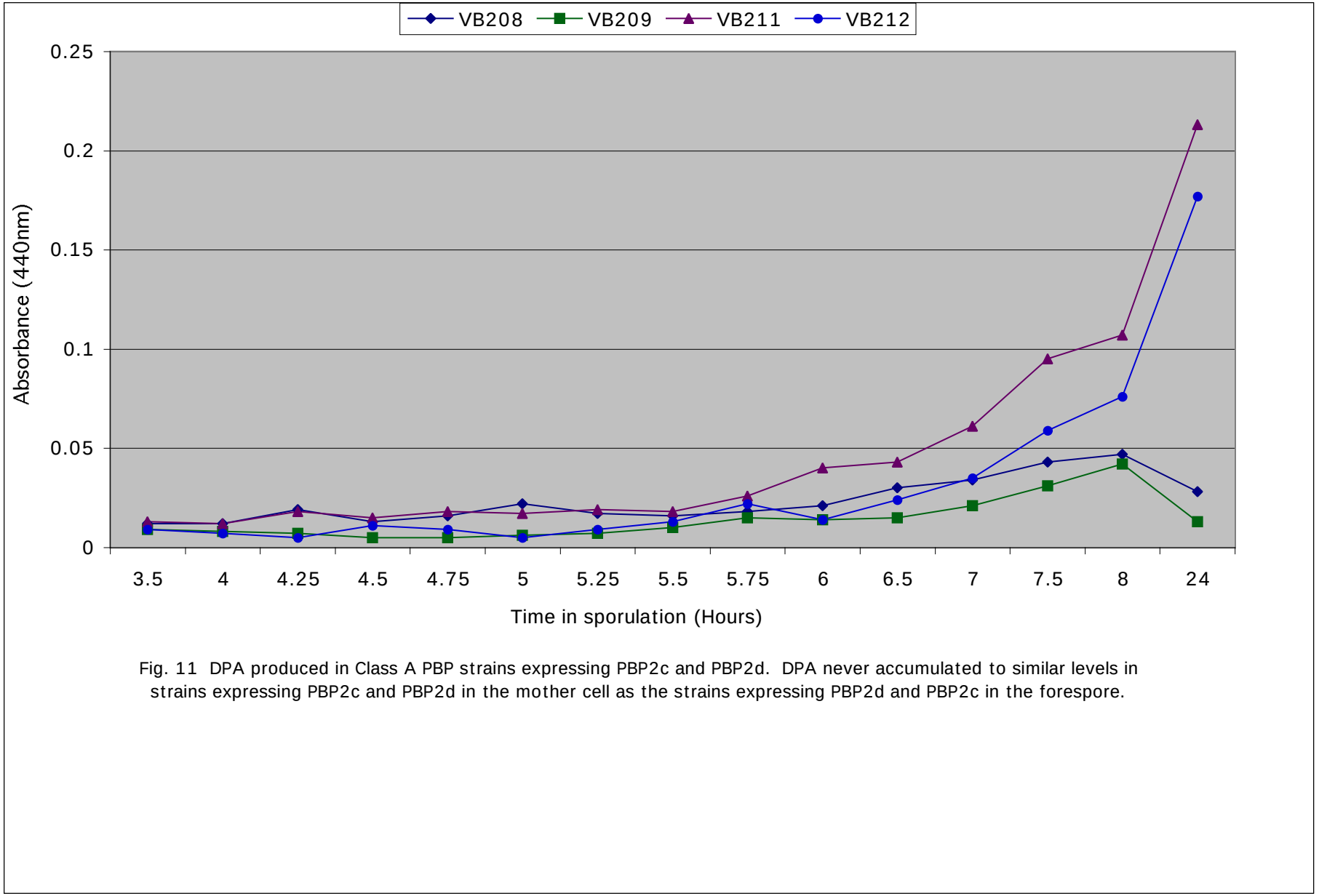

Fig. 11 DPA produced in Class A PBP strains expressing PBP2c and PBP2d. DPA never accumulated to similar levels in strains expressing PBP2c and PBP2d in the mother cell as the strains expressing PBP2d and PBP2c in the forespore.

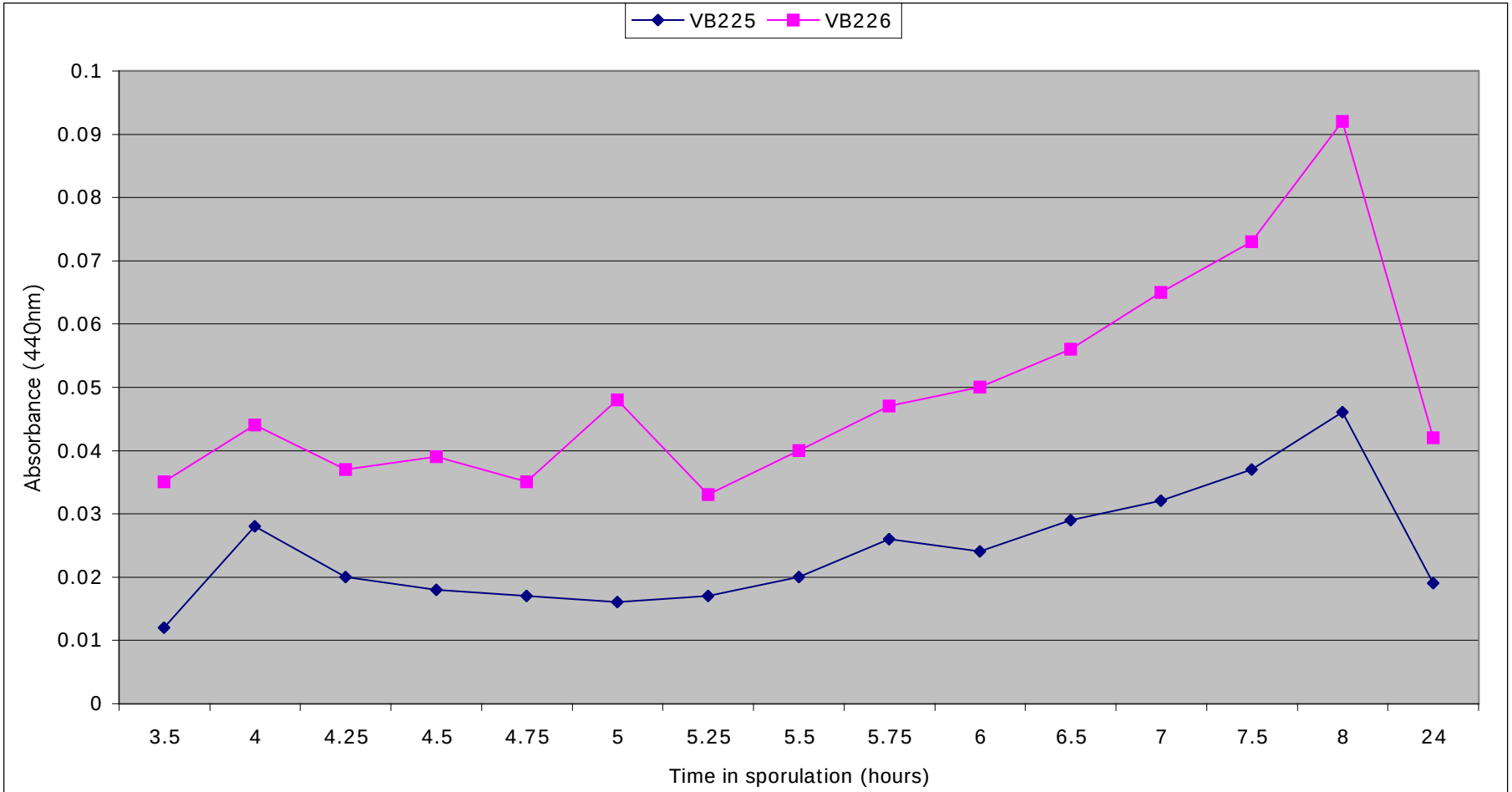

Fig 12. DPA produced in Class A PBP strain expressing PBP4. DPA accumulation was lower in PBP4 strains compared to wild type strains.

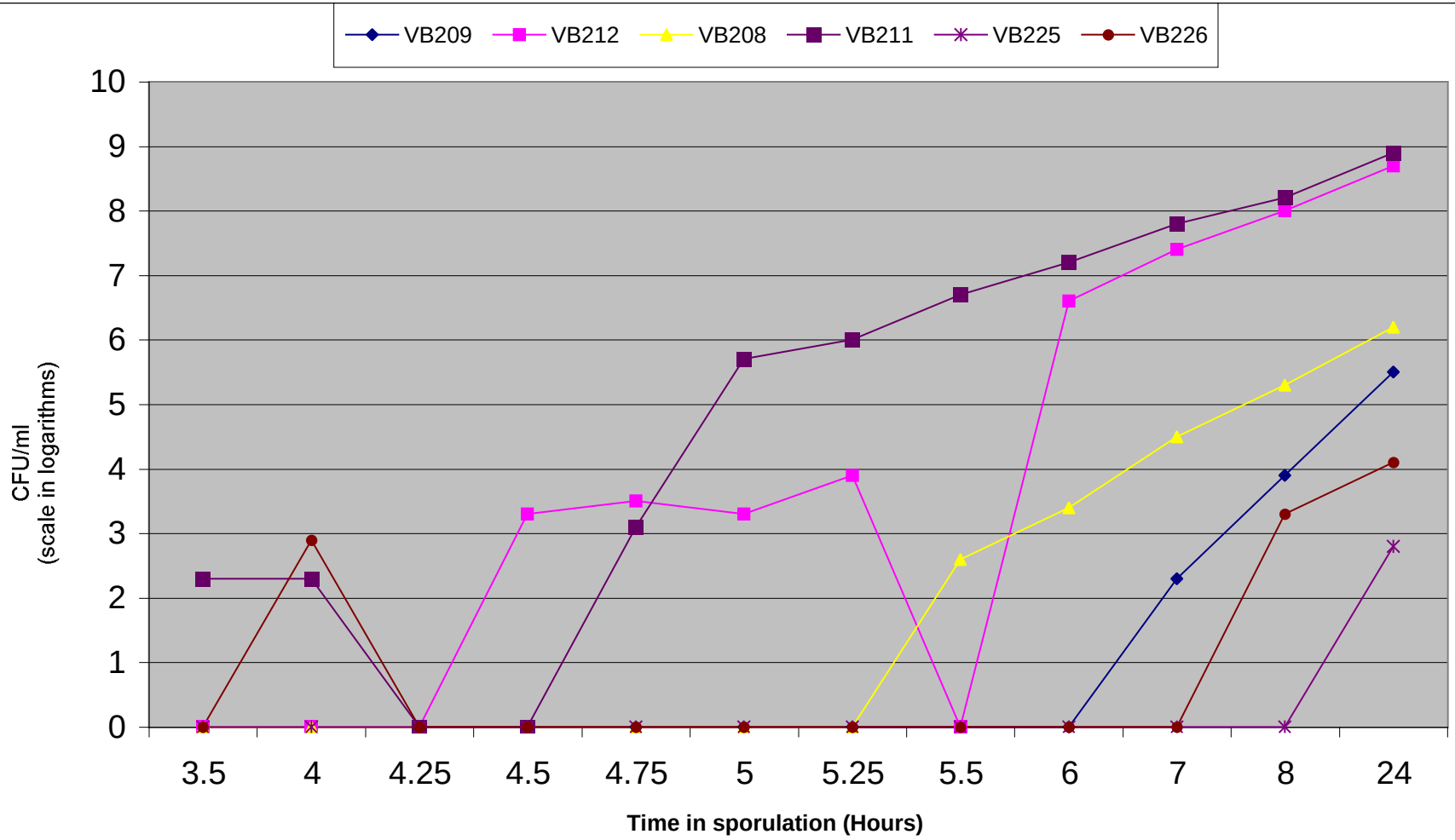

Fig 13. Heat resistant spores formed in Class A PBP strains expressing PBP2c, PBP2d and PBP4. Less heat resistant spores were formed in strains DPVB208,209,225 & 226 than strains expressing PBP2c and PBP2d in the forespore.

Structure of developing forespore PG

The strains constructed with *pbpF, pbpG* and *pbpD* were sampled to determine the structure of the spore peptidoglycan. A *pbpG* single mutant and a *pbpF* single mutant produce normal heat resistant spores and spore cortex structure. The double mutant makes a normal amount of spore peptidoglycan, but the cortex structure is altered. The cortex appears to be made in large masses at the poles of the forespore, instead of evenly placed around the forespore (10). The *dacFp-pbpF* (DPVB211) and *dacFp-pbpG* (DPVB212) control strains produced spore peptidoglycan like that of the wild type and *pbpF* and *pbpG* single mutants (Table 9,10). However, when *pbpF* and *pbpG* were expressed in the mother cell, forespore peptidoglycan similar to that of the *pbpF pbpG* double mutant was observed (Table 11,12), suggesting that *pbpF* and *pbpG* must be expressed in the forespore in order to carry out its specific role during spore peptidoglycan synthesis.

Another Class A high molecular weight PBP, *pbpD*, was cloned into the mother cell-specific and forespore-specific expression vectors and transformed into a *pbpF pbpG* double mutant. This gene encodes PBP4, which plays a minor role in vegetative peptidoglycan synthesis (18,19). It was to be determined if expressing *pbpD,* within the forespore or within the mother cell of the *pbpF pbpG* double mutant, could complement for the loss of *pbpF* and *pbpG* products, PBP2c and PBP2d. PBP4 was chosen because it is the simplest Class A high molecular weight PBP. Results confirmed that when *pbpD* is expressed, either in the forespore or mother cell, the forespore peptidoglycan produced was similar to that of the *pbpF pbpG* double mutant (Table 13, 14), indicating that *pbpD*

does not complement the loss of *pbpF* and *pbpG* for synthesis of normal spore germ cell wall.

Table 9. Structural parameters of forespore PG produced by VB211 (*dacFp-pbpF)*.

Sample	% muramic acid as lactam	% muramic acid with alanine	% muramic acid with tripeptide	% muramic acid with tetra-peptide	% muramic acid with cross-linked peptide
T4.5	15.4*	23.5*	42.3*	18.8*	7.1*
T4.75	17.3 ± 1.3	23.2 ± 2.6	41.8 ± 3.7	17.8 ± 0.2	4.8 ± 3.2
T5	34.4 ± 3.7	31 ± 4	21.4 ± 0.1	13.3 ± 7.8	4.5*
T5.25	40.8 ± 2.8	33.2 ± 3.5	12 ± 1.8	14.1 ± 8	3.5*
T5.5	41.4 ± 4	30 ± 2.1	10.9 ± 1.2	17.8 ± 4.9	3.3 ± 1.8
T5.75	41.4 ± 2.8	29.1 ± 0.1	9.1 ± 1.7	20.6 ± 1.1	3.4 ± 1.1
T6	42.7 ± 2.8	27.2 ± 0.2	7.2 ± 1.6	23 ± 1	3.5 ± 0.9
T6.5	42.9 ± 3.3	26.2 ± 4.2	7.1 ± 1.6	23.9 ± 5.9	3.6 ± 1.7
T7	44.8 ± 2.2	24.5 ± 0.7	5.3 ± 1	25.5 ± 1.9	3.8 ± 0.9
T7.5	45.6 ± 1.3	23.8 ± 1.5	4.5 ± 0.6	26.3 ± 2.2	3.7 ± 0.9
T8	46.7 ± 1.0	23.9 ± 3.4	3.5 ± 0.7	25.9 ± 3.7	3.6 ± 1.5
T24	46.2 ± 3	11.7 ± 9.8	3.6 ± 1.7	38.5 ± 11.2	4.4 ± 1.5

*Data only available for one sampling

Values are averages of two independent analyses with errors of one standard deviation.

Table 10. Structural parameters of forespore PG produced by VB212 (*dacFp-pbpG)*.

Sample	% muramic acid as lactam	% muramic acid with alanine	% muramic acid with tripeptide	% muramic acid with tetra-peptide	% muramic acid with cross-linked peptide
T5.25	27.7*	35.2*	25.8*	11.3*	3.8*
T5.5	31.4 ± 1.9	34.7 ± 1.6	17.7 ± 3.6	16.4 ± 3.3	2.4 ± 1.4
T5.75	37 ± 1.5	34.4 ± 1.6	11.2 ± 2	17.5 ±1.2	2.3 ± 1.1
T6	41.2 ± 1.6	33.8 ± 2	7.6 ± 0.1	17.5 ± 0.4	2 ± 1.3
T6.5	42.8 ± 1.2	32 ± 1.6	5.6 ± 1.1	19.7 ± 1.6	2.8 ± 0.5
T7	44.4 ± 0.4	29.8 ± 1.8	4.4 ± 0.1	21.5 ± 2.3	3.1 ± 0.1
T7.5	43.7 ± 2.8	28.9 ± 4.7	4 ± 0.4	23.4 ± 2.4	3.2 ± 1.1
T8	43.7 ± 1.9	23.6 ± 1.5	3.4**	29.5 ± 0.4	3.7 ± 0.2
T24	47.9 ± 1.1	21.5 ± 2.6	2 ± 0.2	28.7 ± 3.5	3.1 ± 0.2

*Data only available for one sampling
**Values in the multiple analyses were identical

Values are averages of two independent analyses with errors of one standard deviation.

Table 11. Structural parameters of forespore PG produced by VB208 (*dacBp-pbpF).*

Sample	% muramic acid as lactam	% muramic acid with alanine	% muramic acid with tripeptide	% muramic acid with tetra-peptide	% muramic acid with cross-linked peptide
T5.25	6.1*	8.0*	54.6*	31.2*	5.2*
T5.5	7.1 ± 2.9	10 ± 6.1	40.5 ± 4.5	42.5 ± 4.7	7.1 ± 4.2
T5.75	10.6 ± 0.6	11.4 ± 3.7	32.4 ± 0.9	45.6 ± 2.3	7 ± 3.1
T6	14 ± 2.7	9.7 ± 1.4	27.3 ± 0.4	49.1 ± 3.7	8.3 ± 2.7
T6.5	13.7*	5.9*	21.7*	58.8*	10.0*
T7	15.9 ± 0.9	5.6 ± 1.1	17.4 ± 0.3	61.2 ± 0.3	8.9 ± 1.6
T7.5	16.4*	5.9*	14.0*	63.6*	10.2*
T8	16 ± 0.3	7.4 ± 3.6	13.1 ± 1.5	63.6 ± 4.8	7.6 ± 0.1
T24	22.3*	**	5.5*	72.2*	**

*Data only available for one sampling

**Values not available for this time point.

Values are averages of two independent analyses with errors of one standard deviation

Table 12. Structural parameters of forespore PG produced by VB209 (*dacBp-pbpG*).

Sample	% muramic acid as lactam	% muramic acid with alanine	% muramic acid with tripeptide	% muramic acid with tetra-peptide	% muramic acid with cross-linked peptide
T6	11.4*	16.8*	22.2*	49.6*	9.5*
T6.5	9.6 ± 0.7	6.2 ± 0.5	21.7 ± 1.5	62.7 ± 1.2	7.2 ± 1.9
T7	12.2 ± 0.6	6.2 ± 0.1	14.4 ± 1.3	67.3 ± 0.8	5.6 ± 3.5
T7.5	13.2*	10.6*	14.7*	61.6*	10.2*
T8	13.9*	5.6*	9.3*	71.3*	4.3*

*Data only available for one sampling

Values are averages of two independent analyses with errors of one standard deviation.

Table 13. Structural parameters of forespore PG produced by VB226 (*dacBp-pbpD*).

Sample	% muramic acid as lactam	% muramic acid with alanine	% muramic acid with tripeptide	% muramic acid with tetra-peptide	% muramic acid with cross-linked peptide
T5	6.3	21.3	29.3	43.1	11.0
T5.25	5.5	*	31.7	62.8	6.7
T5.5	5.9	4.9	22.6	66.6	8.2
T5.75	5.6	5.9	19.7	68.7	9.8
T6.5	7.4	6.7	15.7	70.2	10.0
T7	15.7	7.4	8.5	68.4	7.6
T7.5	16.7	8.0	8.6	66.7	7.9
T8	15.7	9.0	9.3	66.0	10.5

Only one sampling was performed on this strain.
*No data available for this time point.

Table 14. Structural parameters of forespore PG produced by VB225 (*dacFp-pbpD*).

Sample	% muramic acid as lactam	% muramic acid with alanine	% muramic acid with tripeptide	% muramic acid with tetra-peptide	% muramic acid with cross-linked peptide
T5.5	4.4	6.3	25.4	63.9	8.6
T5.75	4.7	7.1	22.3	65.8	7.7
T6	9.9	7.0	15.7	67.4	10.3
T6.5	13.0	9.2	12.9	64.9	9.0
T7	12.8	6.9	11.5	68.7	11.6
T7.5	17.9	7.5	7.5	67.0	7.3
T8	17.5	7.4	7.2	67.9	7.3

Only one sampling was performed on this strain.

Table 15. Summary of structural parameters of PG produced by Class A High Molecular Weight PBP's.

Strain	Promoters and Genes at *amyE*	% muramic acid as lactam	% muramic acid with alanine	% muramic acid with tripeptide	% muramic acid with tetra-peptide	% muramic acid with cross-linked peptide
PS1869**	*pbpF*$^-$	46.5	22.8	2.3	28.4	3.4
VB45**	*pbpG*$^-$	46.1	23.7	4.2	26.0	3.6
VB56**	*pbpF*$^-$*pbpG*$^-$	19.3	6.0	5.8	68.9	6.0
VB208	*dacBp-pbpF*	16 ± 0.3	7.4 ± 3.6	13.1 ± 1.5	63.6 ± 4.8	7.6 ± 0.1
VB209*	*dacBp-pbpG*	13.9	5.6	9.3	71.3	4.3
VB211	*dacFp-pbpF*	46.7 ± 1.0	23.9 ± 3.4	3.5 ± 0.7	25.9 ± 3.7	3.6 ± 1.5
VB212	*dacFp-pbpG*	43.7 ± 1.9	23.6 ± 1.5	3.4	29.5 ± 0.4	3.7 ± 0.2
VB225*	*dacFp-pbpD*	17.5	7.4	7.2	67.9	7.3
VB226*	*dacBp-pbpD*	15.7	9.0	9.3	66.0	10.5

Samples from t_8

*Only one sampling was performed

**Data for PS1869, VB45 and VB56 is from reference 10.

Values are averages of two independent analyses with errors of one standard deviation. In cases where no error is indicated the values in the multiple analyses were identical.

CHAPTER FOUR

Results from analyses of low molecular weight PBP's

The goal of the low molecular weight PBP research was to determine the functional and compartmental specificity of PBP5* and DacF. A set of strains were constructed that had compartmentalized expression of the low molecular weight PBP encoding genes, *dacB* and *dacF*. Does the loss of DacF from the inner forespore membrane cause the germ cell wall to be made incorrectly and in turn cause abnormal cortex synthesis? Perhaps, DacF does not remain associated with the inner forespore membrane and affects the cortex by entering the intermembrane space. Is DacB functionally equivalent to DacF?

All the constructed plasmids containing *dacB* and *dacF* were inserted into *dacF dacB* double mutants. Each gene, *dacB* and *dacF,* was placed in a vector that would allow expression specifically in the mother cell or the forespore. This was accomplished through the use of a mother cell-specific promoter, *dacBp,* and a forespore specific promoter, *dacFp*. Each gene contained its native ribosome-binding site for expression of the gene in the designated compartment. These new vectors also contained *amyE* sequences that allowed for recombination of a single copy of the gene into the nonessential *amyE* locus located in the *B. subtilis* chromosome.

Timing of biochemical and phenotypic sporulation markers

The methods used for DPA, GDH and heat resistance analysis were applied to the strains expressing low molecular weight PBP's. Experiments were performed in duplicate for the *dacB* strains. One data set from each strain is presented but the other results looked similar. In both DPVB161 (*dacFp-dacB*) and DPVB163 (*dacBp-dacB*) *dacB* strains, GDH activity increased until the 8th hour of sporulation and dropped near zero at t_{24} (Fig. 14). DPA accumulation increased at the 6th hour of sporulation and continued throughout the remainder of sporulation (Fig. 15). Heat resistant spores were produced by both *dacB* strains (Fig. 18).

DPA accumulation and GDH activity results from the *dacF* strains did not suggest a failure of the *dacFp-dacF* complementation. GDH activity of strains DPVB201 and DPVB204 was similar to the activity observed in both of the *dacB* strains (Fig. 16). GDH activity increased until 7.5 hours into sporulation and then dropped near zero at t_{24}. DPA accumulation in the *dacF* strains increased at the sixth hour of sporulation and continued to increase, reaching its maximum at t_{24} (Fig. 17). The DPA accumulation at t_{24} for the *dacF* strains was less than the DPA accumulation in the *dacB* strains at t_{24}. When the heat resistant spores were evaluated, there was a notable difference between the *dacF* and *dacB* strains (Fig. 18). Both *dacF* strains produced less heat resistant spores than the *dacB* strains.

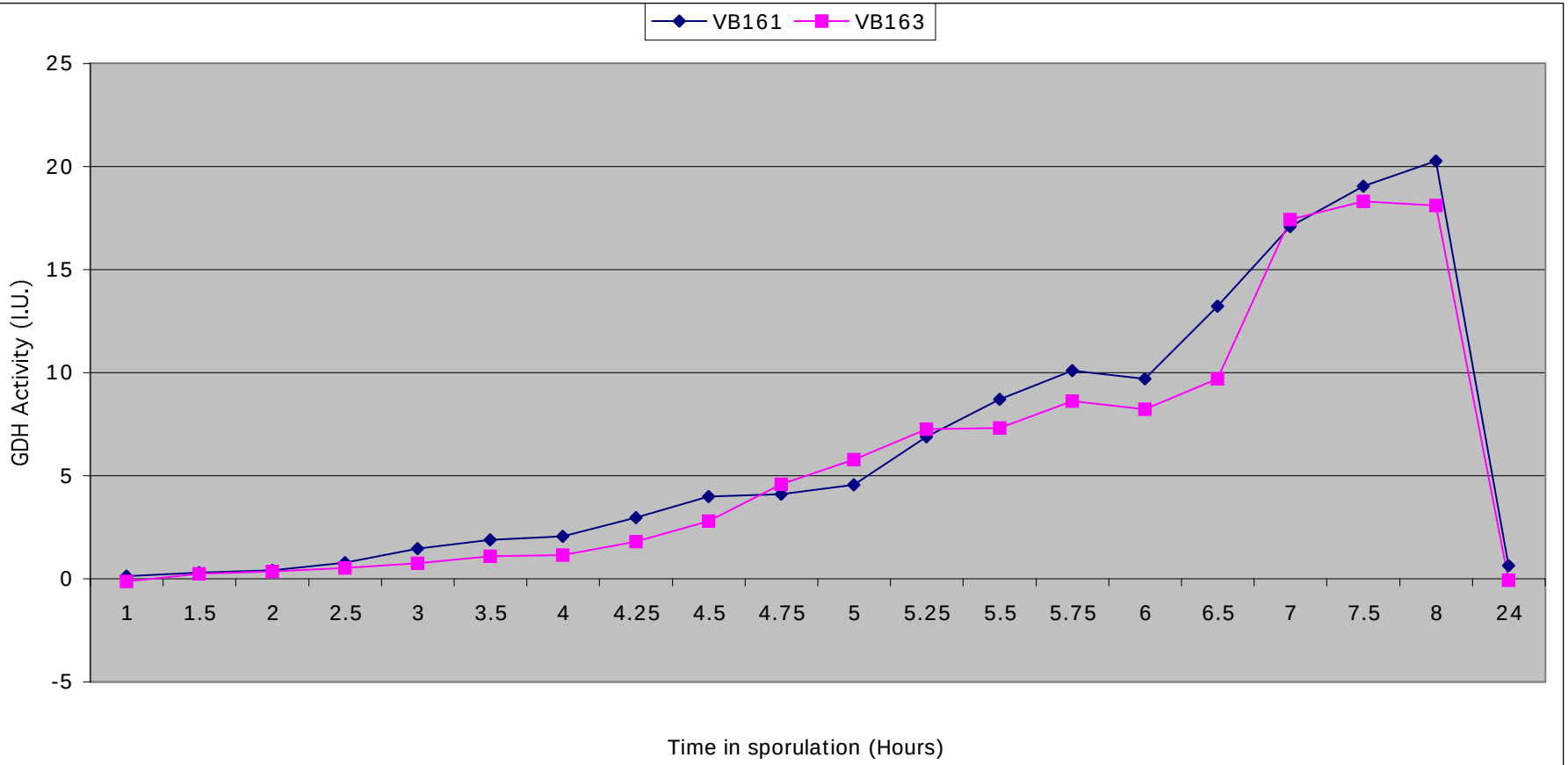

Fig. 14 - GDH activity produced in low molecular weight PBP strain expressing PBP5*. GDH actvity increased until the 8th hour of sporulation and dropped near zero at t24.

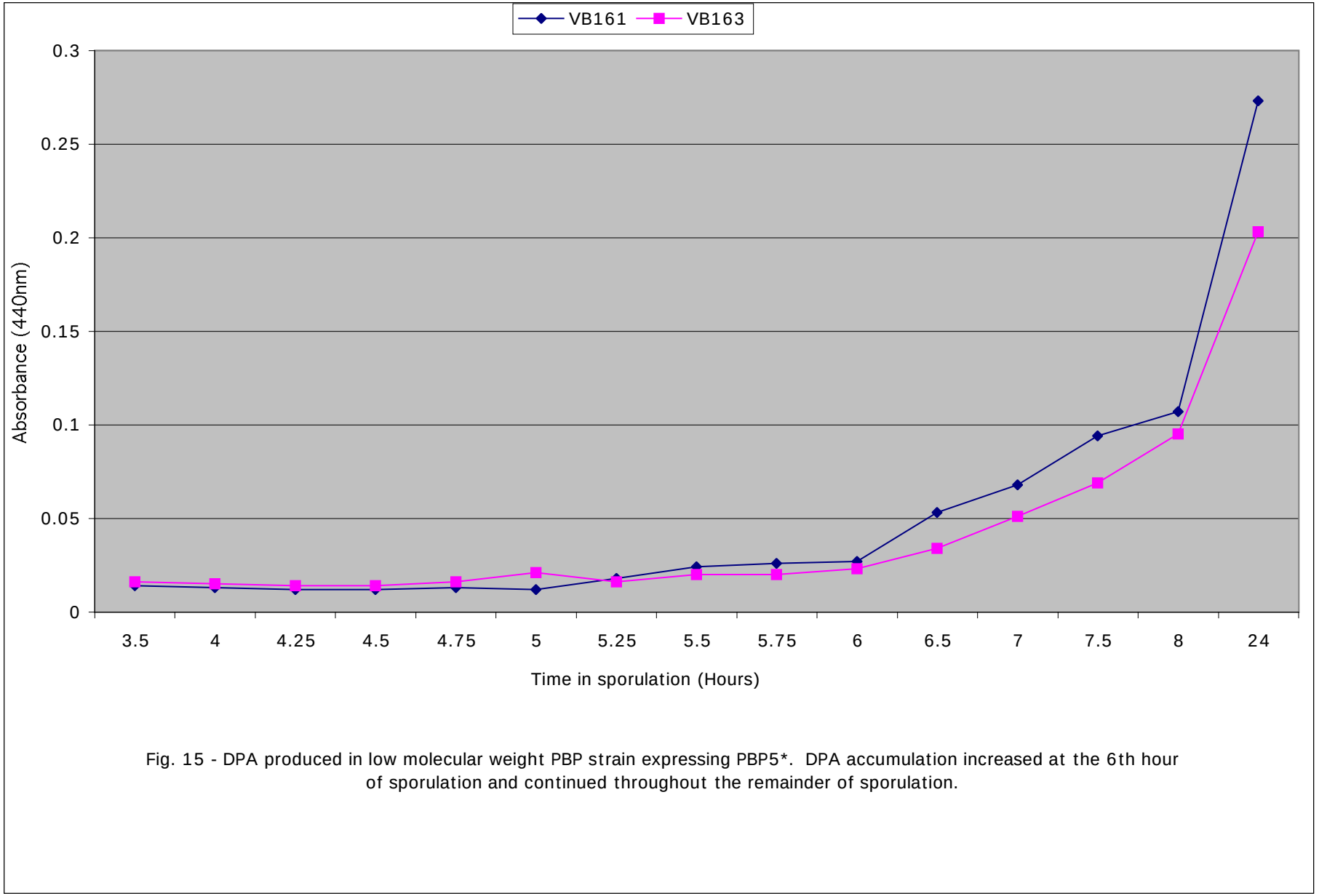

Fig. 15 - DPA produced in low molecular weight PBP strain expressing PBP5*. DPA accumulation increased at the 6th hour of sporulation and continued throughout the remainder of sporulation.

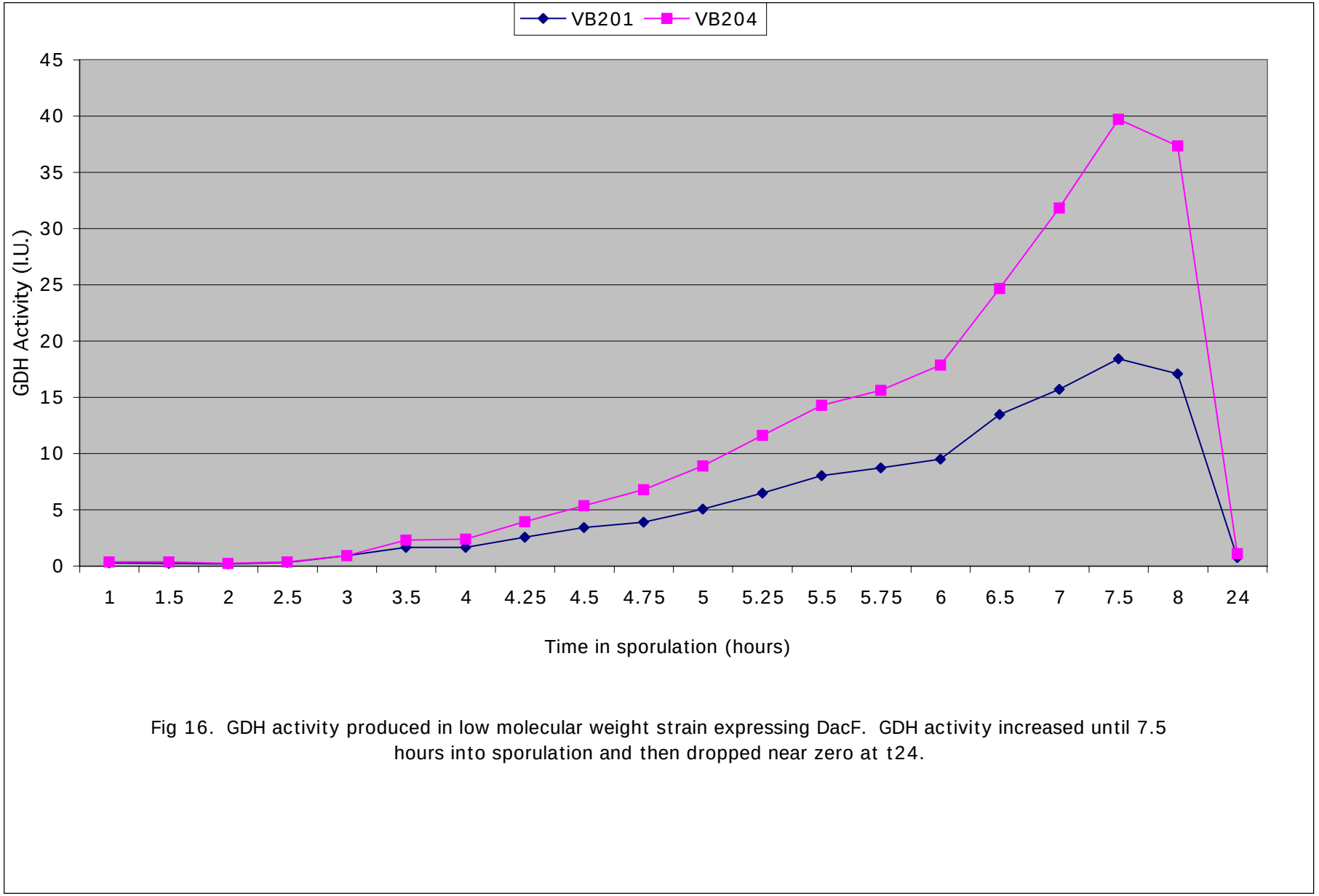

Fig 16. GDH activity produced in low molecular weight strain expressing DacF. GDH activity increased until 7.5 hours into sporulation and then dropped near zero at t24.

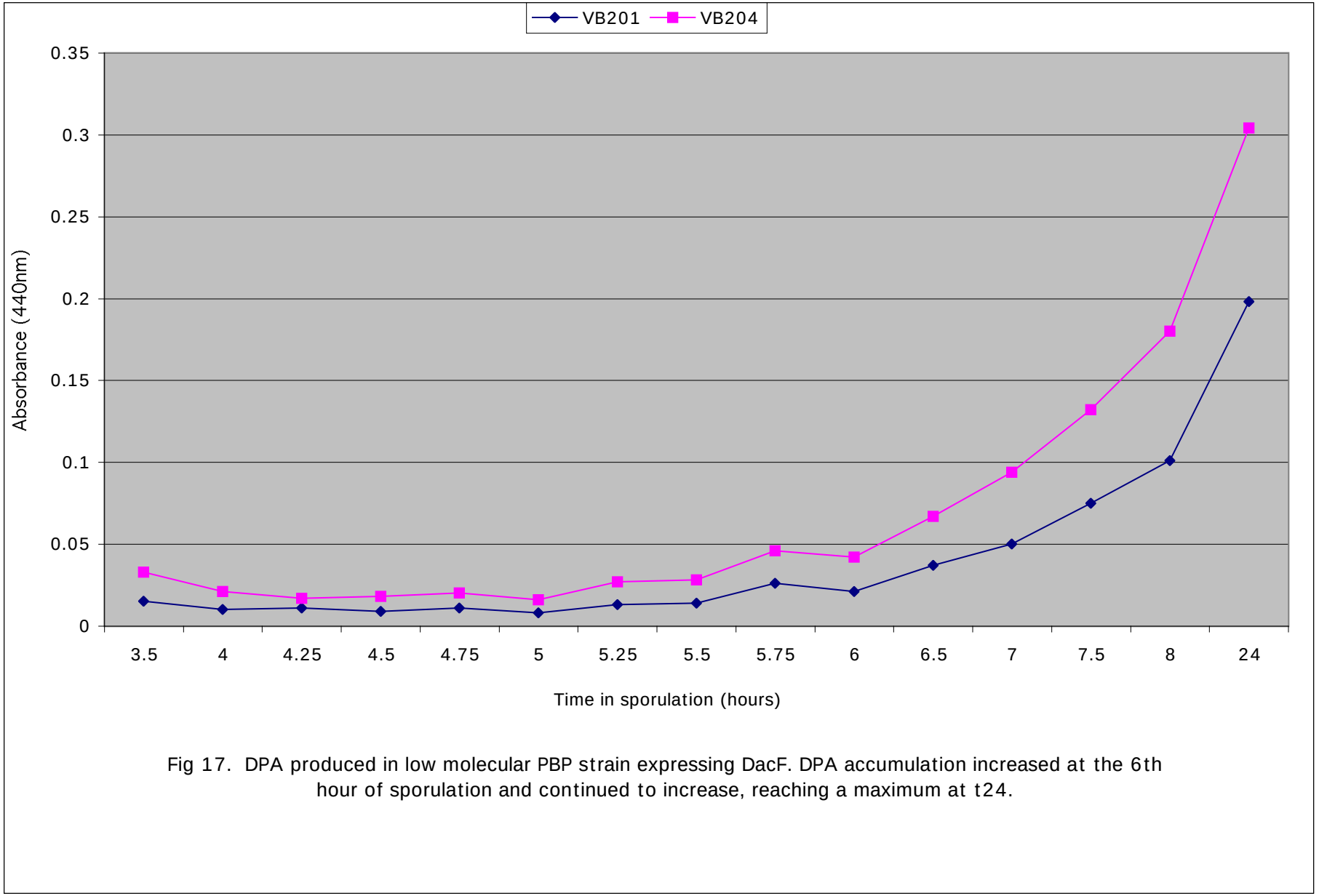

Fig 17. DPA produced in low molecular PBP strain expressing DacF. DPA accumulation increased at the 6th hour of sporulation and continued to increase, reaching a maximum at t24.

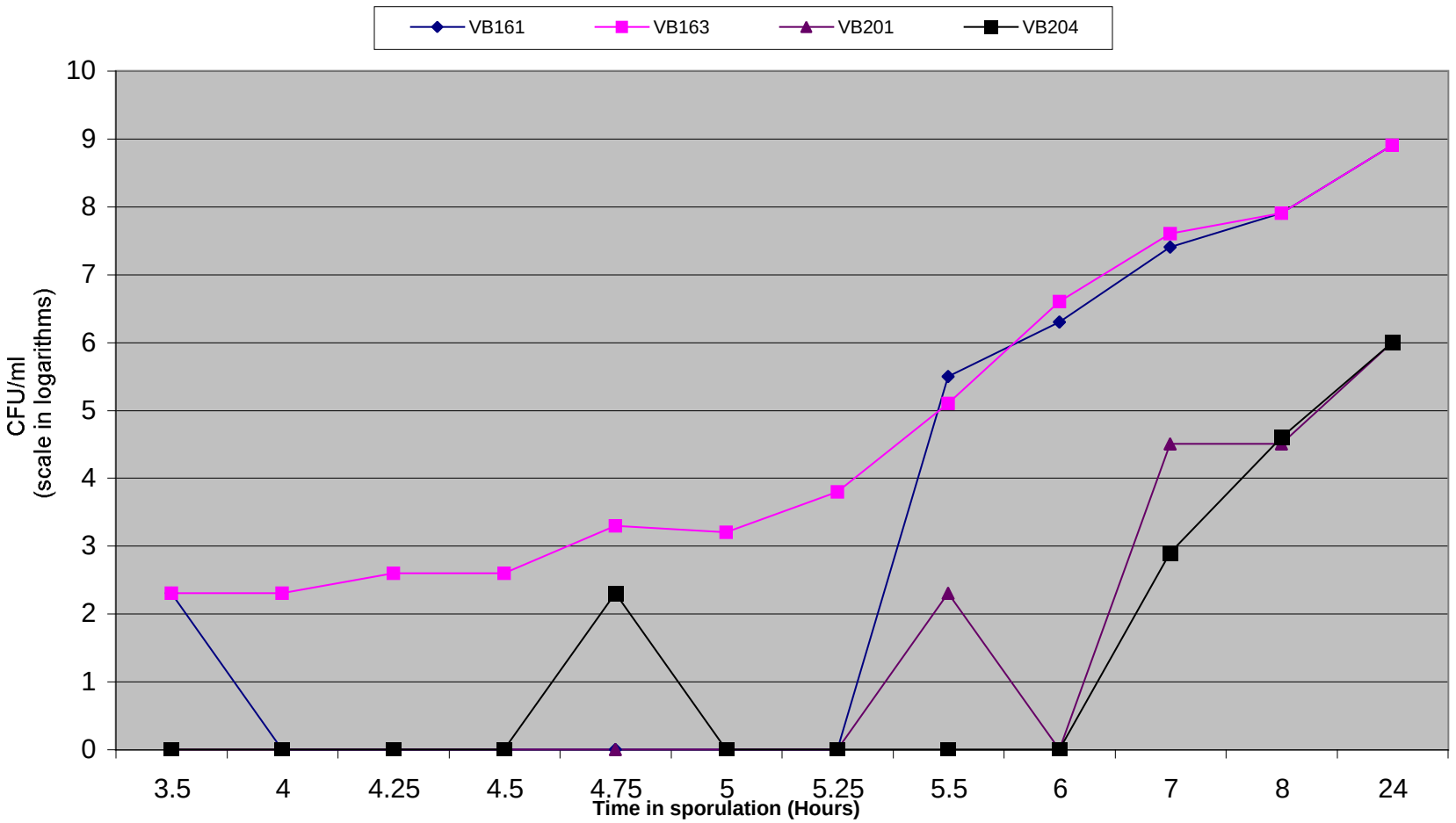

Fig. 18 Heat resistant spores formed in strains expressing PBP5* and DacF. Both *dacF* strains (DPVB201 and DPVB204) produced less heat resistant spores than the *dacB* strains (DPVB163 and DPVB161).

Structure of developing forespore PG

The strains constructed with *dacB* and *dacF* were sampled to determine the spore peptidoglycan structure and phenotypic properties. The experiments conducted using *dacB* and *dacF* were done in a *dacB dacF* double mutant. A *dacF* single mutant has spore peptidoglycan cross-linking identical to wild type peptidoglycan cross-linking, whereas a *dacB* single mutant has increased spore peptidoglycan cross-linking (15). However, the double mutant shows a greater amount of cross-linking in the cortex than the single *dacB* mutant (15). The *dacBp-dacB* (DPVB163) control strain had spore peptidoglycan that resembled that of a *dacF* single mutant (Table 16), which produces wild type germ cell wall and cortex peptidoglycan, as expected. The *dacFp-dacB* strain (DPVB161) displayed a phenotype similar to wild type peptidoglycan also (Table 17). Expressing *dacB* in either the mother cell or the forespore reduced the very high amount of cross-linking from what is seen in a double mutant back to what is typical of wild type peptidoglycan.

After reviewing results from the samplings of DPVB162 and DPVB164, the control *dacFp-dacF* strain (DPVB162) did not change the phenotype of the double mutant. A phenotype that resembled a *dacB* single mutant was expected. Results indicated a high degree of cross-linking similar to the double mutant (Fig. 19).

A possible reason for the apparent failure of the *dacFp-dacF* construct to complement DacF function in the *dacB dacF* double mutant is an effect on *spoIIA* expression. Transcription through *dacF* leads to an increased expression of *spoIIA* (20), therefore, it is possible that a polar *dacF* mutation could affect sporulation through an effect on *spoIIA* expression (Fig. 20). A strain with an in-frame deletion of

Table 16. Structural parameters of forespore PG produced by VB163 *(dacBp-dacB)*.

Sample	% muramic acid as lactam	% muramic acid with alanine	% muramic acid with tripeptide	% muramic acid with tetra-peptide	% muramic acid with cross-linked peptide
T5	27.8*	28.1*	32.2*	11.9*	4.6*
T5.25	33.9*	28.9*	16.4*	20.9*	4.6*
T5.5	40.3*	33.1*	11.1*	15.5*	2.5*
T5.75	42.4*	29.1*	7.2*	21.4*	3.5*
T6	42.9*	31.0*	7.8*	18.3*	2.2*
T6.5	45.3*	33.1*	5.3*	16.3*	1.6*
T7	46.2*	29.9*	4.3*	19.7*	2.2*
T7.5	45.2 ± 3.3	29.6 ± 0.4	4.1 ± 1.2	21.2 ± 2.3	3.7 ± 2.3
T8	46 ± 2.9	27.9 ± 2.2	3.4 ± 0.8	22.8 ± 4.2	3.7 ± 2.5
T24	48 ± 1.6	23.5 ± 0.1	2.3 ± 0.4	26.3 ± 1.3	3.9 ± 1.8

*Data only available for one sampling

Values are averages of two independent analyses with errors of one standard deviation.

Table 17. Structural parameters of forespore PG produced by VB161 *(dacFp-dacB)*.

Sample	% muramic acid as lactam	% muramic acid with alanine	% muramic acid with tripeptide	% muramic acid with tetra-peptide	% muramic acid with cross-linked peptide
T5	29.1*	29.1*	30.8*	10.9*	4.8*
T5.25	40.8*	34.3*	12.0*	12.8*	2.2*
T5.5	41.0*	34.0*	10.1*	15.0*	2.6*
T5.75	43.4 ± 0.1	36.1 ± 2.6	8.8 ± 0.8	11.8 ± 3.5	2.3 ± 0.1
T6	44.5 ± 0.2	36.4 ± 3.3	7.4 ± 0.5	11.8 ± 4	2.3 ± 0.5
T6.5	45.5 ± 1.4	32.9 ± 4	5.5 ± 0.2	16.2 ± 5	2.8 ± 2
T7	46.7 ± 0.9	29.9 ± 3.3	4.5 ± 0.4	19.1 ± 3.7	3.2 ± 2
T7.5	47.5 ± 1.1	28.8 ± 3.6	3.8 ± 0.8	20.1 ± 3.8	3.1 ± 2
T8	47.4 ± 2.1	33.1 ± 4.1	4.4 ± 2.3	15.3 ± 4.4	2.3 ± 0.5
T24	48.7 ± 0.9	19.2 ± 5	4.4 ± 2.3	29.5 ± 4.9	3.9 ± 2.1

*Data only available for one sampling

Values are averages of two independent analyses with errors of one standard deviation.

Table 18. Summary of structural parameters of PG produced by Low Molecular Weight PBP's.

Strain	Promoters and Genes at amyE	% muramic acid as lactam	% muramic acid with alanine	% muramic acid with tripeptide	% muramic acid with tetra-peptide	% muramic acid with cross-linked peptide
PS832**	Wild type	50	26	1.5	23	
PS2066**	*dacB*$^-$	47	12	2.2	43	
PS1901**	*dacF*$^-$	51	28	1.4	20	
PS2421**	*dacB*$^-$*dacF*$^-$	40	6	4.8	57	
VB161	*dacFp-dacB*	47.4 ± 2.1	33.1 ± 4.1	4.4 ± 2.3	15.3 ± 4.4	2.3 ± 0.5
VB163	*dacBp-dacB*	46 ± 2.9	27.9 ± 2.2	3.4 ± 0.8	22.8 ± 4.2	3.7 ± 2.5

Samples from t_8

**Data for PS832, PS2066, PS1901 and PS2421 from reference 15.

Values are averages of two independent analyses with errors of one standard deviation. In cases where no error is indicated the values in the multiple analyses were identical.

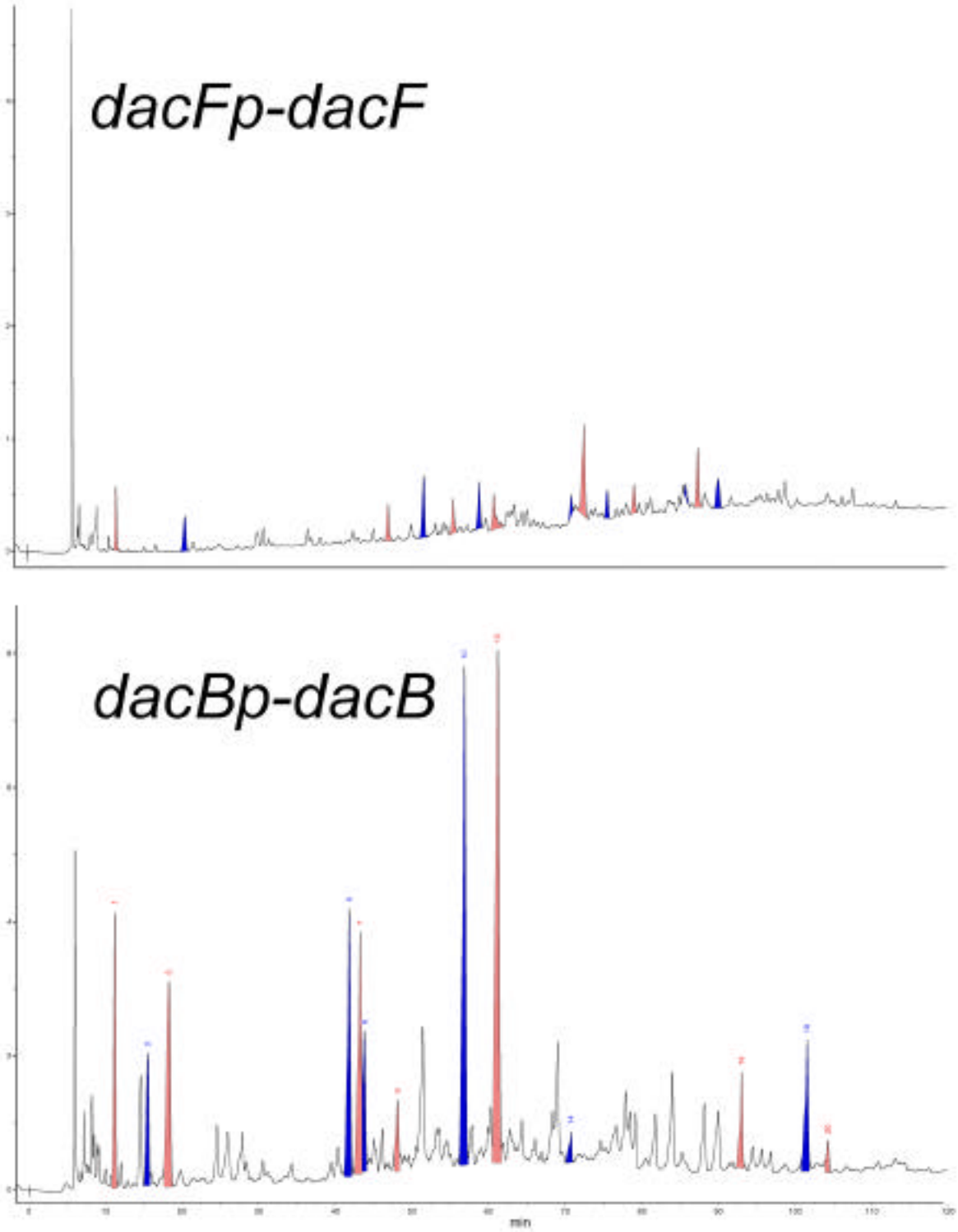

Fig. 19. HPLC traces of low molecular weight PBP control strains. The top panel shows the HPLC data from DPVB201 (dacFp-dacF). These results resemble dacB dacF double mutant peptidoglycan instead of a dacB single mutant which was expected. The lower panel shows the HPLC data from DPVB163 (dacBp-dacB). These results resemble wild type peptidoglycan which was expected.

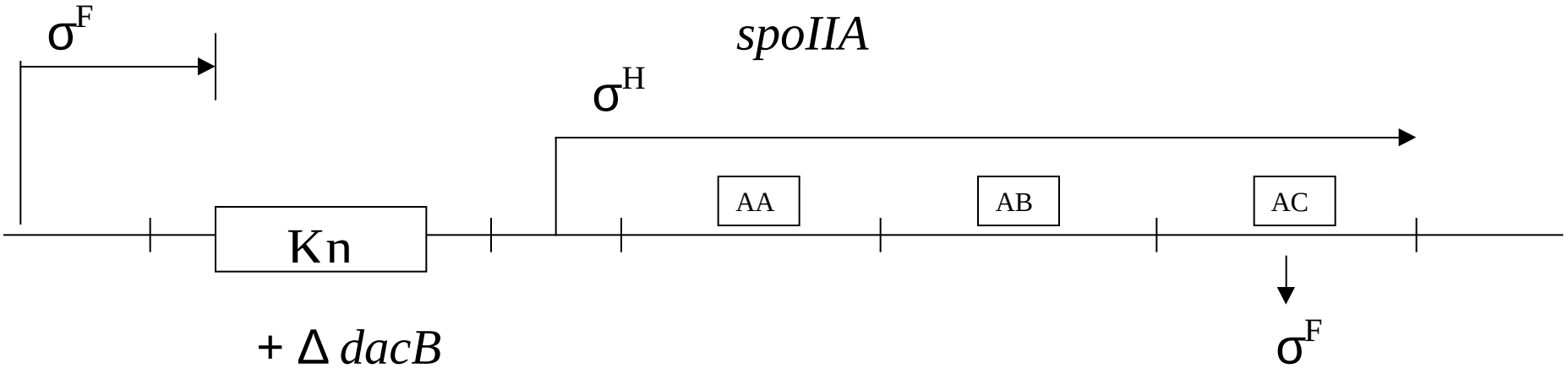

Figure 20: Strain DPVB98 *(ΔdacB, dacF*::Kn) displays a very high amount of cross-linking in the cortex. This dramatic cortex defect could be either due to the loss of *dacF* or a decrease in *spoIIA* expression.

dacF was previously constructed, and it was expected to have no effect on *spoIIA* expression (Fig. 21). When this Δ*dacF* was put into a Δ*dacB* strain, it produced the same phenotype as that seen in the Δ*dacB, dacF* :: Kn strain. This led researchers to believe that the effect of the *dacF* :: Kn mutation was due to the loss of DacF, rather than an effect on *spoIIA* expression (Meador-Parton & Popham, Unpublished).

When the *dacFp-dacF* construct was placed in the *amyE* locus in the Δ*dacB dacF*:: Kn strain, a sporulation defect was seen. This raised the possibility that both *dacF* and increased late expression of *spoIIA* are required for normal sporulation. The construct *dacFp-dacF* was transformed into the Δ*dacB* Δ*dacF* strain (DPVB219) and tested to determine if the construct was able to complement the phenotype produced by the Δ*dacF* in a Δ*dacB* background (Fig. 21). After reviewing the results from the sampling of DPVB201 (*dacFp-dacF*) and DPVB204 (*dacBp-dacF*), the control strain (DPVB201) did not change the phenotype of the double mutant. With the failure of the *dacF* control, additional experiments were needed to determine if sigma F expression was altered in the *dacF*::Kn and Δ*dacF* strains.

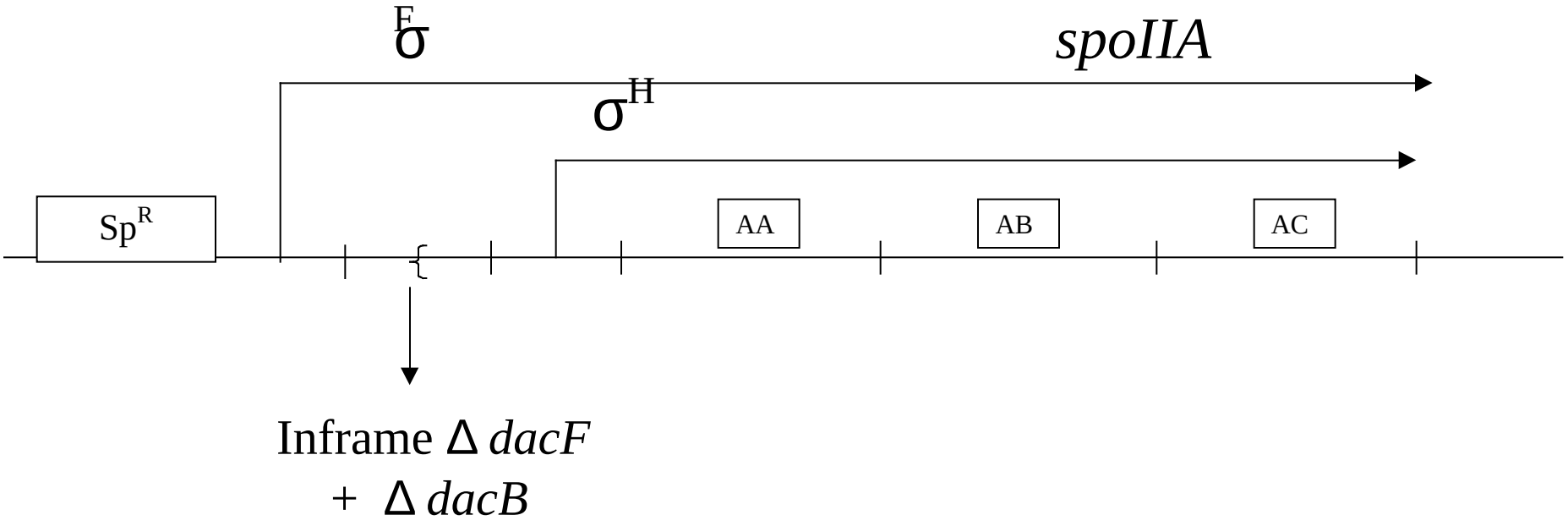

Figure 21: Strain DPVB219 (*dacB⁻dacF⁻)* also displays a very high amount of cross-linking in the cortex. It was assumed that it was due to the loss of *dacF* and not a decrease in *spoIIA* expression.

Results of β-galactosidase assays for sigma F dependent transcriptional fusions

Assays of β-galactosidase expression from these fusions allowed the determination of whether there was decreased sigma F activity in the *dacB dacF* double mutants (DPVB98 and DPVB219). Expression of both *pbpG*- and *pbpI-lacZ* fusions is sigma F dependent.

The results for the *pbpI-lacZ* fusion strains demonstrated that there was approximately a 20% decrease in expression of the fusion in the $dacB^{-}dacF^{-}$ strains, compared to expression in the wild type (Fig. 22). This suggests that the promoter just upstream of *dacF* contributes about 20% and both of the $dacF^{-}$ mutations are polar and block this increased *spoIIA* expression. The polarity is most likely due to the kanamycin resistance cassette in DPVB98. It is unclear why the in frame deletion in DPVB219 is polar.

The results of the *pbpG-lacZ* strains did not show the decrease in expression caused by the *dacF* mutations, as well as the *pbpI-lacZ* strains (Fig. 23). There was a slight decrease in the double mutant strains until the last three data points. A slight decrease in *pbpG* expression resulting from decreased sigma F activity may be masked by sigma G-dependent expression of *pbpG* (14).

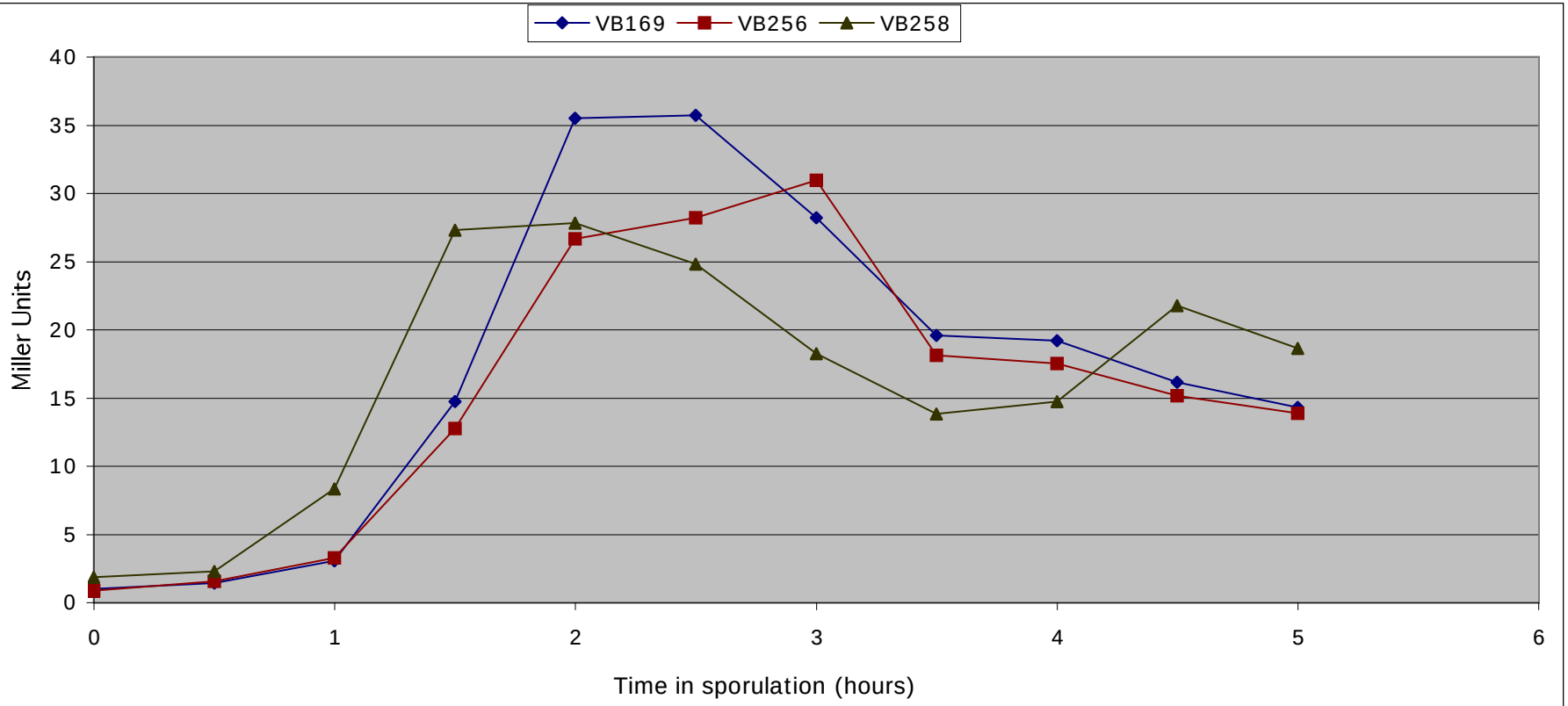

Fig. 22. β-galactosidase Assay of pbpI -lacZ . There was approximately a 20% decrease in expression of the fusion in the double mutant strains, compared to expression in the wild type.

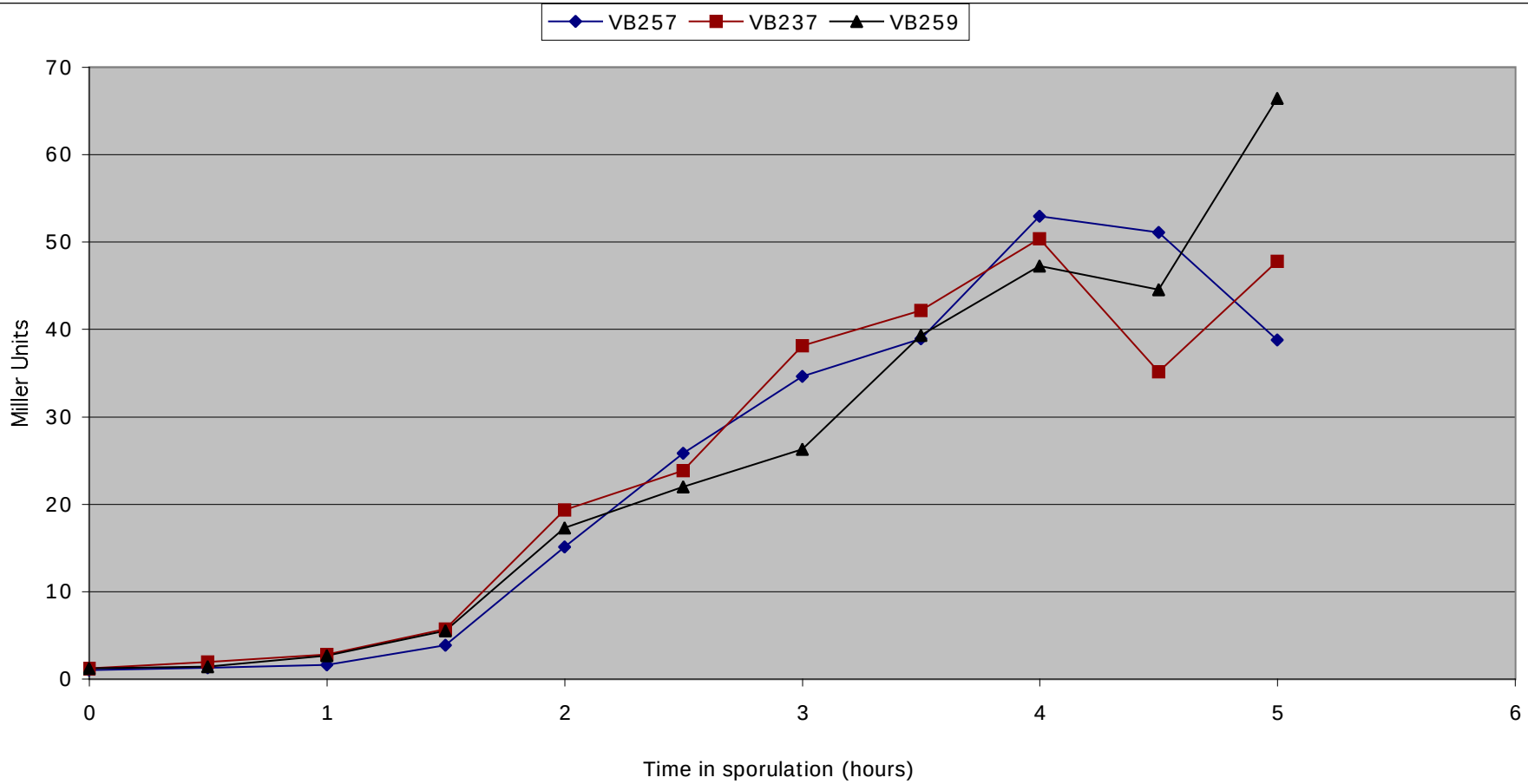

Fig. 23. β-galactosidase Assay for *pbpG-lacZ*. There is a slight decrease in expression in the double mutant strains until the last three data points.

Results of β-galactosidase assays for translational fusion of *spoIIAA*

Assays of β-galactosidase expression from this fusion allowed the determination of the expression of the *spoIIA* operon. The results for the *spoIIAA* fusion strain demonstrated interesting results. When the *spoIIAA* fusion was placed in PS832 (wild type strain) and DPVB98 *(*Δ*dacB, dacF*::Kn strain), an enormous amount of activity was observed (Fig. 24). However, when the *spoIIAA* fusion was placed into DPVB99 (Δ*dacB* Δ*dacF* strain), the activity was significantly lower. It was possible to have observed in the *dacB dacF* double mutant strains with the *spoIIAA* fusion less activity than the *spoIIAA* fusion in PS832, if it was true that both the kanamycin resistance cassette and the in-frame deletion of *dacF* were both polar mutations. It is unclear why DPVB265 (DPVB219 + *spoIIAA*) had a much lower amount of activity compared to DPVB263 and DPVB264.

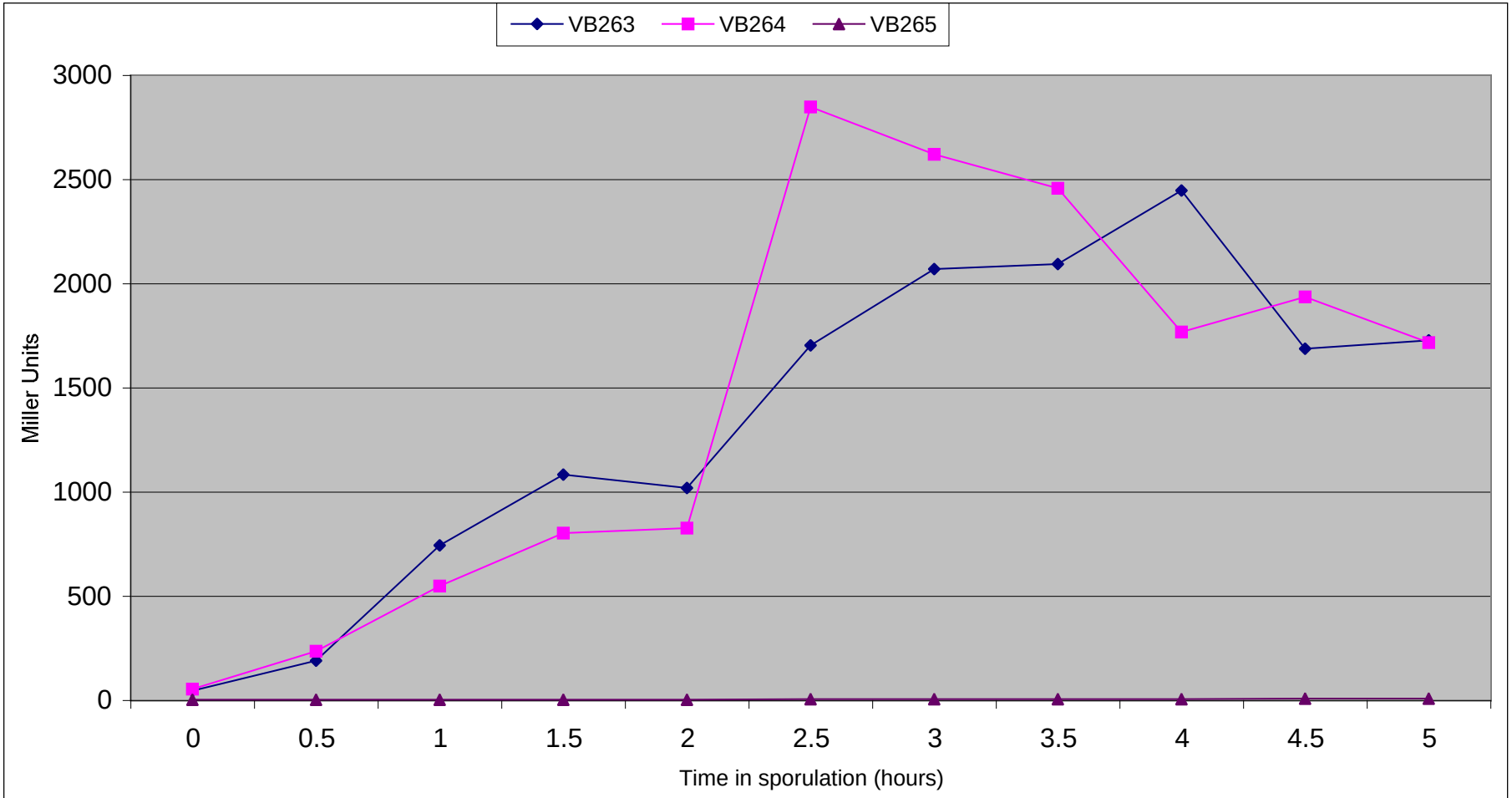

Fig. 24. β-galactosidase Assay for translational fusion of *spoIIAA* . A lower amount of activity was observed in DPVB265 compared to the other two strains.

CHAPTER FIVE

DISCUSSION

Discussion of Class A high molecular weight PBP's

Analysis of the phenotypic properties and the peptidoglycan structures from strains with compartmentalized expression of specific Class A high molecular weight PBP's allowed for the determination of the functional and compartmental specificity of the individual PBP's. Through temporally correlated biochemical tests for DPA accumulation and GDH activity, it was confirmed that all the strains containing compartmentalized expression of Class A PBP's initiated sporulation with efficiencies similar to the wild type strain. DPA never accumulated to similar levels in DPVB208 and DPVB209 because both DPVB208 and DPVB209 have unstable forespores. DPA is pumped into the forespore during sporulation and the DPA is presumably lost from the unstable forespores when preparing the samples for the assay.

The data derived from HPLC analysis of spore peptidoglycan indicate that *pbpF* and *pbpG* must be expressed in the forespore to carry out their specific role during spore peptidoglycan synthesis. According to previous research, PBP2c and PBP2d were believed to be functionally redundant in germ cell wall synthesis (10). Single mutants of *pbpF* and *pbpG* both produce normal heat resistant spores and spore cortex structure (10). However, a *pbpF pbpG* double mutant makes a normal amount of spore peptidoglycan but the cortex appears to be made in large masses at the poles of the forespore, instead of evenly placed around the forespore (10). Only when both proteins are absent, is the germ cell wall made incorrectly (10). All of the strains examined in this study contained *pbpF*,

pbpG and *pbpD*-expressing plasmids recombined into the nonessential *amyE* locus in a *pbpF pbpG* double mutant, which has an altered cortex structure. The *dacFp-pbpF* (DPVB211) and *dacFp-pbpG* (DPVB212) control strains had phenotypes that resembled *pbpF* and *pbpG* single mutants, as expected. Expressing *pbpF* and *pbpG* from the *dacF* promoter in their correct compartment, the forespore, allowed for the complementation of *pbpF* and *pbpG* in the double mutant. However, when *pbpF* and *pbpG* were expressed from the *dacB* promoter, in the mother cell compartment, a phenotype similar to a *pbpF pbpG* double mutant was observed, suggesting that *pbpF* and *pbpG* must be expressed in the forespore in order to carry out their specific role during spore peptidoglycan synthesis.

The structural analysis of the forespore of the *pbpF* and *pbpG*-expressing strains supported the hypothesis that PBP2c and PBP2d do play fully redundant roles in germ cell wall synthesis and PBP2c is not required to be in the outer forespore membrane for cortex synthesis. It has been theorized that an alteration in the germ cell wall peptidoglycan structure could create an altered template for synthesis of the cortex peptidoglycan by proteins on the outer forespore membrane (10). These data with the *pbpF* and *pbpG* strains supports this theory because with *pbpF* and *pbpG* absent from the inner forespore membrane, an alteration in the cortex structure is observed.

The data derived from HPLC analysis of forespore peptidoglycan structure indicate that expressing *pbpD*, which plays a minor role in vegetative peptidoglycan synthesis (18,19), in either the mother cell or the forespore compartment does not change the phenotype of the *pbpF pbpG* double mutant. This indicates that *pbpD* does not complement the loss of *pbpF* and *pbpG* for synthesis of normal spore germ cell wall.

PBP4 has the two known enzymatic domains, glycosyl transferase and transpeptidase (18, 19), which are also found in PBP2c and PBP2d. These two enzymatic activities must not be enough to carry out a specific function needed for germ cell wall synthesis. There must be additional sequence information contained in PBP2c and PBP2d that allows these proteins to carry out their specific role during germ cell wall synthesis. It is possible that PBP2c and PBP2d have a specific interaction with other proteins in peptidoglycan synthetic machinery. This additional sequence information could be in larger N and C-terminal extensions or could be within *pbpF* and *pbpG*'s enzymatic domains.

Discussion of low molecular weight PBP's

Analysis of the phenotypic properties and the peptidoglycan structures from strains with compartmentalized expression of specific low molecular weight PBP's allowed for the determination of the functional and compartmental specificity of the individual PBP's. Temporally correlated biochemical tests for DPA accumulation and GDH activity confirmed that all the *dacB* and *dacF* strains initiated sporulation with efficiencies similar to the wild type strain.

The data derived from HPLC analysis of spore peptidoglycan synthesis indicate that when *dacB* is expressed in either the forespore or the mother cell compartment, it is able to correct the cortex synthesis defect that appears in the *dacF dacB* double mutant. All the constructed strains containing *dacB* and *dacF* were recombined into the nonessential *amyE* locus in a *dacB dacF* double mutant, which has a very high amount of cross-linking in the cortex. A *dacF* single mutant, which should be the situation when *dacB* is expressed in the mother cell, has spore peptidoglycan cross-linking identical to wild type peptidoglycan cross-linking. Strain DPVB163, the *dacBp-dacB* control strain did have a phenotype that resembled a *dacF* single mutant.

Surprisingly, strain DPVB161 (*dacFp-dacB*) also displayed a phenotype of a *dacF* single mutant. These data demonstrate that when *dacB* is expressed in the forespore, the very high amount of cross-linking is reduced from what is seen in a double mutant back to what is typical of wild type peptidoglycan. The results from DPVB161 are surprising because it would be expected that PBP5* expressed in the forespore would affect the germ cell wall, but not much of the cortex. There are two possibilities to explain these results. The first explanation could be that PBP5* can "reach out" from the

inner forespore membrane to affect more of the peptidoglycan layers than DacF. The second possibility is that PBP5* action on the germ cell wall has a greater effect on the cortex than DacF action has on the germ cell wall.

The first set of *dacF* strains (DPVB162 and DPVB164) were sampled and there appeared to be no change in phenotype of the double mutant. The control strain expresses *dacF* in the forespore and a phenotype of a single *dacB* mutant is expected. A *dacB* single mutant has increased spore peptidoglycan cross-linking. When *dacF* was expressed in the forespore, a phenotype that resembled a *dacB* single mutant was expected, but instead a very high degree of cross-linking, similar to that of the *dacB dacF* double mutant, was observed. It was theorized that these results were due to an effect on *spoIIA* expression. Strains DPVB162 and DPVB164 were constructed in a *dacB dacF* double mutant background that had a kanamycin resistant cassette placed in the *dacF* gene. It is possible that this polar *dacF* mutation was affecting sporulation through an effect on *spoIIA* expression. However, when a strain with an in-frame deletion of *dacF* was previously constructed and placed into a Δ*dacB* strain, it produced the same phenotype as the Δ*dacB, dacF*::Kn strain, suggesting that the effect of the *dacF*::Kn mutation was due to the loss of DacF, rather than an effect on *spoIIA*.

Even though these data suggested it was the loss of DacF that was responsible for the increased cross-linking phenotype, the data from the first sampling indicated otherwise. Expression of *dacF* in the forespore was unable to complement the *dacF*::Kn mutation. The results raised the possibility that both *dacF* and increased late expression of *spoIIA* are required for normal sporulation.

The construct *dacFp-dacF* was transformed into the Δ*dacB*Δ*dacF* strain (DPVB219) and sampled to determine if the construct was able to complement the phenotype produced by the Δ*dacF* in a Δ*dacB* background. The results from the samplings of strains DPVB201 (*dacFp-dacF*) and DPVB204 (*dacBp-dacF*) showed again that the control strain (DPVB201) had the same phenotype as the double mutant. There are three possibilities to explain why using the *dacFp-dacF* construct was unable to complement the *dacB dacF* double mutant. The first explanation could be that the *dacFp* was not functioning correctly, however, this does not seem to be the case since the *dacF* promoter apparently worked in the strains expressing *pbpF* and *pbpG*. The second alternative is that *dacF* is not functioning correctly, but this too cannot be explained easily since the sequence of the cloned gene was identical to that in the published genome sequence. The final possibility is that there is not really an effect of *dacF* on spore peptidoglycan synthesis, rather an effect of increased *spoIIA* expression. A *pbpI-lacZ* was used to assay the sigma F activity expressed from the *spoIIA* operon. A slight decrease in sigma F activity in the *dacF*::Kn strain was observed. It is hard to attribute such as large effect on spore peptidoglycan to such a small change, but it is possible. The in-frame Δ*dacF* displayed the same affect on *pbpI-lacZ* expression. This may indicate that Δ*dacF* may not be non-polar. In future experiments, a better non-polar Δ*dacF* will be needed to test and a method is needed to show the production of active DacF.

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