Biological Containment of Recombinant Spores and Dissemination of Pathogenic Spores

A thesis submitted for the degree of Doctor of Philosophy

By

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FOR MY DAD

Abstract

Genetic manipulation of bacterial spores of the genus *Bacillus* has shown potential for vaccination and for delivery of drugs or enzymes. Remarkably, proteins displayed on the spore surface retain activity and generally are not degraded. The heat stability of spores coupled with their desiccation resistance makes them suitable for delivery to humans or to animals by the oral route. Despite these attributes one regulatory obstacle has remained regarding the fate of recombinant spores shed into the environment as viable spores. We have addressed the biological containment of spore GMOs by utilizing the concept of a 'thymine-less death', a phenomenon first reported six decades ago. Using *Bacillus subtilis*, we have inserted chimeric genes in the two thymidylate synthase genes, *thyA*, and *thyB*, using a two-step process. Insertion is made first at *thyA* followed by *thyB* where resistance to trimethoprim enables selection of recombinants. Importantly, this method requires the introduction of no new antibiotic resistance genes. Recombinant spores have a strict dependence on thymine (or thymidine) and in their absence cells lyse and die. Insertions are stable with no evidence for suppression or reversion. Using this system, we have successfully created a number of spore vaccines as well as spores displaying active enzymes.

Despite numerous attempts to reduce the risk of transmission of *C. difficile* still this nosocomial infection presents a considerable problem. BclA1 is a glycosylated protein expressed on the exosporium layer of *C. difficile* spores. So far two types of BclA1 have been identified, a full-length and truncated BclA1 encoding for a 693 and 48 amino acids protein respectively. Previously shown that BclA1 play a role in colonisation as a mutant strain, CD630, that had a deleted BclA1 required 2-logs higher spores to colonise in mice in comparison to the isogenic wild-type strain. In this work, the study on BclA1 has expanded by first Identify different types of *bclA1* gene within different ribotypes and second test

different ribotypes with different *bclA1* for colonisation. The *in vivo* result shows that different non-isogenic strains of *C. difficile*, regardless the type of BclA1 they have, needed 100 spores to colonise in mice. Interestingly R176, a hypervirulent strain of *C. difficile* needed a higher number of spores to colonise. The hypervirulent strain also showed to produces more spores than other strains. The findings of this work are that, first, BclA1 may not play a role in colonisation within non-isogenic strains, and second, higher sporulation of hypervirulent *C. difficile* strains, possibly is a reason for faster dissemination and high incident despite reduced ability to colonise.

List of Publications

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Contents

Chapter 1: Introduction

1.1 Sporulation of *Bacillus subtilis* 1

1.2 *B. subtilis* spore structure 2

1.2.1 Spore coat 2

1.2.2 Spore coat proteins 4

1.3 Microbial cell surface display 5

1.4 Engineering the *B. subtilis* spore coat 6

1.5 Applications of *B. subtilis* wild-type and recombinant spores 8

1.5.1 Probiotics 8

1.5.2 Recombinant spore vaccine 10

1.5.3 Recombinant spores as a drug delivery vehicle 12

1.5.4 Other applications of recombinant *B. subtilis* spores 13

1.6 Regulatory issues regarding the use of recombinant spores 14

1.7 *C. difficile* infection 16

1.7.1 Antibiotics and CDI 16

1.7.2 *C. difficile* spore 19

1.7.3 Virulence factors and pathogenesis mechanisms 21

a) Toxins 21

b) Non-toxin virulence and pathogenesis factors 24

1.7.4 Hypervirulent strains 26

1.7.5 Relapse 27

1.8 Treatment of CDI 28

a) Antibiotics 28

b) Tolevamer 28

c) Faecal microbiota transplantation 29

d) Passive immunisation 30

e) Pre-colonisation with non-toxin-producing *C. difficile* strains 31

f) Vaccine 33

Chapter 2: Materials and methods

2.1 General methods 37

2.2 Bacterial strains 37

2.3 pThyA and pThyB construction 37

2.4 Recombinant *B. subtilis* strains 40

2.4.1 Construction of recombinant strains by the inactivation of *thy* genes 40

2.4.2 Constraction of chloramphenicol resistance PY79 42

2.5 Electroporation 43

2.6 Sporulation and spore purification 44

2.7 Whole-spore ELISA 44

2.8 Expression and purification of His-tagged recombinant proteins 44

a) Protein expression 45

b) Cell lysis and protein extraction 45

c) Purification of His-tagged proteins 45

2.9 Raising antibodies 46

2.10 Western blotting 47

2.11 Titration of thymine and thymidine 47

2.12 Well diffusion assay 48

2.13 Thymine starvation 48

2.14 Stability of double *thy*-insertion 48

2.15 Germination 49

2.16 Minimal inhibitory concentration 49

2.17 Growth curves 50

2.18 Animal experiments 50

2.18.1 Persistence of spores in GI-tract 50

2.18.2 Immunisation in mice 51

2.18.3 Colonisation experiments of different *C. difficile* clades (ID_{50} determination) 52

2.19 Conjugation of anti-TcdA antibody to streptavidin, and detecting the conjugated antibodies by ELISA and immunofluorescence 52

2.20 Toxins subtraction assay (Chapter 4) 53

a) Crude toxin preparation 53
b) Preparation of cells and the assay 54
c) The assay 54
2.21 Enzyme activity 54
2.22 Crude DNA extraction 55
2.23 PCR ribotyping 55
2.24 Amplification of *tcdA* and *tcdB* 56
2.25 Identification of different *bclA* genes 57
2.26 Sporulation of *C. difficile* 57
2.27 Toxin A and toxin B titration 58
2.28 Statistical analysis 59

Chapter 3: Development of a novel spore cloning system

3.1 Introduction 61
3.2 Aim 64
3.3 Results 66
3.3.1 Construction of *B. subtilis* recombinant strains 66
3.3.2 Growth of the *thyA thyB* insertional mutants in different media 69
a) Growth in minimal media 69
b) Growth in rich media 75
3.3.3 Sporulation of *thyA thyB* mutants 81
3.3.4 Different types of colony after the first genetic cross 81
3.3.5 Thymine and thymidine titration 83
3.3.6 Elimination of thymine and thymidine from the growth media 84
3.3.7 Reversion 84
3.3.8 *In vitro* germination 86
3.3.9 Persistence of *thyA thyB* mutant spores in GI-tract 87
3.4 Discussion 88
3.4.1 Rationale 88
3.4.2 Experimental considerations for the use of ectopic insertion at the *thy* loci 91

a) Growth in rich media 91
b) Gene transfer 92
c) Choice of one coat protein anchor 94
3.4.3 Stability of the *thyA thyB* insertion 95
3.4.4 *In vivo* fate of *thyA thyB* insertion mutant spores in the GI-tract 95
3.4.5 Comparison of *thy*-insertion cloning system with other existing techniques that do not require an antibiotic-resistance gene 96
3.5 Conclusion 98

Chapter 4: Application of clones constructed by the *thy*-insertion cloning system

4.1 Introduction 101
4.2 Aim 103
4.3 Results 104
4.3.1 Utility of spore display 104
4.3.2 Vaccine delivery vehicle 104
4.3.3 Conjugation of antibody to the spore surface 105
a) Conjugation of anti-$TcdA_{26-39}$ antibody to SH16 105
b) Subtraction of toxin A from *C. difficile* toxin sample 110
4.3.4 Display of active enzymes 111
a) Subtilisin E 112
b) Amylase E 115
4.4 Discussion 118
4.4.1 Spore display and its use 118
a) Antigen (recombinant spore vaccine) 118
b) Enzymes 119
c) Streptavidin 120
4.5 Conclusion 121

Chapter 5: Are the hypervirulent strains of *C. difficile* less infectious?

5.1 Introduction 123
5.1.1 Bacterial colonisation 123

5.1.2 *C. difficile* spore structure and proteins 124
5.1.3 Colonisation of *C. difficile* 128
5.2 Aim 129
5.3 Results 130
5.3.1 Genotypic characterisation of clinical isolates 130
a) Ribotyping 130
b) bclA typing 130
5.3.2 Identifying the clades of clinical isolates 131
5.3.3 Pattern of *bclA1* in different clades 138
5.3.4 The role of BclA1 in colonisation of *C. difficile* 141
5.3.5 *In vitro* sporulation and cell cytotoxicity of strains from different clades 144
5.4 Discussion 147
5.5 Conclusion 151

Chapter 6: General discussion

6.1 Concerns over GMOs 153
6.2 Therapeutic and commercial advantages of clones constructed by *thy*-insertion cloning system 157
6.3 Role of BclA proteins 159

List of Figures

Figure 1.1: Schematic diagram of different stage involves in spore formation 3
Figure 1.2: Spore surface display of an antigen 7
Figure 1.3: Commensal bacteria mediated defences against *C. difficile* 18
Figure 1.4: Ultrastructural morphotype of a *C. difficile* spore 20
Figure 1.5: Structure and mechanism of cellular intoxication of toxin A and toxin B 23
Figure 2.1: Schematic diagram of pThyA and pThyB 39
Figure 3.1: Schematic diagram of pDG364 62
Figure 3.2: The action of trimethoprim on the folate pathway 65
Figure 3.3: Schematic diagram of *thyA thyB* construction 67
Figure 3.4: Spore coat expression 70
Figure 3.5: Surface expression determined by "Whole Spore ELISA" 71
Figure 3.6: Growth of the PY79, *thyA* and *thyA thyB* strains in minimal media 73
Figure 3.7: Growth of *thyA thyB* mutant in SMM media 74
Figure 3.8: Growth of the *thyA thyB* mutant in SMM media with different amino acids 76
Figure 3.9: Growth of PY79, *thyA* and *thyA thyB* insertion strains in rich media 77
Figure 3.10: Growth of *thyA thyB* insertion strain in rich media with different concentrations of yeast extract 79
Figure 3.11: Growth of *thyA thyB* insertion strain in rich media with different concentrations of tryptone 80
Figure 3.12: Effect of adenosine on the growth of SH14 81
Figure 3.13: Different types of colony after first crossover 83
Figure 3.14: Thymine and thymidine titration 85
Figure 3.15: Viability of SH14 after thymine elimination 85
Figure 3.16: Germination of PY79, SH13 and SH14 87
Figure 3.17: Survival of *thyA thyB* insertion mutant in a murine GI-tract 88
Figure 4.1: Immunogenicity of SH14 spores expressing the *C. difficile* $TcdA_{26\text{-}39}$ antigen 106
Figure 4.2: Examining the conjugation of anti-$TcdA_{26\text{-}39}$ antibody to PY79 and SH16 by Western blotting 108
Figure 4.3: Spore surface conjugation determined by "Whole Spore ELISA" 108
Figure 4.4: Surface display of anti-$TcdA_{26\text{-}39}$ using immunofluorescence imaging of suspensions of SH16 and PY79 109
Figure 4.5: HT29 morphology treated with crude toxin 111
Figure 4.6: Determination of protease activity of PY79 and SH20 112
Figure 4.7: Degradation of casein by SH20 spores 113

Figure 4.8: Tyrosine standard curve 114
Figure 4.9: Degradation of starch by SH18 spores. 116
Figure 4.10: Maltose standard curve 117
Figure 5.1: Spore structure of *C. difficile* 126
Figure 5.2: Schematic representation of different types of BclA protein of CD630 128
Figure 5.3: Schematic diagram of different types of *bclA1* gene 136
Figure 5.4: Nucleotide analysis of different *bclA1* 137
Figure 5.5: Different types of *bclA2* 140
Figure 5.6: CFU determination in infected mice caecum 142
Figure 5.7: Toxin A and toxin B determination of caeca of infected mice 143
Figure 5.8: Sporulation of clade one to five representative strains 145
Figure 5.9: Cytotoxicity effect of clades one to five representative strains 146

List of Tables

Table 2.1: PCR primers for amplification of different genes 40
Table 2.2: Primers used to check insertion into *thyA* and *thyB* genes 42
Table 2.3: Primers used for construction of pET clones 46
Table 2.4: PCR conditions for amplification of *tcdA* and *tcdB* 57
Table 2.5: Primers and PCR conditions for amplification of different *bclA* genes 58
Table 3.1: Phenotypes of *B. subtilis* recombinant strains. 68
Table 3.2: Percentage sporulation of the PY79, *thyA* and *thyA thyB* insertional mutants 82
Table 3.3: Reversion of SH14 upon sub-culture in DSM 86
T**able 4.1:** Determination of reduction in toxicity 110
Table 4.2: Determination of the unit of activity for casein digestion 115
Table 4.3: Determination of the unit of activity for starch digestion 117
Table 5.1: Genotypic characterisation of various *C. difficile* clinical strains 132
Table 5.2: Genotypic characterisation of reference strains of different clades 139
Table 5.3: Infectivity of spores of different *C. difficile* strains in mice 144
Appendices A: *thyA* and *thyB* sequences cloned in pThyA and pThyB 160
Appendices B: Amino acid sequences of the fusion genes 160

Abbreviations

amyE	Amylase E gene
BHI	Brain Heart Infusion
BHIS	Brain Heart infusion + L-cysteine
bp	Base pair
CAA	Casamino acid
CDI	*Clostridium difficile* Infection
CDT	*Clostridium difficile* Transferase
CFU	Colony Forming Unit
Cm^R	Chloramphenicol Resistant
DSM	Difco Sporulation Media
EFSA	European Food Safety Authority
ELISA	Enzyme-Linked Immunosorbent Assay
FDA	Food and Drug Administration
GI-tract	Gasterointestinal Tract
GM	Genetically Modified
GMOs	Genetically Modified Organisms
GRAS	Generally Recognized as Safe
GTP	Guanosine Triphosphate
HMW	High Molecular Weight
HRP	Horseradish Peroxidase
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IgY	Immunoglobulin Y
LMW	Low Molecular Weight
mwt	Molecular Weight
NT	Non-Toxigenic
NTCD	Non-Toxigenic *Clostridium difficile*
OD	Optical Density
PAbs	Polyclonal Antibodies
PBS	Phosphate-Buffered Saline
PCR	Polymerase Chain Reaction
PVDF	Polyvinylidene Difluoride
QPS	Qualified Presumption of Safety

R	Ribotype
RT	Room Temperature
SIgA	Secretory Immunoglobin A
SLPs	Surface-Layer Proteins
SlpA	Surface-Layer Protein A
SMM	Spizizen's Minimal Media
SOC2	Super Optimal Broth 2 (no yeast extract)
spo	Genes Involve in Sporulation
thrC	Threonine Synthase
TLR4	Toll-like Receptor 4
TMB	3, 3', 5, 5'-Tetramethylbenzidine
w/v	Weight/Volume
v/v	Volume/Volume

CHAPTER 1

INTRODUCTION

1.1 Sporulation of *Bacillus subtilis*

Bacillus subtilis (*B. subtilis*), also known as hay bacillus or grass bacillus, was first discovered in 1835 by Christian Gottfried Ehrenberg. It was initially named *Vibrio subtilis* and was renamed by Ferdinand Cohn (Cohn, 1872). It is a Gram-positive spore former and aerobic bacterium commonly found in soil (Siala and Gray, 1974, Kunst *et al.*, 1997). It is rod-shaped and catalase positive, and although it is considered as an obligate aerobic bacterium, it can also grow and function anaerobically in the presence of nitrate and nitrite (Stewart, 1988, Ramos *et al.*, 1995). *B. subtilis* is not toxic or pathogenic and it is considered safe for human consumption (Sorokulova *et al.*, 2008). The size of *B. subtilis* cells is 4-10 μm long and 0.25–1.0 μm in diameter (Allen *et al.*, 2014). It can divide symmetrically, resulting in two daughter cells, or asymmetrically, resulting in the formation of an endospore (Grossman and Losick, 1988).

Spore formation is triggered by changes in environmental conditions that can be lethal to vegetative forms, e.g. nutrient limitation, changes in temperature, desiccation, and exposure to noxious chemicals and radiation. Once the environment is unfavourable for survival, *B. subtilis* vegetative cells will enter a pathway where the cells divide asymmetrically in the pole of the bacterium to form two different compartments, a smaller forespore and a larger mother cell. The mother cell nurtures the developing forespore that is

destined to become a spore. At the early stage of sporulation, the mother cell and forespore lie side by side. However, later in the development, the mother cell entirely swallows the forespore by migrating its membrane around the spore and creating a cell within a cell. The inner forespore will then mature and become a spore, and eventually, the spore will liberate itself from the mother cell by lysis (**Figure 1.1**) (Hilbert and Piggot, 2004). The shape of a mature *B. subtilis* spore is ellipsoidal, and it is approximately 1.2 μm in length (Ricca and Cutting, 2003). Spores can stay inert and survive indefinitely in the environment, and once the proper environmental conditions return, they can germinate and produce vegetative cells (Cano and Borucki, 1995a).

1.2 *B. subtilis* spore structure

B. subtilis spores are made of three different layers, and these layers can be visualised using transmission electron microscopy. The most inner and central part of the spore is the core that contains the chromosome. The internal core is surrounded by a thin layer of peptidoglycan called the cortex, which is involved in the dehydration state of spores. The next layer surrounding the cortex is called the coat layer, which sub-divides into an inner coat and an outer coat (**Figure 1.2**). The inner coat is a thin layer approximately 70 nm wide, though the outer coat is thicker and ranges from 70 to 200 nm wide (Driks, 1999).

1.2.1 Spore coat

The coat layer is a proteinaceous shell surrounding the spore that is vital for protection and survival of the spore. This layer is build up of components that synthesised, during forespore maturation, in the mother cell compartment (Henriques, 2004, Kim *et al.*, 2006). The coat layer consists of a lamella-like inner coat layer and an electron-dense outer coat layer. It protects the spores against environmental insults including lytic enzymes,

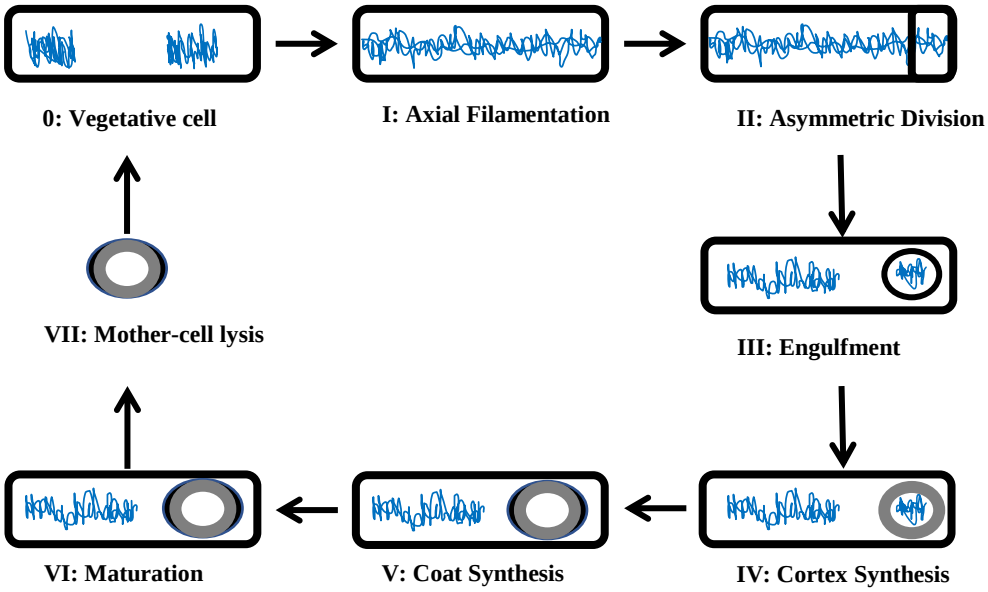

Figure 1.1: Schematic diagram of different stage involves in spore formation. In stage 0 (vegetative cell), the DNA will replicate, and the cell contains two complete chromosomes. The two chromosomes must be segregated into two cells. In stage I, the two chromosomes are remodelled by an axial filament, where they form an elongated filament that stretches across the long axis and anchors to the pole of each axis. Stage II is where the asymmetric division occurs that divides the cell into a small forespore and large mother cell. The DNA translocase will then pump the remaining chromosome into the forespore. The mother cell (stage III) will fully swallow the forespore by migrating its membrane around the forespore. Here, the forespore can mature into spore. Synthesis of cortex and spore coat in stages IV and V respectively will then take place. Stage VI is where the spore is fully matured, with a cortex and coat layer, and has acquired its full resistance properties. Finally, in stage VII, the mother cell will lyse, liberating the spore from the environment.

oxidizing agents and toxic molecules (Setlow, 2003, Setlow, 2011). A large number of spore coat proteins, >70 proteins, have been identified and only a small number of these proteins are essential for coat morphogenesis including CotX, CotY, CotZ and CotE. The coat architecture is severely disrupted if any of these morphogenic proteins are absence (McKenney *et al.*, 2013). CotE is a 181-amino-acid morphogenic protein required for the

assembly of the coat's outer layer of the endospore (Zheng *et al.*, 1988, Bauer *et al.*, 1999, Driks, 1999). It also regulates the expression of different coat proteins such as CotB, CotC and CotH (Zheng *et al.*, 1988). The spores of *B. subtilis* strain that have mutated *cotE* (unable to express functional CotE) lack the electron-dense outer coat and thus show higher sensitivity to lysozyme, that has peptidoglycan-degrading property (Nash *et al.*, 2006) and targets the cortex, compared to wild-type spores that have both an inner and outer coat (Driks, 1999). The coat layer also protects the spore from being digested once it has been ingested by other microorganisms. For example, when wild-type *B. subtilis* spores (Strain PS533) and spores from a *cotE* mutant strain were incubated with *Tetrahymena thermophile* – which consumes bacteria through ingestion – there was no decrease in wild-type spore titer after 48 hours of incubation, whereas *cotE* mutant spores showed a 100-fold reduction in spore titer (Klobutcher *et al.*, 2006). Moreover, the coat layer plays a role in changing the state of the spore form dormancy back to a vegetative form through a process called germination. The correct assembly of the inner coat layer mainly depends on a protein called GerE. *gerE* mutant spores lack the inner coat and have a severe defect in the outer coat layer (Driks, 1999). Spores of the *cotE* mutant strain and spores of the strain with mutant *gerE* are highly deficient in germination compared to their wild-type strain (James and Mandelstam, 1985, Driks, 1999).

1.2.2 Spore coat proteins

25% of total proteins of *B. subtilis* spores are coat proteins, which make up 10% of the total weight of a single dry spore. At least 70 different proteins on both layers of the spore coat of *B. subtilis* have been identified (Henriques and Moran, 2007). CotA (65 kDa), CotB (59 kDa), CotG (24 kDa), CotC (11 kDa), and CotF (8 kDa) are the principle polypeptides that belong to the outer coat layer. CotB, CotC, and CotG are possibly the most

abundant proteins on the outer coat layer, and their assembly depends on the CotE protein (Potot *et al.*, 2010). Some of these proteins were shown to be useful for a strategy referred to as the microbial cell surface display system , wherein their role as carrier proteins is to display a heterologous protein on the spore surface (Ricca and Cutting, 2003).

1.3 Microbial cell surface display

Microbial cell surface display system allows for proteins and peptides to be displayed from different sources on the surface of living cells or spores by genetically fusing them with anchoring motifs. Numerous strategies for presenting proteins or peptides on microbial cell surfaces have been established since the first report of surface display in 1985, wherein small proteins were fused with the phage protein pIII and expressed on the surface of bacteriophage (Smith, 1985). Since then, expressing peptides or proteins has been performed on the surface of both prokaryotes and eukaryotes such as bacteria, yeast, and insect and mammalian cells. Both Gram-negative and Gram-positive bacteria can be used to display proteins on their surface (Desvaux *et al.*, 2006, van Bloois *et al.*, 2011). The display of proteins at the cellular surface used in a large number of biomedical and biotechnical applications proved their effectiveness in the development of bioadsorbents, biocatalysts, and the delivery of drugs (Han and Lee, 2015). Microbial cell surface display system provides additional characteristics to the host without affecting the cell metabolism and causing metabolic abnormalities. Display proteins, as well as single or multiple epitopes on the cell or spore surfaces, can be used to develop recombinant vaccines that can be taken orally (Kramer *et al.*, 2003). Additionally, bacteria can be engineered to express and display recombinant enzymes on their surface; therefore, they can be used as biofactories with a large number of biotechnological applications (Jose *et al.*, 2002, Jose and von Schwichow, 2004). Currently, spore-based display systems, especially those using *B. subtilis* spores, have found many

applications (Samuelson *et al.*, 2002, Potot *et al.*, 2010, Permpoonpattana *et al.*, 2011a). Examples of spore-based display are display proteins such as urease B that has a potential to be used as oral vaccination against *Helicobacter pylori* (Zhou *et al.*, 2015a) or display enzymes such as phytase that can be used in animal feed for the purpose of better digestion (Potot *et al.*, 2010).

The surface display consists of a carrier protein that is anchored on the cell or spore surface, a heterologous protein, and a host. Successful expression and presentation of any protein or peptide highly depends on the type of anchoring motif used. The choice of incorrect anchoring can destabilise the cell envelope and therefore cause growth defects (Han and Lee, 2015). An efficient signalling peptide, strong anchoring motifs, and resistance to protease are the necessary features of a successful carrier protein. The signalling peptide is vital in allowing the fusing protein to pass through the inner membrane. Avoiding detachment from the surface of the cell or spore enables the carrier protein to have a strong anchoring motif. Finally, the presence of proteases in the periplasmic space or extracellular media requires a carrier protein to be protease resistant (Ricca and Cutting, 2003).

1.4 Engineering the *B. subtilis* spore coat

Engineering *B. subtilis* spores to express a heterologous protein on its spore surface was first shown by Isticato and colleagues (2001). The strategy for producing recombinant spores is illustrated in **Figure 1.2**. To display a heterologous protein on the surface of *B. subtilis* spores, two coat proteins, *cotB* and *cotC*, were initially used as the carrier protein, since these proteins were not necessary for the formation of typical spores. To be able to display a peptide or protein fused to *cotC* and *cotB*, two points must be considered. First and foremost, for the construction of a translational fusion, the promoter and gene of *cotB* or

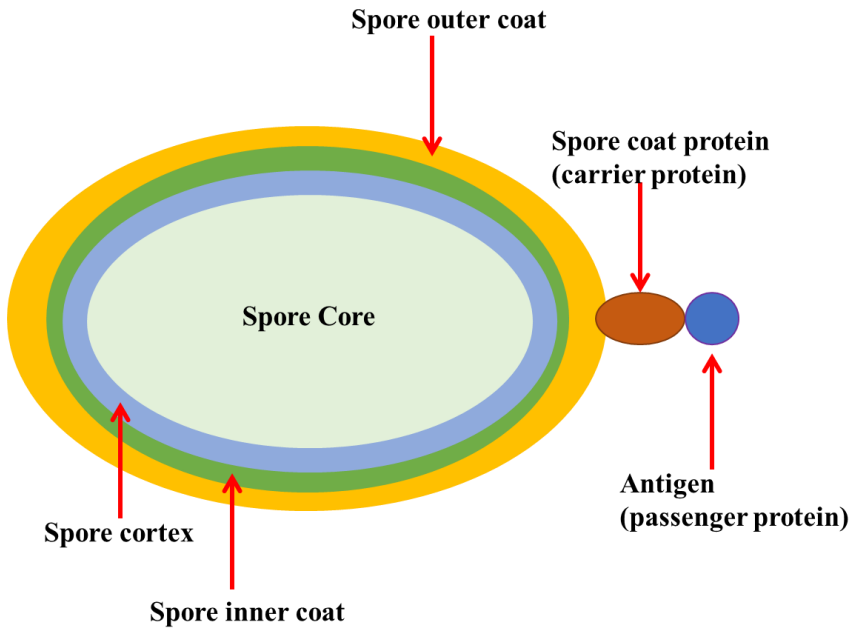

Figure 1.2: Spore surface display of an antigen. The internal core (light green) is encased by the cortex (light blue), and the cortex is surrounded by the coat layer, which is subdivided into the inner coat (dark green) and outer coat (yellow). An antigen protein (dark blue) can be expressed and displayed on the spore surface by fusing it to a spore coat protein (brown).

cotC must be used. Second, the fusion gene must be integrated into the coding sequence of a non-essential gene (Ricca and Cutting, 2003). However, it is also possible to incorporate the fusion into the coding sequence of an essential gene if the product of the mutated gene is supplemented to the growth media in which the bacteria grow (Iwanicki *et al.*, 2014). The correct assembly of a chimeric protein depends on which part of the carrier protein the heterologous passenger protein is genetically fused to. The protein can be fused to the N-terminus, C-terminus, and/or the middle (sandwich fusion) of the carrier protein. However, in *B. subtilis*, when 459 amino acids of a C-terminal fragment of the tetanus toxin (TTFC), 51.8 kDa, was fused to the C-terminal of *cotB*, it failed to assemble on the surface of the

spore correctly (Ricca and Cutting, 2003). It was suggested that the misfolded protein could be due to three 25-amino-acid repeats that are present in the C-terminus of CotB. The problem was bypassed by deleting the three 25-amino-acid repeats (CotBΔ105 have 275 amino acids compared to the wild-type, which has 380 amino acids), and when TTFC was fused to it (CotBΔ105-TTFC), it resulted in the correct assembly of the protein on the spore surface. In addition, a fusion of TTFC to the N-terminus and the middle of CotBΔ105 showed the correct assembly of TTFC on the spore surface. This has proven that to create a stable fusion with CotB as a carrier protein, the three 25-amino-acid repeats that make up half of the C-terminus of CotB must be removed. CotC is another protein that can be used as an anchoring motif. This was shown by Ricca and Cutting (2003), who fused TTFC to the C-terminus of CotC, which resulted in the correct assembly on the spore surface.

1.5 Applications of *B. subtilis* wild-type and recombinant spores

1.5.1 Probiotics

Probiotics are living microorganisms that are non-toxic, non-pathogenic and resistant to hydrolysis by mammalian enzymes, which can be formulated into various products, including drugs, foods, and dietary supplements (Heller, 2001). It has been shown that they can provide health benefits to the host by improving colonic balance, produce substances with systemic effects, and improve immune function (Guo *et al.*, 2017). Probiotics can produce a variety of components, such as lactic acid by fermenting sugar molecules, peptides, acetaldehydes and bacteriocins that can prevent or inhibit the growth of pathogenic bacteria (Holzapfel *et al.*, 2001, Gorbach, 2002). These compounds, especially peptides and bacteriocins, are involved in increasing the permeability of pathogenic cells, leading to depolarisation of the membrane and therefore cell death (Simova *et al.*, 2009). Two genera, *Lactobacillus* and *Bifidobacterium*, are commonly used as probiotics against pathogens in

the gut. However, they are limited through their sensitivity to temperature, gastric acid and slow growth (Del Piano *et al.*, 2008). Hence, it is necessary to search for probiotics with better efficiency and resistance to hydrochloric acid in the stomach, which protects the body from pathogens.

Bacillus species, especially *B. subtilis* and some of its close relatives, have been widely used as probiotics. *B. subtilis* spores are robust and resistant to stomach hydrochloric acid, making them attractive as probiotics. As these spores arrive in the small intestine, they germinate and proliferate once they sense that the environment is favourable, and it is here that they convey benefits to the host. They prevent intestinal inflammation, have an antidiarrheal effect, produce antimicrobial substances against pathogens, exclude pathogens, and normalise colonic flora (Mazza, 1994, Foligné *et al.*, 2012, Ramachandran *et al.*, 2014). *B. subtilis* is also recognised as safe for human consumption by the Food and Drug Administration (FDA) and the European Food Safety Authority (EFSA) (Suva *et al.*, 2016). These advantages make *B. subtilis* one of the most intriguing probiotic species for treatment of different clinical diseases.

B. subtilis spores as probiotics have benefits for both human and animals. They have been used to treat many conditions in humans caused by pathogens or food allergies. Mice experimentally infected with *Clostridium difficile* (*C. difficile*), a pathogen that causes diarrhoea, when dosed with probiotic *B. subtilis* spores PXN21 both pre and post infection, showed attenuated symptoms of the disease, although the administration of PXN21 spores post infection produced better suppression of the *C. difficile* infection (CDI). The mechanism of protection was suggested to be through the innate immunity by upregulation of the Toll-like receptor 2 (TLR2) once the PXN21 spore germinated and the peptidoglycan was carried

by the cortex, inducing the release of TLR2 (Colenutt and Cutting, 2014). *Clostridium perfringens* is a pathogenic bacterium that causes a common poultry disease called necrotic enteritis. This condition has a considerable effect on the profitability of commercial broiler chicken production. Traditionally, to overcome the pathogenic impact of necrotic enteritis, antibiotic feed supplements were administered. However, the growth-promoting antibiotic was restricted in the European Union; therefore, the search for an alternative treatment was necessary. In one study, *B. subtilis* spores of strain QST 713 were tested to determine their effect in broiler chickens that had necrotic enteritis (Tactacan *et al.*, 2013). The authors showed that the necrotic enteritis-infected broiler chickens that were not dosed with *B. subtilis* QST 713 spores had high mortality, whereas the necrotic enteritis-infected birds that were administered the QST 713 spores had a substantially reduced mortality rate. The group believe that the reason for that could be due to development of immunological tissue in the ileal mucosa of broiler chickens dosed with *B. subtilis* spores as reported by Molnár *et al.* (2011) increased gut-associated lymphoid tissue development paralleled the increasing *B. subtilis* concentration in the feed.

1.5.2 Recombinant spore vaccine

Currently, there is considerable interest in recombinant spores that offer benefits as a live vaccine vector. The intestinal mucosa is continuously exposed to foreign antigens and is an essential line of defence against the enteric pathogen. Vaccination is a method of triggering an immune response in an individual to promote development of adapted immunity to microbes. Since most pathogens first infect the mucosal surface, there is an increasing interest in the development of vaccines that induce protective mucosal immunity via mucosal routes. So far, most systemically administered vaccines are ineffective against mucosal infections. An ideal mucosal vaccine should provide protection both systemically

and at the mucosal delivery site. Mucosal secretory Immunoglobulin A (SIgA) and systemic immunoglobulin G (IgG) are two types of antibodies that are produced by an immune response via mucosal vaccination (Amuguni and Tzipori, 2012). SIgA is the primary antibody found at the mucosal site and predominantly exists in dimeric form, whereas IgG is found in peripheral blood and tissue spaces. In a mucosal system, the primary effector for adaptive immune defence is SIgA. Recently, it has been shown that mucosal immunity and especially the role of SIgA is essential for protection against CDI (Hong *et al.*, 2017b). Another advantage of mucosal vaccines is that they do not require injection and are easily administered, e.g. via the nasal route. An efficient delivery system is essential for the development of mucosal vaccines. *B. subtilis* spores are used in the generation of the orally administered mucosal vaccine by acting as a platform for the presentation of heterologous proteins (as antigen) on their spore surface, and this species has attracted noticeable attention (Batista *et al.*, 2014, Ricca *et al.*, 2014).

Many animal studies have been conducted that use the recombinant *B. subtilis* spore as a mucosal vaccine. Tuberculosis, caused by a pathogen called *Mycobacterium tuberculosis* (*M. tuberculosis*), is an infectious disease that mainly affects the lungs and has high morbidity and mortality in different parts of the world. A vaccine called Bacillus Calmette-Guérin (BCG) was designed to protect against tuberculosis through the attenuation of live bovine tuberculosis bacillus, *Mycobacterium bovis*, which is unable to cause disease in humans (Simona and Mihaescu, 2013). The BCG vaccine was developed more than 90 years ago, and it is still the only vaccine available today against tuberculosis. However, BCG is incapable of creating full protection against the disease (Colditz *et al.*, 1995). Thus, development of a more efficient vaccine was necessary. Using the *B. subtilis* spore as a delivery vehicle, a strategy was developed to produce recombinant spores, in which a

significant immunodominant antigen, Ag85B from *M. tuberculosis* was displayed on the spore surface. Mice that were dosed with recombinant spores showed an increasing level of the Ag85B-specific IFN-γ producing cell and a higher level of Ag85B-specific IgG antibodies in the serum compared to mice that were dosed with naked wild-type spores (Das *et al.*, 2016). IFN-γ has been shown to prevent the growth and replication of *M. tuberculosis* (Szabo *et al.*, 2002). Therefore, the delivery of the *M. tuberculosis* antigen on the surface of the *B. subtilis* spore proves that it can create an immune response, and this can be a potential vaccine strategy against TB. Recombinant *B. subtilis* spores can also be used as a treatment for food allergies. Immunised peanut allergic C3H/HeJ mice with *B. subtilis* spores, which display the mucosal adjuvant cholera toxin B subunit, fused with the peanut major allergen Ara h2 tp, showed protection against peanut-induced anaphylaxis. Results indicated that the immunotherapeutic effect of peanut-specific IgA was induced by the recombinant spores (Zhou *et al.*, 2015b).

1.5.3 Recombinant spores as a drug delivery vehicle

Despite the potential of using recombinant spores to express drugs, it has not generated much attention for specific disease treatment. *B. subtilis* can be used to deliver anti-tumour compounds. Nguyen and colleagues (2013) constructed killed *Bacillus* spores that could be engineered to display cetuximab, which is a monoclonal antibody that recognises the epidermal growth factor receptor expressed on cancer cells. These spores could therefore be loaded with an anti-cancer drug called paclitaxel on their surface and could specifically target cancer cells *in vitro*, resulting in inhibition of cancer cell growth (Nguyen *et al.*, 2013).

1.5.4 Other applications of recombinant *B. subtilis* spores

Industry plays a vital role in developing the world, yet it can result in various negative consequences such as water pollution and gases. It can also release different types of hazardous chemicals and heavy metals such as lead, mercury, zinc, silver, gold, cadmium, copper, arsenic, and chromium, which can be hazardous to most living creatures. To overcome these problems, several technologies have been developed, including precipitation-dissolution, ion-exchange, and reverse osmosis. However, due to hazardous sludge, removal of specific ions, high costs, and high energy requirements (Ahalya *et al.*, 2003), alternative methods are required. The best way to overcome these problems is to use enzymes that can degrade toxic pollutants. Many enzymes from fungi, bacteria, and plants are involved in the biodegradation of poisonous and carcinogenic pollutants (Karigar and Rao, 2011). For instance, the enzyme nitrilase converts nitriles, which are toxic and can pollute water, into non-toxic products, including carboxylic acid, in the grass (Gong *et al.*, 2012). Some of the disadvantages of using enzymes in industrial applications are the high cost of isolation and purification and sensitivity to various denaturing conditions such as pH and temperature since enzymes are proteins. Additionally, most enzymes dissolve in water, which causes product contamination; therefore, their recovery in an active form for reuse is not feasible (Homaei *et al.*, 2013). Immobilisation of enzymes is a technique that fixes enzymes to solid supports (van de Velde *et al.*, 2002). Immobilisation makes the enzymes more robust and increases their resistance to environmental changes that result in their denaturing. Several studies have reported that *B. subtilis* spores can be used to immobilise enzymes (Potot *et al.*, 2010, Chen *et al.*, 2015) they can re-germinate and re-sporulate both *in vitro* and in the gastrointestinal tract (GI-tract), the active enzymes can be displayed continuously (Tam *et al.*, 2006). Recombinant *B. subtilis* spores have become a fundamental tool for the processes of bioremediation. If histidine amino acids are displayed on the spores

surface, they can absorb heavy metals such as nickel significantly more effectively than the wild-type spores (Hinc *et al.*, 2010a). Finally, the spore surface expression of feed enzymes can potentially improve the digestion of animals. For example, xylanases, amylases, and gluconases that are from the carbohydrase class help to break down carbohydrates such as starch and fibre into simple sugars in the guts of animals (Jacela *et al.*, 2009).

1.6 Regulatory issues regarding the use of recombinant spores

Organisms with changes in their gene pool that cannot occur naturally are regarded as genetically modified organisms (GMOs). GMOs include genetically modified (GM) animals, microorganisms, and plants. The deliberate modification and the resulting entities, besides the benefits, have always been considered a threat both to humans and the environment. For instance, GMOs can be used in agriculture as a biopesticide, nitrogen fixative, or plant growth promoter. However, when introduced into the environment, they can have environmental consequences and have more pronounced ecological roles in comparison to wild-type organisms (Heuer and Smalla, 2007). For example, weeds are a constant problem in farming. To overcome this problem through the use of genetic engineering, herbicides and pesticide-tolerant plants were produced. However, the chemical pesticides and herbicides can potentially result in the evolution of pesticides and herbicide-resistant pests and weeds (Owen and Zelaya, 2005). Therefore, an increased quantity or higher strength pesticides and herbicide need to be used to eliminate them. This could cause damage to biodiversity and ecosystem health (Casida, 2009, Carvalho, 2017).

In addition, as a result of DNA modification, it is possible that the organism acquires other characteristics, and this may not be limited to a feature of the replaced gene (Prescott *et al.*, 2005). It is crucial to ensure that when GMOs are released into nature, they are not

harmful to both humans and the environment. Thus, the environmental risks that may be caused by recombinant organisms once they are introduced into the natural environment must be assessed.

Insertion of a single gene into a microorganism genome could affect the entire genome of the host, resulting in different unintended characteristics, and not all these features can be recognised at the same time. Prediction of all types of risks after a gene insertion is difficult. Some examples of the different kinds of threats identified when using GMOs are: i) the possibility of GMOs cross-breeding with the wild-type, resulting in disappearance of the novel traits in the wild-type organism; ii) GMOs can have an advantage over other organisms as a result of faster growth, which could possibly allow them to spread (become invasive) into a new habitat and cause damage to the economy and ecology; iii) horizontal transfer, via transformation or conjugation, of recombinant genes to other microorganisms; this can be particularly problematic when an antibiotic-resistance gene is transferred or conjugated to a pathogen that can cause disease in human sand animals (Bennett *et al.*, 2004); iv) GMOs can have adverse effects on human or animal health by increasing the pathogenicity or emergence of new diseases.

The scientific panel on GMOs (EFSA GMO) published a risk assessment guideline in 2006. It was used to identify and evaluate the potential adverse effects of GM microorganisms on humans, animals, and the environment and whether these adverse effects are direct or indirect and immediate or delayed (Committee, 2007).

1.7 *C. difficile* infection

1.7.1 Antibiotics and CDI

Approximately 15% of hospitalised patients treated with antibiotics will develop antibiotic-associated diarrhoea, and it is estimated that *C. difficile* is responsible for 15% to 20% of the cases (Hurley and Nguyen, 2002, Beaugerie *et al.*, 2003). *C. difficile* is a spore-forming, Gram-positive, and strictly anaerobic bacterium that is the most common cause of antibiotic-associated diarrhoea in developed countries. *C. difficile* infection (CDI) is a toxin-mediated intestinal disease with clinical features ranging from asymptomatic colonisation, mild to severe diarrhoea in the acute form, and pseudomembranous colitis (Beaugerie *et al.*, 2003, Khan and Elzouki, 2014, Tao *et al.*, 2016). The significant risk factor for developing CDI is antibiotic treatment, though other factors such as advanced age and weakened immune system due to conditions such as diabetes could also increase the susceptibility of the host to the disease (Cloud and Kelly, 2007, Rupnik and Janezic, 2016). Approximately 500,000 and 18,000 cases of CDIs are reported each year in the USA and England respectively (Borren *et al.*, 2017).

The human gut microbiota consists of a complex population of microbial species that play a vital role in human health and disease. A healthy microbiota is required to resist and prevent the colonisation of pathogens in the host gut. More than 500 species exist in the human gut, with healthy flora that prevents *C. difficile* from establishing disease. A decrease in indigenous intestinal microflora such as lactobacilli and bifidobacterial strains that have an antagonistic role against *C. difficile*, could create an empty environment for *C. difficile* to fill, colonise, and cause infection (Naaber *et al.*, 2004, Wei *et al.*, 2018). The use of broad-spectrum antibiotics that are used to treat a primary infection will affect the host microbiota, causing damage and disruption of protective microbiota and increase susceptibility of the

host to infection via opportunistic bacterial pathogens. Therefore, two events are required for CDI to occur; the first is the disruption of the protective gut microbiota, and the second is the acquisition of *C. difficile* spores, which are resistant to the antibiotics that are taken for treating the primary infection.

Microbiota can prevent *C. difficile* colonisation through various mechanisms. To cause the disease, the spores of *C. difficile* must germinate in the host GI-tract. In the GI-tract, the presence of primary and secondary bile acids can hugely affect the germination and the growth of *C. difficile* (**Figure 1.3**). For example, chenodeoxycholate, one of the major primary bile acids, can inhibit *C. difficile* spore germination (Sorg and Sonenshein, 2009). *Clostridium scindens* (*C. scindens*), a commensal species, is highly associated with resistance to CDI by encoding dehydroxylating enzymes, which are essential to converting primary bile acid into secondary bile acids that have inhibitory effects on *C. difficile.* The *C. scindens* inhibitory effect on *C. difficile* is completely abolished by the addition of cholestyramine, which is a bile acid sequestrant to the culture (Buffie *et al.*, 2015). This indicates that the mediators of *C. difficile* growth inhibition were likely the secondary bile acids (**Figure 1.3**). In the gut, commensal bacteria exist that produce sialidase, an enzyme that cleaves the sugars from glycosylated proteins. The glycosylated proteins are bound to the epithelial cell membrane, and cleaving them by sialidases will result in releasing free sialic acid into the lumen (Sonnenburg *et al.*, 2005). In addition, short-chain fatty acids, such as succinate, are produced from complex carbohydrates, specifically fibre and resistant starches, which are broken down by primary fermenters (Wong *et al.*, 2006). Commensal bacteria rapidly consume these metabolites as energy sources. *C. difficile* is also able to metabolise succinate and sialic acid since it has genes for both succinate transporter and sialic acid catabolism. Thus, disrupting the commensal bacteria allows *C. difficile* to have

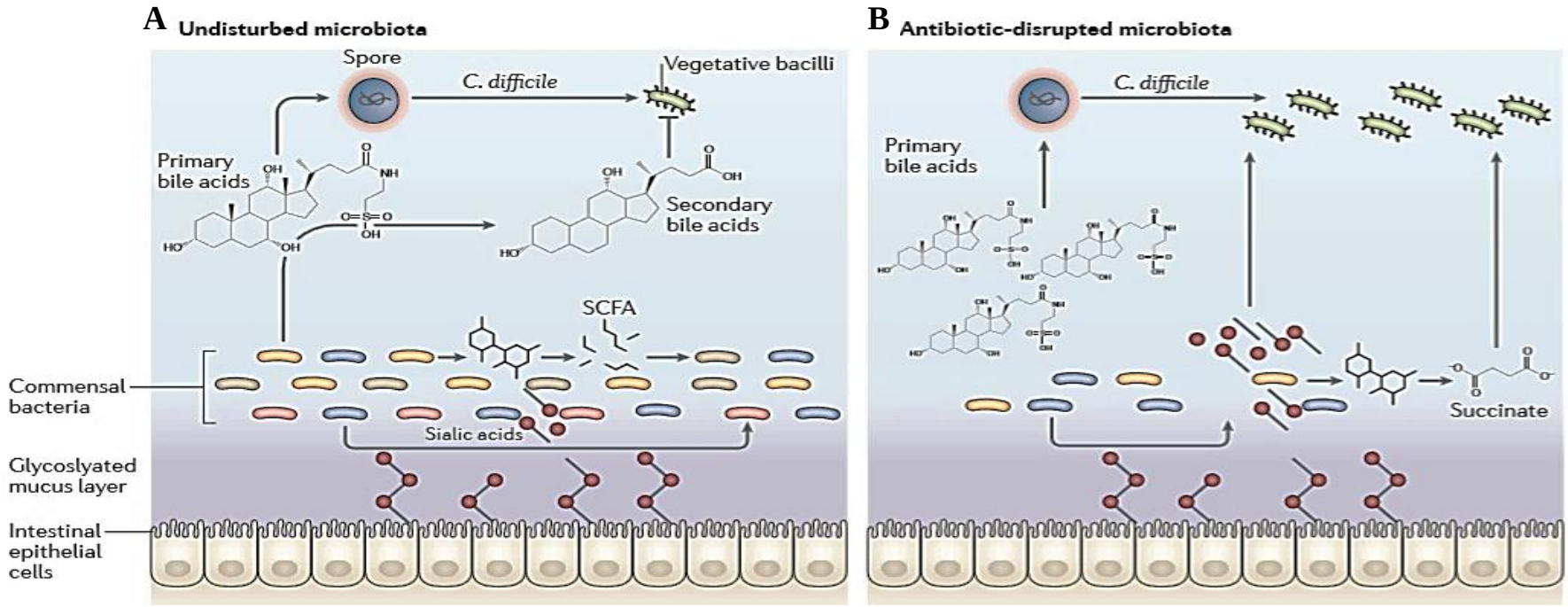

Figure 1.3: Commensal bacteria mediated defences against *C. difficile*. A) the conversion of primary bile acids into secondary bile acids by intact microbiota results in several disturbances that are toxic to *C. difficile* vegetative cells and inhibit its growth. Conversion of carbohydrate into small-chain fatty acids (SCFA), including succinate, is done by fermenting commensal bacteria. Commensal bacteria encode sialidases, which cleaves the sugar molecule from the glycosylated proteins that are attached to epithelial cells into free sialic acids that will be consumed by bystander commensal bacteria population as energy sources. B) Antibiotics disrupt the microbiota, which results in depletion of the primary bile acid converter. It can also deplete sialic acid and succinate consumers. *C. difficile* can therefore germinate, grow, and use the sialic acid and succinate as energy sources. Figure taken from Abt *et al.* (2016).

easy access to these nutrients, and this results in better growth and proliferation of the cells (Ng *et al.*, 2013, Ferreyra *et al.*, 2014). The growth of *C. difficile* is also prevented by direct interaction with bacteria that have antimicrobial activity. A few different bacteriocins have been identified, and these bacteriocins were reported to have antimicrobial properties against Gram-positive pathogens, including *C. difficile* (Rea *et al.*, 2011, Trzasko *et al.*, 2012).

1.7.2 *C. difficile* spore

Due to the strictly anaerobic nature of the vegetative form of *C. difficile*, the primary agent of infection and transmission of CDI, are *C. difficile* spores. Mutant strains of *C. difficile* that cannot produce Spo0A protein, a regulator responsible for the activation and regulation of sporulation genes in response to nutritional stress, failed to persist and transmit CDI (Deakin *et al.*, 2012). Environmental contamination and survival of *C. difficile* spores, especially in hospitals, is a significant contributor to the spread of CDI among patients (Guerrero *et al.*, 2012, Sunkesula *et al.*, 2013). Once *C. difficile* infects the susceptible host, the spores can persist in the colonic environment and can survive the host's innate immune system (Paredes-Sabja *et al.*, 2012, Barra-Carrasco and Paredes-Sabja, 2014). The outgrowth and proliferation of *C. difficile* in a susceptible host results in the shedding of large numbers of spores in the faeces (Songer and Anderson, 2006). Approximately 50% of patient may become asymptomatic shedders of *C. difficile* spores for up to 4 weeks after the treatment and recovery from a CDI episode (Sethi *et al.*, 2010), and are therefore a source of transmission (Sunkesula *et al.*, 2013).

Similar to *B. subtilis* spores, *C. difficile* spores also consist of the core, cortex and coat layer, and as reported for *B. subtilis*, the coat layer of *C. difficile* spores is resistant to heat and proteolytic enzymes such as proteinase k and trypsin (Escobar-Cortés *et al.*, 2013).

However, *C. difficile* spores also consist of an electron-dense layer called exosporium which is the outermost layer (Barra-Carrasco *et al.*, 2013, Díaz-González *et al.*, 2015) (**Figure 1.4**). The exosporium contributes to the spore's hydrophobicity and to the ability of *C. difficile* to adhere to surfaces (Joshi *et al.*, 2012). The exosporium layer has been found in the spores of other species such as *B. anthracis* (Gerhardt, 1967), *B. cereus* (Gerhardt and Ribi, 1964), *B. megaterium* (Stewart, 2015) and *Clostridium sporogenes* (Hodgkiss et al., 1967). The stability of *C. difficile* spore's exosporium is a matter of controversy as some studies have reported that this layer is fragile and easily lost (Permpoonpattana *et al.*, 2011b, Permpoonpattana *et al.*, 2013), while other reports provide evidence that this layer is stable and stays attached to the spores (Barra-Carrasco *et al.*, 2013, Paredes-Sabja *et al.*, 2014). The exosporium seems to be more stable as Joshi *et al.* (2012) reported that the exosporium of *C. difficile* spores contribute to the spore's adherence to inert surfaces such as stainless steel and consequently to the persistence of spores on the surfaces of health care environment.

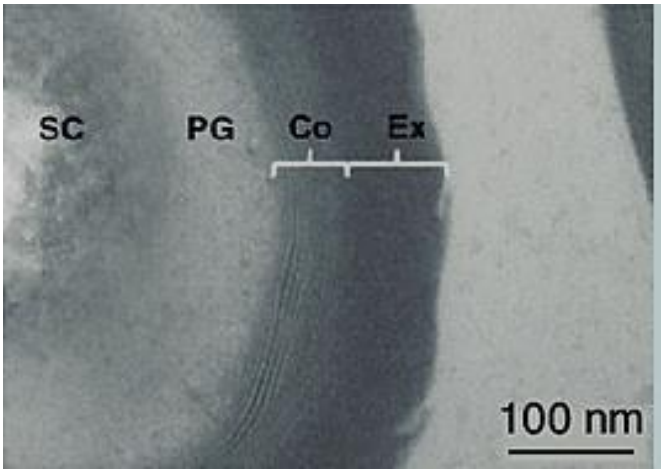

Figure 1.4: Ultrastructural morphotype of a *C. difficile* spore. Electron microscopy image of *C. difficile* stain 630. SC: spore core; PG: Peptidoglycan cortex; Co: coat layer; Ex: Exosporium. Image is taken from Barra-Carrasco and Paredes-Sabja (2014).

1.7.3 Virulence factors and pathogenesis mechanisms

a) Toxins

C. difficile produces three toxins, toxin A, toxin B, and binary toxin. The structurally similar toxin A and toxin B are the main virulence factors of *C. difficile*. These toxins are encoded by *tcdA* and *tcdB* genes located within a 19.6 kbp region called the pathogenicity locus. The pathogenicity locus region also contains three other genes, *tcdD, tcdC,* and *tcdE*, which are involved in regulation and transportation of toxin A and B (Monot *et al.*, 2015).

These toxins are proinflammatory, enterotoxic, and cytotoxic, with a molecular weight of 308 kDa (toxin A) and 270 kDa (toxin B) respectively (Drudy *et al.*, 2007). Both toxins structurally consist of four functional domains: The N-terminus glucosyltransferase domain, a delivery and pore-forming domain, an auto-protease domain, and a combined repetitive oligopeptides domain located in the C-terminus, which is involved in receptor binding (**Figure 1.5**). In favourable conditions in the host gut, *C. difficile* spores colonise and germinate, and the vegetative cells proliferate and produce both toxins A and B, which enter the epithelial cell cytosol and cause the cytotoxic effect. Transferring both toxins into the host cell cytosol is a multistep process (**Figure 1.5**). The process starts with the binding of both toxins to receptors on the host cell surface via their receptor binding site. It seems that the toxins bind to different receptors. Two receptors that are expressed on the human colonocyte apical membrane, glycoprotein 96 and sucrose-isomaltase, are reportedly toxin A receptors that enable the entry of the toxin into the cell and facilitate its cytotoxicity (Pothoulakis *et al.*, 1996, Na *et al.*, 2008). In addition, two receptors, poliovirus receptor-like 3 and chondroitin sulphate proteoglycan 4, have been identified as important for toxin B-mediated cytotoxicity (LaFrance *et al.*, 2015, Yuan *et al.*, 2015). To evoke the cytotoxic effect, toxins must be internalised into cell cytosol. They become internalised by receptor-

mediated endocytosis (Florin and Thelestam, 1983). Once the endosome enters the cell cytosol, its pH reduces and becomes acidic. The endosome acidification is necessary to alter the toxins' structure, which leads to the formation of pores in the endosome membrane. These pores will allow the translocation of the glucotransferase domain into the host cytosol (Giesemann *et al.*, 2006, Genisyuerek *et al.*, 2011). The glucotransferase will then mono-glucosylate the Rho GTPases, which leads to their functional inactivation. The Rho family of GTPases, a sub-group of superfamily Ras proteins, are GTP-binding proteins that are located in the cell cytosol and have multiple regulatory functions, including regulation of actin cytoskeleton, transcriptional regulation, and apoptosis (Aznar and Lacal, 2001, Gerhard *et al.*, 2008). The inactivation of Rho GTPases results in the breakdown of the actin cytoskeleton, which leads to a change in cell morphology (cells become round) and destruction of intestinal barrier function. Glucosylation of Rho GTPases also activates caspases, which are proteolytic enzymes and play an essential role in apoptosis (Gerhard *et al.*, 2008).

The production of binary toxin or *C. difficile* transferase (CDT) has been associated with increasing severity and high mortality of CDI (Bacci *et al.*, 2011). The presence of CDT has been reported in a minority of *C. difficile* PCR ribotypes (Gerding *et al.*, 2014). The toxin belongs to the binary ADP-ribosylating toxin family, and it comprises two components: the enzymatic ADP-ribosyltransferase (CDTa) and a separate binding/translocation component (CDTb) that binds to the host cell and enables passage of the CDTa into the cell cytosol. The CDTa and CDTb are encoded by *cdtA* and *cdtB* genes located on 6.2 kbp region, known as CDT locus or CTLoc (Perelle *et al.*, 1997).

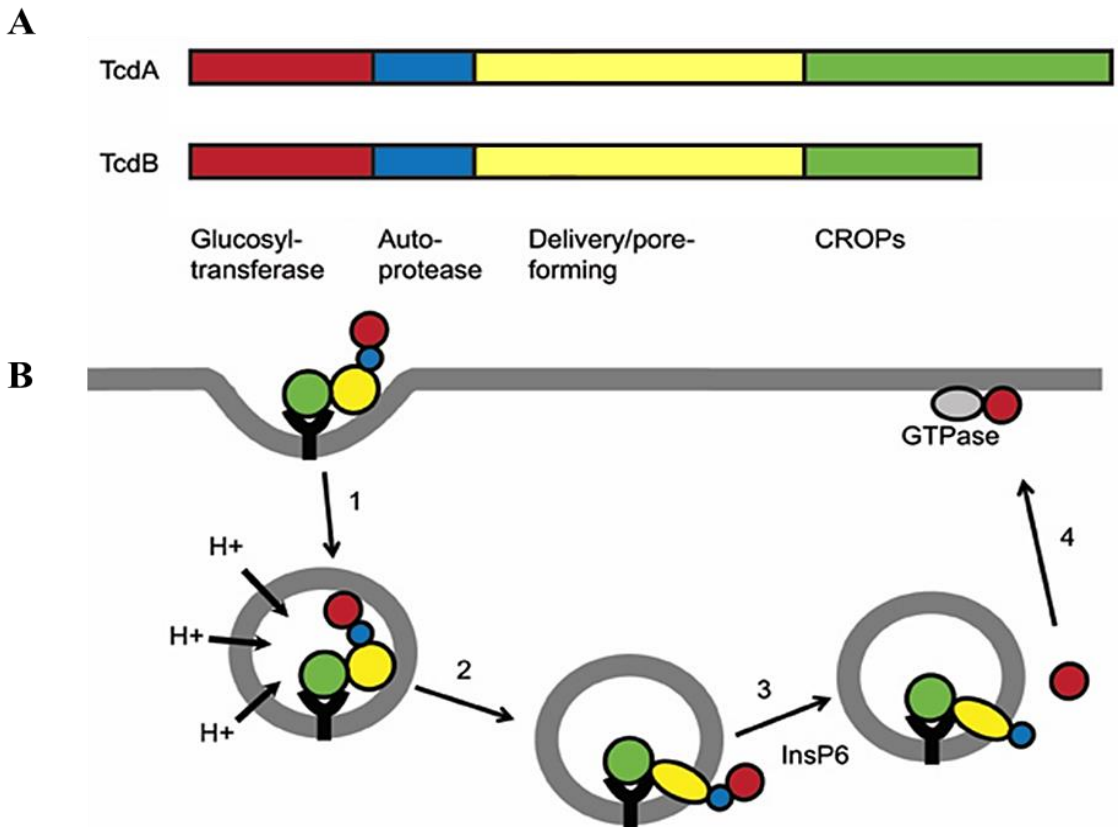

Figure 1.5: Structure and mechanism of cellular intoxication of toxin A and toxin B. A) Both toxins consist of four domains. The N-terminal glucosytransferse domain (red), combined repetitive oligopeptides domain (CROPs) in C-terminal (green), autoprotease domain (blue), and delivery or pore-forming domain (yellow). B) For the delivery of the toxin into the cell, the toxins bind to the cell surface receptor via the combined repetitive oligopeptide domain, and they are internalised by receptor mediated endocytosis (1). Pores form once the endosome has acidified, and this results in glucotransferase translocation (2). Inositol hexakisphosphate (InsP6) dependent autoproteolysis will release the glucotransferase into the cytosol (3). Finally, glucotransferase inactivates the Rho family GTPases by glucosylation, which eventually results in cell death (4). Figure taken from Pruitt et al, (2012).

To determine whether CDT influences the severity of CDI, a strain of *C. difficile* that does not possess *tcdA* and *tcdB* genes but has an intact *cdt* gene was tested on hamsters. This

strain was dosed to hamsters following clindamycin. Although it showed successful colonisation in the hamsters' GI-tract, it did not cause diarrhoea or death (Geric *et al.*, 2006). Another group, however, showed that 3 out of 8 hamsters died when they were dosed with a strain that had deactivated toxin A and toxin B but active CDT, although they did not show any typical hamster CDI symptoms. However, isolates with either toxin A or toxin B remained fully virulent. When hamsters were dosed with a strain that had toxin B inactivated, expression of toxin A and CDT caused significantly more death which suggested that the binary toxin may have an important role in increasing pathogenicity (Kuehne *et al.*, 2014).

Despite extensive knowledge of the structure and enzymatic function of the CDT, its role in increasing the severity of CDI disease was uncharacterised until recently. Cowardian and colleagues (Cowardin *et al.*, 2016) examined the influence of CDT on host immune response and showed that suppressing protective colonic eosinophilia by the binary toxin enhances the *C. difficile* virulence. The authors showed that mice infected with R20291 (*tcdA*$^+$, *tcdB*$^+$, *cdt*$^+$), a hypervirulent strain, had higher mortality and weight-loss in comparison to R20291 that lacked the CDTa or CDTb domains. Their work proved that eosinophils that have innate immunity play a protective role against CDI and that CDT, via a Toll-like receptor 2-dependent pathway, suppresses the eosinophils' protective function in blood or within the colon.

b) Non-toxin virulence and pathogenesis factors

Despite the toxins that play a significant virulence factor in CDI, other putative non-toxin virulence factors exist, including the proteins that are important for adherence and colonisation. A bifunctional coat protein, CotE, was shown to affect colonisation and virulence of *C. difficile* (Hong *et al.*, 2017a). CotE consists of two domains, an N-termini

peroxiredoxin and a C-termini chitinase domain (Permpoonpattana *et al.*, 2011b). Spores are often considered as biochemically dormant, yet Hong and colleagues (2017) showed, both *in vitro* and *ex vivo* using isogenic mutants, that CotE is vital for adherence and binding of the spore to the mucus and that it also has a role in mucus degradation. Hamsters that were dosed with spores with mutated *cotE* showed significantly lower colonisation and clinical symptoms in comparison to hamsters that were infected with an isogenic strain with intact *cotE* (Hong *et al.*, 2017a).

C. difficile cells have an outermost proteinaceous layer called the surface layer (S-layer) that surrounds the bacterium and plays a role in adhesion, complement resistance, and protection from bacterial parasites (Sára and Sleytr, 2000). The *C. difficile* S-layer consists of two protein subunits, low molecular weight (LMW) and high molecular weight (HMW) proteins. These subunits are formed via proteolytic cleavage of their polypeptide precursor, surface-layer protein A (SlpA), by cysteine protease (Cwp84) (Dang *et al.*, 2010). By using an antibody against SlpA, the adherence of *C. difficile* to cultured cells was blocked. In addition, the *C. difficile* adhesion to epithelial cells significantly reduced when the monolayers were pre-treated with crude or purified SlpA (Merrigan *et al.*, 2013). Furthermore, surface layer proteins (SLPs) were involved in immune recognition and the inflammatory response. It has been reported that SLPs are recognised by Toll-like receptor 4 (TLR4), and the activation of TLR4 by SLPs leads to maturation of dendritic cells following by activation of the T-helper cell response (Ryan *et al.*, 2011). Cwp84, as well as being involved in processing the S-layer, has also been shown *in vitro* to degrade extracellular matrix protein and this proteolytic activity could play a role in host tissue degradation resulting in the dissemination of the infection (Janoir *et al.*, 2007).

In addition to the latter non-toxin virulence agents, many other factors that increase the virulence of *C. difficile* have been identified. This includes the flagella, heat shock protein GroEL, fimbriae, type IV pili, and fibronectin binding proteins. The role of these factors in increasing virulence varies within different *C. difficile* strains (Awad *et al.*, 2014).

1.7.4 Hypervirulent strains

There are different strains of *C. difficile*, hypervirulent and not hypervirulent. Since 2004, the occurrence of CDI has markedly increased, and in most cases, the identified *C. difficile* strain was PCR ribotypes 027 (R027) (Morfin-Otero *et al.*, 2016). The most virulent strain of *C. difficile*, an R027 strain called R20291, was isolated in 2006 following a hospital outbreak (Stabler *et al.*, 2009). Hypervirulent *C. difficile* strains are more infectious than other *C. difficile* strains, and they are associated with high severity and mortality. Many studies were performed to identify the factors that make R027 the most virulent strains of *C. difficile*. It has been reported that hypervirulent strains produce more toxins than other strains *in vitro* (Warny *et al.*, 2005). The *tcdC* gene is a negative regulator of toxin expression through destabilising the TcdR-holoenzyme. Initially, it was thought that a change in reading frame or deletion of *tcdC* could explain the high toxin production of R027. The *tcdC* gene has been found to have an 18-bp in-frame deletion or a single base pair (bp) deletion that results in the formation of a stop codon and truncation of the protein (Dupuy *et al.*, 2008). However, by characterising the *tcdC* in other non-epidemic strains, various deletions have been identified, which suggests that increased toxin expression of R027 is not caused by these mutations alone (Spigaglia and Mastrantonio, 2002).

Apart from high toxin production, other factors that may have a role in increasing the severity of R027 have been investigated. R027 shows higher resistance to antibiotics,

especially fluoroquinolone antibiotics, and this is associated with the higher global spread of these strains (He *et al.*, 2013). Some studies reported that the R027 also exhibits increased sporulation and high sporulation could explain the high level of transmission of R027 (Åkerlund *et al.*, 2008, Rupnik *et al.*, 2009, Dingle *et al.*, 2011). However comparison of different *C. difficile* isolates by Burns *et al.* (2011) indicated that neither the total sporulation capacity nor the sporulation rate of the different R027 types were higher than that of non-R027 strains. Furthermore, they observed significant variation in sporulation deficiency of different R027 types. The overrepresentation of R027 is also due to competing with the endemic strains, which has been noted both *in vivo* and *in vitro* (Robinson *et al.*, 2014).

1.7.5 Relapse

The most common complication of CDI infection is its recurrence. Approximately 25% of successfully treated patients with CDI will experience the recurrence of the disease. Recurrence mostly occurs within 30 days, either by the original infecting strain (relapse) or by a new strain (re-infection) from the contaminated local environment (Kamboj *et al.*, 2011). The antibiotics only eliminate vegetative cells and do not affect the *C. difficile* spores, which means that once these antibiotics have been discontinued, spores will have a chance to germinate; this results in proliferation of *C. difficile* in the gut and the symptoms re-occur (McFarland *et al.*, 2002). The recurrence of CDI is mostly in elderly (>65 years) Caucasian patients, females, patients with the severe initial disease, and patients with current antibiotic use (Garey *et al.*, 2008).

1.8 Treatment of CDI

a) Antibiotics

Various antibiotics have been introduced that can treat CDI. Oral administration of metronidazole has been recommended as the first-line treatment for mild, non-severe CDI. Vancomycin is another antibiotic that is used for more severe CDI and recurrence of the disease, although there is evidence that metronidazole can be as effective as vancomycin (Tonna and Welsby, 2005). Another successful antibiotic for treating CDI that was approved by the FDA in 2012 is fidaxomicin. An advantage of fidaxomicin is that once it has been taken orally, because of its low absorption, a high chronic concentration of this drug can be present. Fidaxomicin, like vancomycin, is also used for moderate to severe CDI (Al-Jashaami and DuPont, 2016).

The same antibiotics that are used for treating the primary disease can be used for the first recurrence of CDI. Fidaxomicin and vancomycin were similar in achieving an initial clinical response for patients with the first recurrence of CDI, despite the fact that the rate of subsequent recurrence with fidaxomicin was 19% in comparison to vancomycin, which had a rate of 35% (Cornely *et al.*, 2012).

b) Tolevamer

Tolevamer is a non-antimicrobial, soluble, and anionic polymer that is orally administrated to treat CDI. It has a HMW (> 400 kDa). The unique mechanism of the action of tolevamer is noncovalent, with high-affinity binding to *C. difficile* toxin A and toxin B (Louie *et al.*, 2006). The binding of tolevamer to toxins effectively neutralises them. Thus, unlike the traditional antibiotic that affects both the pathogen and the gut bacteria, tolevamer interacts neither with pathogen nor the gut bacteria and therefore does not further disrupt the

microflora (Kurtz *et al.*, 2001). The original tolevamer was GT160-246; it markedly attenuated the CDI severity and was associated with lower recurrence compared to standard therapy with vancomycin (Davidson *et al.*, 2004, Louie *et al.*, 2006). Different preclinical studies suggested tolevamer for the treatment of mild to moderate CDI. A disadvantage of tolevamer is that it is associated with an increased rate of hypokalaemia (Louie *et al.*, 2006).

c) Faecal microbiota transplantation

Patients with CDI have a reduction in the diversity of microbiota due to antibiotic therapy, and this reduction in the microbiome can be restored by faecal microbiota transplantation (Avila *et al.*, 2016). Faecal microbiota transplantation therapy works by repopulating the patient's protective microbiome of natural colonic flora that has been suppressed or killed as a result of antibiotic treatment. Restoring the commensal bacteria in the gastrointestinal tract can, theoretically, suppress the growth and colonisation of *C. difficile*. This method has been shown to be an adequate way of treating CDI and the recurrence of the disease. More than 90% of patients suffering from the relapse have been cured by faecal microbiota transplantation (Rohlke and Stollman, 2012). The stool from a healthy human donor can be delivered to the intestine by colonoscopy, enema, nasogastric route, or as capsules (Al-Jashaami and DuPont, 2016). Very recently, it was found that bacteriophage transfer during faecal microbiota transplantation is associated with improved CDI treatment outcomes (Zuo *et al.*, 2017). This group showed that a higher *Caudovirales* (tailed bacteriophages) richness in the donor than the recipient resulted in curing all recipients.

Although faecal microbiota transplantation is an effective treatment for CDI, there are some critical risks, such as screening the donor stool for infectious agents, as there is a

possibility that the tests fail to detect a pathogen. The use of faecal microbiota transplantation could also cause inflammatory bowel disease, obesity, and functional gastrointestinal disorders (Sbahi and Di Palma, 2016).

d) Passive immunisation

The duration and severity of CDI largely depends on the level of immune response to *C. difficile* colonisation. It has been reported that the asymptomatic *C. difficile* carriers had a high level of the IgG anti-toxin A antibody and the risk of *C. difficile* diarrhoea was much lower in comparison to carriers who had a low level of the antibody against toxin A (Kyne *et al.*, 2000). Prior to that, another study used the anti-toxin A and toxin B immunoglobin Y (IgY) derived from egg yolk to treat hamsters with CDI. Their results showed that hamsters that were administered both anti-toxin A and B IgY were protected from the CDI, relapse, and subsequent *C. difficile* reinfection. Using the IgY against toxin A, however, hamsters were only protected against CDI but not against the relapse (Kink and Williams, 1998).

In the past decade, few studies have shown that introducing an anti-toxin antibody can prevent CDI and relapse. Administering the human monoclonal antibodies against toxin B alone or with anti-toxin A resulted in 100% protection of piglets, yet administration of anti-toxin A antibody alone developed severe GI and systemic disease in the animals (Steele *et al.*, 2012). These results were unexpected as a previous study had shown that both toxin A and toxin B are cytotoxic and each toxin individually can cause the CDI (Kuehne *et al.*, 2010). In humans, the administration of intravenous immunoglobin from healthy human plasma donors to patients with CDI has successfully treated the disease (Salcedo *et al.*, 1997, Cone *et al.*, 2006). However, some studies cast doubt on the effectiveness of intravenous

immunoglobin in treating CDI (Juang *et al.*, 2007, Abougergi *et al.*, 2010).

The most successful study on passive immunisation for treating CDI was conducted by Lowy and colleagues (2010). They intravenously administered fully human monoclonal antibodies against both *C. difficile* toxins in combination with antibiotics and showed that as well as treating CDI, this combination was effective in reducing the *C. difficile* relapse from 25% to 7% (Lowy *et al.*, 2010). Recent phase-III clinical evaluations using a human monoclonal antibody showed effective treatment of CDI. In this clinical trial, an anti-toxin B human monoclonal antibody called bezlotoxumab resulted in significant reduction of CDI relapse (Wilcox *et al.*, 2017).

e) Pre-colonisation with non-toxin-producing C. difficile strains

For any *C. difficile* strain to cause the infection, it must at least produce either toxin A or toxin B. Not all the strains of *C. difficile* are toxigenic, and a strain that does not produce any toxin is termed non-toxigenic (NT). The NT strains were first isolated in 1980 (Shuttleworth *et al.*, 1980). A few years after the isolation of these strains, Wilson and colleagues (1983) reported that NT *C. difficile* (NTCD) strains could protect hamsters against the toxigenic strains. In their report, 93% of hamsters that were dosed with NTCD before a toxigenic *C. difficile* challenge survived. In comparison, survival of animals that were administered both toxigenic and NT isolate together and hamsters that were only dosed with toxigenic isolates was 32% and 21% respectively. Further investigation proved that protection against CDI by NT requires the NTCD to be present and alive at the time of toxigenic strain exposure. The protective effect of the non-toxigenic strains vanishes if they are killed either by heat or by an antibiotic such as vancomycin (Borriello and Barcley, 1985). Although hamsters that were successfully pre-colonised with NTCD showed

protection against the toxigenic strain, eventually, they were infected by the toxigenic strain and developed CDI, indicating that the protection was short term. To increase the effect and durability of protection, another group have analysed different NTCD strains (Sambol *et al.*, 2002). They tested the efficacy of pre-colonization of hamsters with 3 nontoxigenic *C. difficile* strains for preventing CDI. Groups of hamsters that were pre-colonised with the NTCD isolates, survived up to 106 days.

In another study, pre-colonising hamsters with a NTCD called M3 resulted in 100% protection when they were challenged with the most virulent strain of *C. difficile* (R027) (Nagaro *et al.*, 2013). Realising the potential of the M3 strain for preventing toxigenic *C. difficile* colonisation, Gerding *et al.* (2015) has investigated the protective effect of this strain further by testing it on humans in a randomised clinical trial. Their results produced several key findings. First, they showed that NTCD was well tolerated and safe in patients who received it. Second, the relapse rate in patients who were successfully colonised with the M3 strain compared to patients who were not colonised was 2% and 31% respectively. Finally, no NTCD was detected in patients that received M3 after detection of the toxigenic strain in their stool, which suggests that the NTCD cannot compete with the toxigenic strain that has already colonised.

The mechanism by which NTCD prevents relapse is not clear. One possibility is that NTCD occupies the same adherence niche in the GI-tract as toxigenic strains, and once it is colonised, it is able to compete with the newly ingested or resident toxigenic *C. difficile* strains. There are concerns about using NTCD strains, as there is a possibility that these strains could acquire the pathogenicity locus containing the genes encoding toxin A and B via horizontal transfer (Brouwer *et al.*, 2013).

f) Vaccine

Vaccines can result in long-term protection by stimulating adaptive immunity. Several groups have been working on different types of vaccine against *C. difficile*, and their results for protection against the disease were promising (Ghose *et al.*, 2007, Permpoonpattana *et al.*, 2011a, Bruxelle *et al.*, 2017). The adaptive immune response results in recruitment of the T cells and B cells and the production of the antibody IgA (secreted into the gut) and IgG or immunoglobulin M (IgM) in the blood. The production of SIgA and IgG has been shown to prevent CDI either by neutralising toxins or by preventing *C. difficile* colonisation in the gut. First demonstrated by Libby and colleagues (1982), the concomitant injection of toxoids A and B to hamsters resulted in significant protection against CDI. This has prompted many scientists to further explore the efficiency of toxoid-induced immunity by evaluating the parameters that influence the vaccine efficiency, such as the use of an adjuvant, choice of antigen delivery system, and routes of immunisation. Another group showed that transcutaneous immunisation with *C. difficile* toxoid A resulted in induction of mucosal and systemic immune responses in mice (Ghose *et al.*, 2007). They showed that immunisation of mice resulted in the production of anti-toxin A-specific IgG and IgA in serum and anti-toxin A IgA in faeces and reported that these antibodies neutralise toxin A *in vitro*. Recently, a human clinical trial using a toxoid vaccine by Sanofi Pasteur entered phase III. However, the study was terminated, as they concluded that the probability of success would be low.

As well as the development of toxoid-based vaccines, there are different research groups focused on developing a recombinant protein vaccine. It was proposed that the use of the recombinant toxin sub-domain can have many advantages, such as overcoming the complexity of producing a toxoid that requires the purification of a large protein and

chemical inactivation, with the intrinsic risk of incomplete inactivation (Nencioni *et al.*, 1991). Lyerly and colleagues (1990) were the first to report that 33 repeating units of the receptor binding site of toxin A can induce an immune response. When hamsters were subcutaneously immunised with this recombinant peptide, they were partially protected from CDI. In another study, with the aim of inducing anti-toxin A immunity, a recombinant fusion protein consisting of 14 repeat units of toxin A along with immunogenic fragment C of the tetanus toxin was transferred into an attenuated strain of *Salmonella typhimurium* (Ward *et al.*, 1999b). The authors demonstrated that intranasal and intragastric administration of this strain induced an adaptive immunity and a significant level of anti-toxin A IgA in the gut and anti-toxin A IgG in the serum. Mice that were administered the recombinant strain intranasally, consistently generated higher anti-toxin A IgA antibody, suggesting that the route of administration is important for an optimum immune response.

A mucosal spore vaccine is a further strategy for generating an immune response. Recently, it was found that hamsters that were orally administered *B. subtilis* spores that expressed a segment of the C-terminus of the *C. difficile* toxin A were protected against CDI (Hong *et al.*, 2017b). No toxins and spores of *C. difficile* were detected in the faeces and caecum of the hamsters. The key finding in the above study was that in addition to recognising toxin A, the generated anti-toxin A IgG and SIgA cross-reacted with toxin B and a few epitopes on both vegetative cells and spores of *C. difficile*. It was suggested that the lack of *C. difficile* colonisation in the gut was due to the attachment of the produced antibodies against the toxin A C-terminus segment to both cells and spores of *C. difficile*, blocking them from attachment to the epithelial cells. This spore vaccine is currently in phase I of a human clinical trial.

Thesis objectives

1. To develop a cloning system that results in *B. subtilis* recombinant spores expressing heterologous protein on their surface without using an antibiotic-resistance gene as a selection marker. The gene(s) of interest will be fused with either *cotB* or *cotC* (CotB and CotC will serve as anchor protein) and will be inserted into two thymidylate synthase genes (*thyA* and *thyB,* required for thymine synthesis) of *B. subtilis* strain PY79 which results in the expression and display of chimeric proteins on the surface of PY79 spores. Ultimately the recombinant strains become thymine auxotroph. The constructed clones will be characterised for their growth in different media, sporulation, germination and their persistence in animal gut.

2. To produce and test different recombinant spores for industrial and treatment purposes, especially treatment of CDI. Antigen proteins or enzymes will be expressed and displayed on the surface of PY79 spores and these clones will be tested to find out whether the expressed and displayed proteins are functional.

3. To identify the ribotypes, and *bclA* genes located on the exosporium of *C. difficile* spores, in 45 human clinical isolates of *C. difficile* and to characterise the sporulation, *in vitro* cell-cytotoxicity and colonisation of different toxigenic ribotypes with different types of *bclA1* gene (full-length, truncated, and deleted).

CHAPTER 2

MATERIALS AND METHODS

2.1 General methods

The general methods for work with *B. subtilis*, including the 'two-step transformation procedure', were performed as described previously (Harwood and Archibald, 1990). Cloning in *E. coli* was done as described previously (Sambrook and Russell, 2001).

2.2 Bacterial strains

PY79 is a prototrophic strain of *B. subtilis* derived from the strain 168 type (Zeigler *et al.*, 2008). PP108 (*amyE::cotC-tcdA*$_{26\text{-}39}$ *thrC::cotB-tcdA*$_{26\text{-}39}$) has been described elsewhere (Permpoonpattana *et al.*, 2011a). SH250 is a prototrophic derivative of PY79 carrying the *cat* gene (encoding resistance to chloramphenicol) inserted at the *amyE* locus. DSM (Difco Sporulation Media) is a standard media for growth and sporulation of *B. subtilis* (Nicholson *et al.*, 2000).

2.3 pThyA and pThyB construction (Chapter 3)

pThyA (4,274 bp) carried a 1,910 bp segment comprising the left (900 bp) and right (950 bp) homology arms of the *B. subtilis thyA* gene surrounding a multiple cloning site (MCS) cloned into pMA-RQ (2,556 bp; Genscript, USA). Both arms carried additional proximal and distal DNA sequences adjacent to *thyA*. Similarly, pThyB (4,973 bp) carried a 2,057 bp segment comprising the left (900bp) and right (1.1 kb) arms of the *B. subtilis thyB*

gene surrounding an MCS cloned into pBluescript SK (+) (2,958 bp). Plasmids carried the ampicillin resistance gene (*bla*), and the nucleotide sequences of the *thyA* and *thyB* segments are given in **Appendices A** and shown schematically in **Figure 2.1**. pThyA and pThyB plasmids were constructed which carried chimeric genes inserted at the MCS sites of each vector.

Chimeric genes (not optimised for codon usage) containing an in-frame fusion between the 5' segment of *B. subtilis cotB* or *cotC* with the *vp28*, *vp26*, $tcdA_{26\text{-}39}$ and *streptavidin* coding ORFs were first synthesised with suitable 5' and 3' ends for sub-cloning in the MCS of the pThyA and pThyB vectors. The subtilisin E (*aprE*) and alpha-amylase (*amyE*) genes were PCR amplified from a *B. subtilis* strain (SG115, Sporegen limited collection) and *B. amyloliquefaciens* (SG277, Sporegen limited collection) respectively (**Appendices B**). The amplified *aprE* coding segments lacked the N-terminal regions involved in protein secretion (pre) and activation (pro). For *amyE*, the amplified coding segment lacked the N-terminal regions involved in protein secretion (pre). Both these genes were cut at their 5' end using HindIII restriction enzyme and fused, by ligating, with *cotB* (cut using HindIII at the 3'). The resulting *cotB-aprE* /*cotB-amyE* were ligated to pThyA via BamHI and the EcoRI restriction site. **Table 2.1** shows the primers used to amplify above genes.

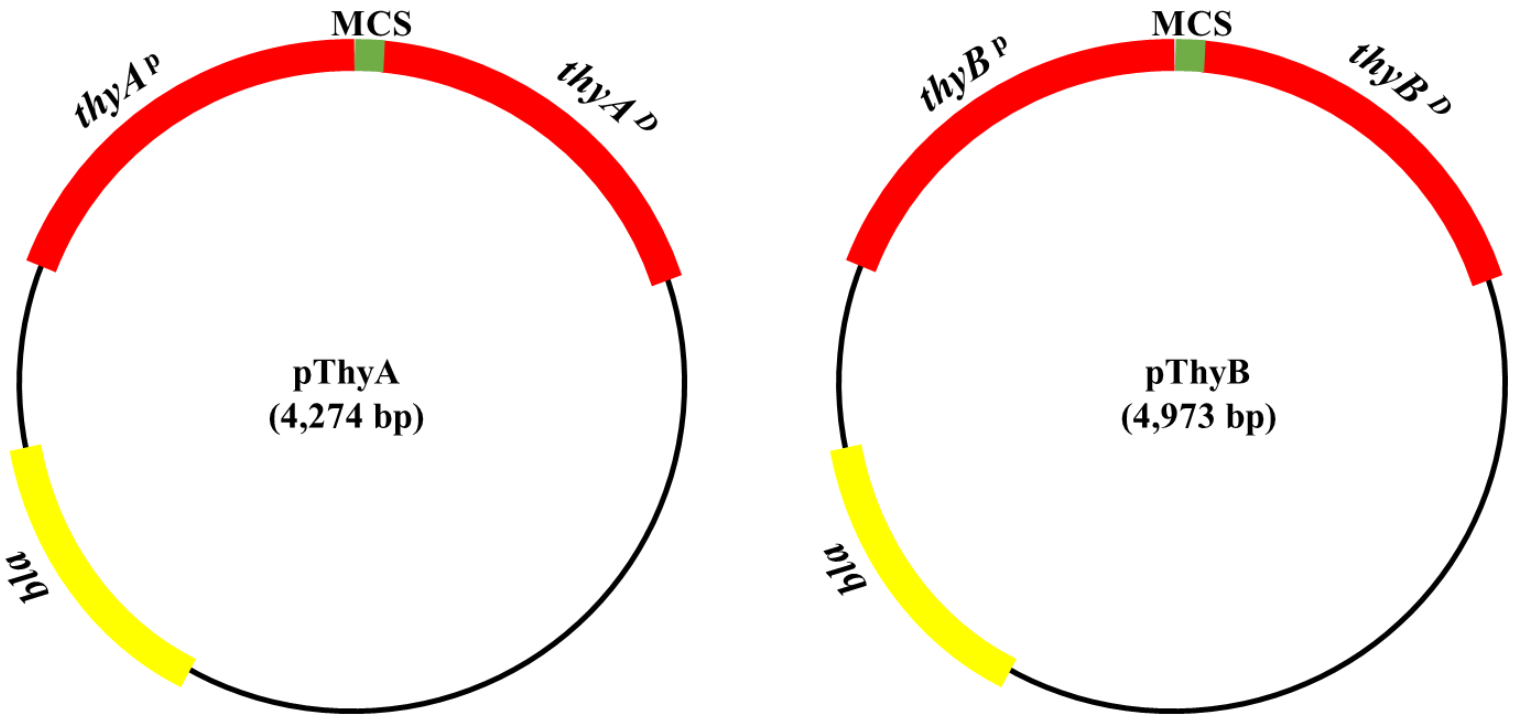

Figure 2.1: Schematic diagram of pThyA and pThyB. A total of ~900 bp proximal (P) and distal (D) of both *thyA* and *thyB* has been synthesised in an *E. coli* plasmid, pMA. Both *thyA* and *thyB* are interrupted at the midpoint with an MCS. pThyA and pThyB have a total of 4,274bp and 4,973bp respectively. The antibiotic-resistant gene on both plasmids is *bla*.

Table 2.1: PCR primers for amplification of different genes

Primer	Direction	Sequence[1]	Restriction site
cotB			
CotB_F	Forward	TATA***GGATCC***ACGGATTAGGCCGTTTG	BamHI
CotB_R	Reverse	TATA***AAGCTT***GGATGATTGATCATCTGAA GATTTTAG	HindIII
cotC			
CotC_F	Forward	TATA***GGATCC***TTCACAAAAATACTCGTTAT TTTG	BamHI
CotC_R	Reverse	TATA***AAGCTT***GTAGTGTTTTTTATGCTTTTT ATACTC	HindIII
amyE			
AmyE_F	Forward	TATA***AAGCTT***GAAACTGCAAACAAATCGAA	HindIII
AmyE_R	Reverse	TATA***GAATTC***TTAATGCGGAAGATAACCGTT TAA	EcoRI
aprE			
AprE_F	Forward	TATA***AAGCTT***GTGAGAGGCAAAAAGGTATG	HindIII
AprE_R	Reverse	TATA***GAATTC***TTACTGAGCTGCCGCCTGTAC	EcoRI

2.4 Recombinant *B. subtilis* strains (Chapter 3)

2.4.1 Construction of recombinant strains by the inactivation of *thy* genes

The procedure developed here consisted of two steps. In the first stage cells, of a wild-type recipient strain (in the work described here the prototrophic strain PY79 was used) were made competent using a 'two-step transformation' procedure described by Dubnau

(Dubnau and Davidoff-Abelson, 1971) and in common use in *Bacillus* labs (Dubnau and Davidoff-Abelson, 1971, Cutting, 1990). In brief, a single colony of the *B. subtilis* strain PY79, grown overnight on LB at 30°C, was used to inoculate 20 ml of SPC media (T-base [$(NH_4)_2SO_4$; 2 g, $K_2HPO_4 \cdot 3H_2O$; 18.3 g, KH_2PO_4; 6 g, trisodium citrate$\cdot 2H_2O$; 1g]; 20 ml, 50% (w/v) glucose; 0.2 ml, 1.2% (w/v) $MgSO_4 \cdot 3H_2O$; 0.3 ml, 10% (w/v) yeast extract; 0.4 ml, 1% (w/v) casamino acids; 0.5 ml) and allowed to grow at 37°C, with vigorous aeration, to an OD_{600} of 0.5-0.6. Then 0.2 ml of the culture was used to inoculate 20 ml of pre-warmed SPII media (T base; 20 ml, 50% (w/v) glucose; 0.2 ml, 1.2% (w/v) $MgSO_4 \cdot 3H_2O$; 1.4 ml, 10% (w/v) yeast extract; 0.2 ml, 1% (w/v) casamino acids; 0.2 ml, 0.1 M $CaCl_2$; 0.1 ml) and allowed to grow for s further 90 min at 37°C with aeration. Cells were pelleted by centrifugation (8,000 g, 5min) at RT. The supernatant was decanted into a sterile container and cells were gently resuspended in 1.6 ml of the supernatant and 0.4 ml of 50% glycerol. Aliquots of the competent cells (0.3 ml) were made and stored at -80°C.

pThyA plasmids carrying the chimeric gene were linearised with either ApaLI or ScaI digestion. Then ~500 ng of linearised plasmid was added to 300µl of competent cells and was incubated for an hour stirring at 37°C. After that, cells were plated on SMM (Spizizen's Minimal Media (SMM)) (Harwood and Archibald, 1990) agar, supplemented with thymine (50 µg/ml) (Sigma; T0376) and trimethoprim (3 µg/ml) (Sigma; T7883). After 96h of growth at 37°C, single colonies were colony purified and checked for growth at 37°C and 46°C on SMM agar supplemented with ± thymine (50 µg/ml) and trimethoprim (3 µg/ml). Cells carrying an insertion at the *thyA* locus would grow at 37°C with or without thymine but were unable to grow at 46°C unless supplemented with thymine. A further verification was to amplify, using PCR, the presence of the chimeric gene from transformants using primers annealing to the *thyA* sequences (**Table 2.2**). In the second

stage, a linearised (ApaL1 or Sca1) pThyB plasmid carrying a chimeric gene was introduced into cells of the *thyA* insertion strain by electroporation. Electroporated cells were plated on SMM+CAA (SMM containing 0.2% (w/v) casamino acids (CAA)) supplemented with thymine (50 μg/ml) and trimethoprim (6 μg/ml) and incubated at 37°C for 48h. To confirm the presence of both *thyA* and *thyB*, insertion colonies were streaked on SMM+CAA agar ± thymine (50 μg/ml) and grown at 37°C. Cells carrying two insertions were unable to grow at both 37°C and 46°C unless supplemented with thymine. A final verification was made using PCR primers that amplified the two insertions (**Table 2.2**). Using electroporation, integration frequencies were about 1 X 10^3/μg of linear DNA with ~20% of trimethoprim-resistance colonies carrying two insertions (*thyA* and *thyB*).

Table 2.3: Primers used to check insertion into *thyA* and *thyB* genes

Primer	Direction	Sequence
thyA		
ThyA_F	Forward	GTCTAAATGGAGAAAAAGTGGATC
ThyA_R	Reverse	GTTAAGGCCATTGCGTCTAATTC
thyB		
ThyB_F	Forward	GATATTAAAACAAATCCGAACTC
ThyB_R	Reverse	GTCAGACACATAGAATTG

2.4.2 Constraction of chloramphenicol resistance PY79 (Chapter 3)

Plasmid DG364 (pDG364) that had left and right homology arms (to enable double cross-over) of the *amyE* locus and also a chloramphenicol resistance gene (surrounded by the two arms), were linearised and transformed into PY79 competent cells. Transformants

were confirmed by their ability to grow on LB supplemented with 5 μg/ml of chloramphenicol and also by their inability to break down starch on a starch agar plate.

2.5 Electroporation (Chapter 3)

The procedure used here was modified from established methods (Xue *et al.*, 1999) for electroporation in *Bacillus* primarily with the use of an SOC2 media that contained no yeast extract. The SOC2 was tryptone (2% w/v), NaCl (10 mM), KCl (2.5 mM), $MgCl_2$ (5 mM), $MgSO_4.7H_2O$ (5 mM) and glucose (20 mM). An overnight culture of the strain carrying a *thyA* insertion was sub-cultured in 25 ml of the SOC2 media (supplemented with 0.5M sorbitol) to give a starting OD_{600} of 0.2. The culture was grown at 37^oC to an OD_{600} of 1.4, cooled on ice for 10 min, and then harvested by centrifugation at 4^oC (5,000 g, 5 min). Cells were washed four times in ice-cold electroporation solution (0.5M sorbitol, 0.5M mannitol, 10% (v/v) glycerol) and suspended in 1.6 ml of the same ice-cold solution. The cells were then electro-competent and ready for immediate use. Cells were kept on ice and used within 30 min, although aliquots can be stored at -80^oC indefinitely. 1μl (~50ng) of linearised plasmid DNA was added to 60μl of electrocompetent cells and the mixture transferred to a pre-chilled cuvette (1 mm gap width) and incubated for 1.5 min on ice. The cuvette was then placed inside the electroporator (BioRad GenePulser Xcell) and the following parameters used for electroporation: voltage – 2,100V, resistance – 200W, time – 5 milliseconds and number of pulses – 1. After electroporation, 1 ml of the recovery media (SOC2 media containing 0.5M sorbitol and 0.38M mannitol) was added to the cuvette and the mixture was transferred to 2 ml Eppendorf tubes and incubated for 3h at 37^oC after which cells were serially diluted and plated SMM containing 0.2% (w/v) CAA), thymine (50 μg/ml) and trimethoprim (6 μg/ml) and incubated at 37^oC for 48h.

2.6 Sporulation and spore purification (Chapter 3 and 4)

Sporulation was carried out using the exhaustion method. For sporulation of thyminless *B. subtilis*, thymine (50 μg/ml) was supplemented in all media. Spores were harvested 24h after the initiation of sporulation. As previously described, spore suspensions were purified using lysozyme to break the residual sporangial cells followed by washing in 1 M NaCl-1 M KCl and water (three-times) (Nicholson *et al.*, 2000).

2.7 Whole-spore ELISA (Chapter 3 and 4)

An ELISA method was used to detect surface exposed proteins as described previously (Permpoonpattana *et al.*, 2013). Microplate wells (Greiner, high binding) were coated with 50μl of a suspension of pure spores (2 X 10^8 spores/well) and left overnight at 4^oC. Plates were blocked with 2% (w/v) BSA for 1h at 37^oC. Rabbit polyclonal antibodies (PAbs), recognising either the heterologous antigen expressed on the spore surface or the whole *B. subtilis* spore, were used as primaries with incubation for 2h at RT. Anti-rabbit IgG-horseradish peroxidase (HRP) conjugate (1:5,000 in PBS plus 0.05% Tween-20) was used as a secondary with 1h incubation at RT. TMB (3, 3', 5, 5'-tetramethylbenzidine) was used as the substrate. After addition of TMB and developing the ELISA, the reaction was stopped using 2M H_2SO_4 and measured at 450nm.

2.8 Expression and purification of His-tagged recombinant proteins (Chapter 3 and 4)

The pET28b expression vector was used to express recombinant proteins (VP26 of white spot syndrome virus). The amplified products were cloned in-frame into pET28b and the plasmid was transformed into expression *E. coli* strain BL21. Primers used for the construction of pET28b clones are shown in **Table 2.3**.

a) Protein expression

100 ml of LB containing 30 μg/ml of kanamycin was inoculated with an overnight culture of BL21 containing the plasmid to an OD_{600} of 0.05. The culture was grown with agitation (200 rpm) at 37°C to an OD_{600} equal to 0.4-0.6. IPTG (Thermo Fisher; R0392) was added to the culture to the final concentration of 100 μM to induce the protein expression, and the culture was incubated further for at least 3h. The cells were harvested by centrifugation at 4,500 x *g* for 10 min at 4°C. The supernatant was discarded and the pellet was stored at -20°C till assay.

b) Cell lysis and protein extraction

Cell pellet was resuspended in lysis buffer (NPI-10 [50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0], protease inhibitor, 2% Triton X100, lysozyme 1 mg/ml, 1 mM dithiothreitol, 1% sarkosyl, 1μl per 10 ml of 10% glycerol benzonase) and incubated for 30 min at 4°C with slow agitation. Then the cells were sonicated on ice for 1.30 min (1 sec on, 1 sec off, amplitude 30%). The sample was further incubated for 30 min on ice before it was centrifugated (13,000 x g, 20 min) at 4°C. The supernatant was filter-sterilised (0.45-μm pore size) and transferred to a clean tube.

c) Purification of His-tagged proteins

The cell lysate was injected into an FPLC NGS-purifier system (BioRad) and purified, according to the BioRad manual, by passing the cell lysate through an HiTrap chelating HP column and removing the cell debris with washing buffer NPI-20 (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, pH 8.0). The His-tagged proteins were then eluted using elution buffer NPI-250 (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, pH 8.0), different fractions were collected, and the fractions were checked for the presence

of protein by SDS-PAGE.

Table 2.4: Primers used for construction of pET clones

Primer	Direction	Sequence [1]	Restriction site
vp26			
Vp26_NcoI_F	Forward	TATA***CCATGG***AATTTGGCAACCTAACA	NcoI
Vp26_notI_R	Reverse	TATA***GCGGCCGC***CTTCTTCTTGATTTCGTCCTTGAT	NotI

[1] Restriction site in bold and italics

2.9 Raising antibodies (Chapter 3 and 4)

PAbs to VP28 and $TcdA_{26\text{-}39}$ were raised in rabbits using four sub-cutaneous injections (1 μg/dose, every 14 days). VP26 polyclonals were raised in mice using purified rVP26 protein (4 intra-peritoneal doses at 14-day intervals, 10 μg/dose [0.1 ml/site]). Recombinant proteins were complexed with Freund's adjuvant (Sigma; F5881). After the third dose of protein, mice were monitored for the production of antibodies. By day 50, the whole blood was collected from the mice and transferred into sterile tubes. The blood samples were left for 2h at 4^{o}C to separate cells from serum. Cells were then further removed by refrigerated centrifugation (2,000 rpm, 10 min). The top layer (serum) was removed into a clean tube. The serums were then incubated in a 56°C water bath for 15 min to destroy the complement system. Obtained serums were purified using protein A chromatography protocol from BioRad.

2.10 Western blotting (Chapter 3)

For Western blotting, purified spores were prepared and 2 X 10^8 suspended in 40 µl of Bolt LDS buffer (Life Tech.) and incubated at 95ºC for 10 min. The spore suspensions were centrifuged (18,000 g, 10 min) and 20 µl of each supernatants was run on 12% SDS-PAGE gels. Then the proteins from the gels were blotted onto an Immobilon-P PVDF Membrane (Merck; IPVH00010) using a Trans-Blot® Turbo™ Transfer System (Bio-Rad). Membranes were then blacked in methanol and allowed to dry for 1h at RT. After that, membranes were exposed to the relevant primary antibody in PBST (1M PBS, pH 7.4; 0.05% Tween 20) plus 0.3% skimmed milk, recognising the heterologous antigen; they were then incubated for an hour at RT. Membranes were washed with PBST three times at 1 min intervals. Then, they were incubated with appropriate secondary HRP-conjugate antibodies in PBST with 0.3% skimmed milk for 1h at RT. After washing the membranes three times with PBST (3 min intervals), signals were visualised by addition of an Amersham ECL prime Western blotting detection reagent (GE healthcare; RPN2232) and the membranes were developed on Amersham hyper-film ECL (GE healthcare; 28906836).

2.11 Titration of thymine and thymidine (Chapter 3)

To find the lowest concentration of both thymine and thymidine that is needed for the *thyA thyB* mutant growth, PY79 and SH14 (*thyA::cotB-tcdA; thyB::cotC-tcdA*) were grown in SMM supplemented with 0.2% (w/v) CAA and thymine (50 µg/ml) for 8h. Then both PY70 and SH14 were sub-cultured (1:1,000) into different tubes containing fresh SMM supplemented with 0.2% (w/v) CAA and different concentrations of thymine and thymidine (Sigma; T9250). Tubes were incubated O/N at 37ºC stirring. The optical density 600nm (OD_{600}) of the cultures was measured after 16h.

2.12 Well diffusion assay (Chapter 3)

An SMM agar supplemented with 0.2% (w/v) CAA and thymine (50 μg/ml) was prepared and the agar was flooded with the 8h culture of SH14 grown in SMM broth supplemented with 0.2% (w/v) CAA and thymine (50 μg/ml). Wells were made in the agar and into each well, 50 μl of different concentrations (0, 100, 200, 500 μg/ml) of adenosine (Sigma. A9251) was placed. Plates were incubated at 37^{o}C for 48h.

2.13 Thymine starvation (Chapter 3)

An overnight SH14 culture in an SOC2 media supplemented with trimethoprim (6 μg/ml) and thymine (50 μg/ml) was sub-cultured (1:100) into fresh SOC2 (2 X 20ml) supplemented with thymine (50 μg/ml) only and incubated at 37^{o}C. Once the OD_{600} reached from 0.5-0.6, the cultures were spun down (6,000 g, 10 min) and washed three times with sterile PBS (pH 7.4). After washing, pellets were resuspended in the same amount of SOC2 media (20 ml) ± thymine. At different time intervals, 1 ml of each culture was removed, serially diluted and plated on both DSM and DSM supplemented with thymine (50 μg/ml) and trimethoprim (6 μg/ml).

2.14 Stability of double *thy*-insertion (Chapter 3)

A single colony of double *thy* mutant, SH14, was used to inoculate 20 ml of DSM supplemented with thymine (50 μg/ml). After 24h, 1 ml of the culture was removed and plated on both DSM supplemented with thymine (50 μg/ml) and DSM only. The same culture was sub-culture into two fresh DSM (20 ml each) one of which was supplemented with thymine and the other which wasn't. This procedure was continued for five days.

2.15 Germination (Chapter 3)

Purified spores of PY79, SH13, and SH14 were resuspended in distilled water to OD_{600} of 3 before activating them using heat at 70°C for 30 min. Heat activated spores were diluted 10-fold in 10mM Tris-HCl, with a pH of 8.4, to give a final OD_{600} of 0.3. After 15 min incubation at 37°C, germination was initiated by addition of L-alanine (Sigma; 05130) to 10mM. At different time intervals (including time-point 0), 0.1 ml of the spore suspension was transferred to 10 ml of distilled water at 70°C. Spores were incubated at 70°C for 30 min. Spores were then serially diluted and plated on DSM and DSM supplemented with thymine (50 μg/ml) and trimethoprim (6 μg/ml).

2.16 Minimal inhibitory concentration (Chapter 3)

To assess the minimal inhibitory concentration of *Bacillus* strains, the guideline proposed by the EFSA was used (with a slight modification for the double *thy* mutant).

a) Preparation of culture

20 ml of LB in 250 ml of Bellco (for the double *thy* mutant SOC2 was used) was inoculated with a single colony (1-4 days old) and incubated in a water bath shaker at 37°C until the OD_{600} of the culture reached from 0.6-0.8 (mid-log phase).

b) Preparation of antibiotic dilution plates

936μl of Muller-Hinton broth (Oxoid; CM0405) was added into Eppendorf tubes followed by the addition of 64 μl of 1 mg/ml of different antibiotics resulting in a final antibiotic concentration of 64 μg/ml. Next, 300 μl of each antibiotic (64 μl/ml) was added to the first column of wells, for the 96 wells ELISA plate, and two-fold serial dilutions of each tested antibiotic in MH broth were prepared.

c) The minimal inhibitory concentration assay

Once the OD_{600} of the culture reached from 06-0.8, 1 ml of the culture was removed and diluted in pre-warmed Muller-Hinton broth to OD_{600} of 0.15. Finally, 15 μl of the diluted strain culture was added to each well of the ELISA plate described in *panel b* and was incubated O/N at 37°C. After O/N incubation, the OD_{600} of each well, using a microplate reader was measured.

2.17 Growth curves (Chapter 3)

To assess the fitness of the constructed strains, growth curves were plotted for each strain and were compared to the wild-type. Strains were grown overnight and sub-cultured into LB, DSM and SMM with a starting optical density 600nm (OD_{600}) of 0.05 and then grown for 8 hours at 37°C. All media were supplemented with 50 μg/ml of Thymine. OD_{600} was measured every hour.

2.18 Animal experiments

All animal work was performed under the UK Home Office project license PPL 70/8276.

2.18.1 Persistence of spores in GI-tract (Chapter 3)

For this study, Balb/c mice (females, aged 7-8 weeks) were used. Mice (n = 5) were administered a single dose of pure spores (2×10^9) of SH14 or SH250 by oral gavage. At different times thereafter, freshly voided faeces were collected (3-4 pellets) and homogenised and serial dilutions of SH14 were plated on (i) DSM and (iii) DSM + trimethoprim (6 μg/ml) + thymine (50 μg/ml) agar plates. SH250 colonies were plated on

DSM plates containing chloramphenicol (5 μg/ml). Individual SH14 colonies were randomly checked for the presence of *thy*-insertions using PCR.

2.18.2 Immunisation in mice (Chapter 4)

For the immunogenicity studies, pathogen-free mice (C57 BL/6, females, aged 7 weeks) were used. Immune responses to $TcdA_{26\text{-}39}$ in serum and faecal samples were tested as described previously with some modifications (Permpoonpattana *et al.*, 2011a). Groups of pathogen-free mice were dosed orogastrically (0.2 ml) with a dose of 5×10^{10} spores of PY79 or SH14 every 14 days (each group received four doses in total). A naïve group (unimmunised mice) was included in this experiment. On day -1, 34 and 51, blood and faeces were collected from the mice and stored at -80°C. Faeces extraction was done by resuspending (one-fifth w/v) in an extraction buffer (2% fetal calf serum [FCS], Dulbecco's modified Eagle's media [DMEM] plus protease inhibitor cocktails, trypsin [0.1 mg/ml], benzamide [1 μg/ml], phenylmethylsulfonyl fluoride [1 mM], and EDTA [0.05 mg/ml]). The resuspended faeces were gently shaken for 30 min at 4°C to disrupt solid material. Then the samples were centrifuged (13,000 rpm for 15 min) and the supernatants were filtered (0.45-μm pore size) before analysis. The sera were separated from the cells and collected as described earlier (**Section 2.9**). The presence of anti-$TcdA_{26\text{-}39}$ antibodies from faeces and sera were determined by indirect ELISA. 96-wells ELISA plates were coated with $rTcdA_{26\text{-}39}$ (10 mg/ml) in PBS and incubated overnight at 4°C. The next day plates were blocked for 1 h at 37°C with 2% BSA. Next, 2-fold serially diluted samples were added, starting at a dilution of 1/100 for IgG or 1/5 for IgA, in diluent buffer (0.01 M PBS [pH 7.4], 0.5% [w/v] BSA, 5% [v/v] fetal bovine serum [FBS], 0.1% [v/v] Triton X-100, 0.5% [v/v] Tween 20) and incubated for 2h at RT. The preimmune serum was used as a negative control. Horseradish peroxidase-conjugated anti-mouse antibodies in conjugate a buffer (5% FBS

[v/v], 1% BSA [w/v], 0.05% Tween 20 in 0.01 M PBS) was added, and plates were further incubated for 1 h at RT. The plates were then developed using tetramethylbenzidine substrate, and the reactions were stopped using 2 M H_2SO_4. The ODs (optical densities) were read at 450 nm.

2.18.3 Colonisation experiments of different *C. difficile* clades (ID_{50} determination) (Chapter 5)

Groups of 4 C57BL/6 mice received a single dose of clindamycin orally (30 mg/kg) at day 1 and day 3. Mice were kept in IVC cages. At day 8, mice were infected with different amounts of spores ranging from 10^2 to 10^4 per mice. After 24h mice were culled, and the caeca removed aseptically. Caeca were reconstituted in a PBS buffer supplemented with protease inhibitor tablets (Thermo Scientific) using a ratio of 1:5 [weight of faeces (g): volume (ml)]. Caeca were then homogenised and incubated at 4^{o}C for 2h. Finally, the caeca were spun down (10,000 g, 10 min, 4^{o}C) and the supernatants which contained the toxins were removed into fresh tubes. Faeces were resuspended in dH2O, heated at 67^{o}C for 30 min to kill the vegetative cells before they were serially diluted and plated on BHISS. Toxins were extracted by homogenising caecum in extraction buffer as described by Hong *et al.* (2017a) and toxins were detected by ELISA as mentioned in **Section 2.18.2**.

2.19 Conjugation of anti-TcdA antibody to streptavidin, and detecting the conjugated antibodies by ELISA and immunofluorescence (Chapter 4)

Polyclonal antibodies (rabbit; 100 μg) raised to $rTcdA_{26\text{-}39}$ protein were biotinylated using the Lightning-Link Rapid biotin conjugation kit type A (Innova Biosciences). Purified spores (1 X 10^9) of strain PY79 or spores expressing CotB-Streptavidin (SH16) in 200 μl of PBS were mixed with 1 μg of biotinylated antibody and incubated overnight at 4^{o}C. Spores

were then washed four times with PBS (pH 7.4) and suspended in 1 ml of PBS. About 3 X 10^8 of conjugated spores were used to coat microplate wells which were then probed with an anti-rabbit IgG-horseradish conjugate (1:5,000 in PBS plus 0.05% Tween-20) which was used as a secondary with 1h incubation at RT then washed three times followed by undergoing TMB (3,3',5,5'-tetramethylbenzidine) colour development. For immunofluorescence, microscope coverslips were first treated with 0.01% poly-L-lysine overnight. ~5 X 10^6 spores of SH16 or PY79 were added to the microscope slides and allowed to air dry (each microscope slide was placed in a 24 well plate). After three washes with PBS, slides were blocked with PBS containing 2% (w/v) BSA plus 0.05% (v/v) Tween-20 for 45 min at 37ºC. Biotinylated anti-$TcdA_{26\text{-}39}$ antibodies (1:300 dilution. 200µl) were added to slides incubated for 30 min at RT and then washed three times with PBS + 0.05% (v/v) Tween-20. Rabbit FITC serum (Sigma F0382 at 1:200 dilution.) was added and the slides were incubated for 30 min at RT. Image analysis was done using an EVOS fl LED microscope.

2.20 Toxins subtraction assay (Chapter 4)

a) Crude toxin preparation

C. difficile strain R176 (*tcdA*$^+$ *tcdB*$^+$) was grown in TY broth (3% w/v tryptose, 2% w/v yeast extract and 0.1% w/v sodium thioglycolate) for 24h at 37ºC. The cell-free supernatant was filter-sterilised and kept at 4ºC till the assay. The minimum lethal concentration of supernatant required to cause 100% toxicity to HT29 cells was determined using 2-fold dilution and addition of the diluted lysate to HT29 cells using a cell rounding assay (Torres *et al.*, 1992) to determine cell toxicity.

b) Preparation of cells

The cell culture media for HT29 was freshly prepared by mixing McCoy's 5A Modified Media without L-glutamine (Sigma: M8403), 2mM L-glutamine (Sigma: G7513), 1% (v/v) solution of Penicillin-streptomycin (Sigma: P4333) and 10% FBS. A monolayer of HT29 cells in the T75 flask (70-90% confluent) was washed with 10 ml of Dulbecco's phosphate buffered saline (DPBS) (Sigma: D8537). To detach the cells from the flask, 5 ml of Trypsin-EDTA (Sigma: T4049) was added and the cells were incubated for 4 min at 37^{o}C. After incubation, 10 ml of freshly complete McCoy media was added to cells to inactivate the trypsin. The cell suspension was removed from the flask, transferred to a sterile 15 ml conical tube and centrifuged (200 g, 5 min) at RT. Then, the supernatant was removed, and the cells were resuspended in 10 ml of complete McCoy media. Once cells were counted using a haemocytometer, they were seeded (2 X 10^4) in 96-well plates (100µl/ well). The plate was incubated in a humidified chamber (5% CO_2) at 37^{o}C for 24h.

c) The assay

For the assay, 10^9 pure spores of conjugated SH16 spores were added to 200 µl of 2% McCoy's media containing the toxins (typically a 1/4000 dilution). The mixture was incubated for 5 min at RT and then cytotoxicity was assessed using HT29 cells and incubation for 24h. As a control, PY79 spores that had been mixed with $TcdA_{26\text{-}39}$ antibodies (as described for SH16) were used in parallel.

2.21 Enzyme activity (Chapter 4)

Casein degradation activity was determined using the "Universal protease assay", described by Sigma Aldrich, using casein as a substrate. Casein agar was 1% (w/v) casein, 1% (w/v) skimmed milk and 1.2% (w/v) agar technical No. 2, Oxoid. The agar was

supplemented with a cocktail of antibiotic (trimethoprim; 10 μg/ml, Chloramphenicol; 30 μg/ml, Erythromycin; 30 μg/ml). After 24h incubation at 37°C, the plate was flooded with 2 ml of bromocresol green and incubated for 30 min at RT. Amylase activity in the liquid was measured as described (Bernfeld, 1955). Production of active amylase was tested by applying suspensions of spores (volume of 20 μl) to the agar plates carrying only soluble starch (1% w/v) and beef extract (0.3% w/v) and, also, three antibiotics (trimethoprim 10 μg/ml, chloramphenicol 30 μg/ml and erythromycin 30 μg/ml). Antibiotics were used to prevent any bacterial growth on the plates ensuring that activity arose from dormant spores only. Plates were incubated for 48h at 37°C after which the plate was flooded with Lugol solution (Sigma) for 2 min to reveal zones of starch degradation. Units of amylase activity were determined as described by Bernfield (Bernfeld, 1955).

2.22 Crude DNA extraction (Chapter 5)

A two-day-old *C. difficile* colony, grown on BHIS agar, was resuspended in 300μl of nuclease-free water containing 5% Chelex® 100 (Sigma; C7901). The sample was then incubated at 100°C for 20 min. After that, it was spun down (18000 g, 10 min) and the top 50μl, containing the crude DNA was removed into a fresh eppendorf.

2.23 PCR ribotyping (Chapter 5)

This method used here is first described by Bidet (Bidet *et al.*, 1999) with slight modification (Bidet *et al.*, 1999). Two primers that amplify the intergenic spacer region between 16S and 23S of ribosomal RNA were used. Primer sequences were 5'-GTGCGGCTG-GATCACCTCCT-3' (16S primer) and 5'-CCCTGCACCCTTAATAACTTGACC-3' (23S primer). PCR reaction mixes were made in 50μl of volume using GoTaq® G2 Hot Start Polymerase (Promega; M7401). The PCR reaction mix contained 10μl of 5X Colourless

GoTaq® Flexi Buffer, 4µl of $MgCl_2$ (25µM solution), 1µl of PCR Nucleotide Mix (10mM each), 1µl of both up and downstream primers (10mM), 3µl of crude DNA, and 1µl of GoTaq® G2 Hot Start Polymerase. The conditions for amplification were as follows: 1 cycle of 6 min at 94°C, followed by 35 cycles of 1 min at 94°C, 1 min at 57°C and 1 min at 72°C and a final extension of 5 min at 72°C. Then using 3% agarose gel containing 0.1% SYBR safe DNA gel stain (Invitrogen; S33102), the amplified products were fractionated. A gel image was taken using a BioRad Gel Doc system. The gel profiles were then analysed using "Applied math" software and different profiles were sent to Leeds (Reference Laboratory, Leeds General Infirmary) to identify the ribotypes.

2.24 Amplification of *tcdA* and *tcdB* genes (Chapter 5)

A PCR reaction amplifying ~3kbp of *tcdA* using primer forward 5'TTATCAAACATATATTTTAGCCATATATC-3' and primer reverse 5'-TATTGA-TAGCACCTGATTTATATACAAG-3', and ~3kbp of *tcdB* using primer forward 5'-CAGA-TAATGTAGGAAGTAAGTCTATAG-3' and PCR reverse 5'-AGAAAATTTTATGAG-TTTAGTTAATAGAAA-3' was performed. GoTaq® G2 Hot Start Polymerase (Promega; M7401) was used to amplify these regions. PCR reactions were set up like PCR ribotyping reactions. Two-step PCR conditions for both *tcdA* and *tcdB* are summarised in the table below (**Table 2.4**).

Table 2.6: PCR conditions for amplification of *tcdA* and *tcdB*

Steps	*tcdA*	*tcdB*
Initiation	93°C, 3 min	93°C, 3 min
Annealing & extension	35 cycles 47°C, 8 min 93°C, 3 sec	35 cycles 57°C, 8 min 93°C, 3 sec
Final extension	47°C, 10 min	47°C, 10 min

2.25 Identification of different *bclA* genes (Chapter 5)

To identify different types of *bclA* genes, each *bclA1, 2* and *3* has been amplified from crude DNA, using the same polymerase and reaction mix stated in section 2.23. The primers used to amplify these genes and the PCR conditions are summarised in the table below (**Table 2.5**). The amplified products were checked using agarose electrophoresis and DNA sequencing.

2.26 Sporulation of *C. difficile* (Chapter 5)

O/N cultures of different *C. difficile* strains in BHIS were sub-cultured (1 in 1,000) into 20 ml fresh BHIS. After 8h incubation, the cultures were sub-cultured into fresh 20 ml BHIS to ensure no spores were present at time point 0. At different time points, 1 ml of each culture was removed, heated (68ºC, for 30 min), serially diluted and plated on BHIS agar containing 0.1% of sodium taurocholate.

Table 2.7: Primers and PCR conditions for amplification of different *bclA* genes

Genes	Primers sequence	PCR condition	Region amplified
bclA1 (set 1)	5'-AGCTAAGCCAGTCAAGG-3' 5'-CAATTAAGCTGTCTTCTGC-3'	95°C, 5 min 35 cycles 95°C, 30 sec 45°C, 30 sec 72°C, 30 sec 72°C, 5 min	Amplification of 621 bp, also to check the truncation of *bclA1*
bclA1 (set 2)	5'-AAATCTGTTACTGTAGAAA-3' 5'-CAATTAAGCTGTCTTCTGC-3'	95°C, 5 min 35 cycles 95°C, 30 sec 40°C, 30 sec 72°C, 3 min 72°C, 5 min	Amplification of 3189 bp, also to check the deletion of *bclA1*
bclA2	5'AGTGATATTTCAGGTCCAAGTT TATATC-3' 5'TTGTATTCTATAAACTGATACA TATCCAGC-3'	95°C, 5 min 35 cycles 95°C, 30 sec 50°C, 30 sec 72°C, 1 min 40 sec 72°C, 5 min	Amplification of 1671 bp to check the presence of *bclA2*
bclA3	5'-GACCATTTGATGATAATGATTAC-3' 5'- CGCTCCTGTTGGACCTATTAATCC-3'	95°C, 5 min 35 cycles 95°C, 30 sec 43°C, 30 sec 72°C, 40 sec 72°C, 5 min	Amplification of 557 bp to check the presence of the *bclA3*

2.27 Toxin A and toxin B titration

Cell were prepared according to section 2.20b (except where Vero cells DMEM was used). Different strains were grown O/N in 10 ml TY broth (3% w/ v bacto-tryptose, 2% w/v yeast extract and 0.1% w/v thioglycolate, adjusted to pH 7.4) under anaerobic conditions. Before centrifugation and sterilisation, the cell density of different cultures was standardised.

The filtrates were diluted in two-fold series and were added onto monolayers of Vero and HT29 cells. Cytotoxicity was recorded after 24 h.

2.28 Statistical analysis

To compare between the groups, an unpaired *t* test was used. A p value of >0.05 was considered non-significant.

CHAPTER 3

DEVELOPMENT OF A NOVEL SPORE CLONING SYSTEM

3.1 Introduction

Genetic manipulation of bacterial spores of the genus *Bacillus* has shown potential for vaccination and delivery of drugs or enzymes (Potot *et al.*, 2010, Ning *et al.*, 2011, Nguyen *et al.*, 2013). Remarkably, proteins displayed on the spore surface retain activity and generally are not degraded (Ning *et al.*, 2011). Despite the potential utility of *Bacillus* spores for industrial applications, there remain a number of obstacles relating to the deliberate release of genetically modified *Bacillus* into the environment. First, is the use of an antibiotic-resistance gene used in the engineering of stable recombinant strains. The majority of procedures requiring insertion of heterologous DNA into a host require ectopic insertion using an antibiotic-resistance gene for positive selection (Shimotsu and Henner, 1986). Plasmid vectors carrying chloramphenicol-resistance or erythromycin-resistance genes are typically used for ectopic insertion at a genetic locus that is redundant for cell growth, commonly the amylase E (*amyE*) or threonine C (*thrC*) genes (Guérout-Fleury *et al.*, 1996). For instance, pDG364 (6257 bp) is a suicide vector that has been designed to integrate inserts into the *amyE* locus of the *B. subtilis* 168 chromosome (Cutting, 1990). It contains the left and right homology arms for *amyE*, interrupted between the two arms, a multiple-cloning site and a chloramphenicol-resistance gene (**Figure 3.1**). Once the plasmid is linearised, it enables the insertion of cloned DNA via double-crossover, at the *amyE* locus. The *cat* gene

is used as a selection marker. Also, the insertion at the *amyE* locus creates an Amy$^-$ phenotype (Cutting and Vander Horn, 1990).

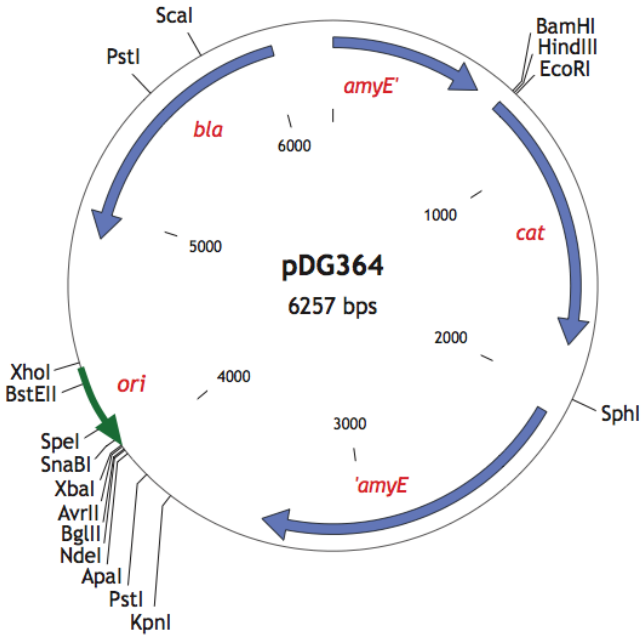

Figure 3.1: Schematic diagram of pDG364. The two *amyE* are the left and right homology arm of complete *amyE* gene. The *cat* (chloramphenicol acetyltransferase) and *bla* (β-lactamase) represent the chloramphenicol and ampicillin resistance genes respectively. The multiple-cloning site consisting of BamHI, EcoRI and HindIII is located in between the left homology arm of *amyE* and the chloramphenicol-resistance gene. *ori* is the origin of replication. The plasmid, in total, is 6257 bp. (Figure taken from *Bacillus* genetics stock centre website, http://www.bgsc.org).

The use of antibiotics in cloning procedures as a selection marker gene can have various consequences such as dissemination in the environment, causing the spread of resistant bacteria and emergence of new resistant or multi-resistant pathogenic bacterial strains (Mignon *et al.*, 2015). The potential risk of antibiotic-resistance gene transfer following release is recognised and so now there are at least two systems in *B. subtilis* that

have been described which enable insertion of heterologous genes without the introduction of an antibiotic-resistance gene (Bloor and Cranenburgh, 2006, Iwanicki *et al.*, 2014).

The second, and most challenging, hurdle is the ultimate fate of recombinant spores; once released as spores, they have been shown to be able to survive indefinitely in the environment (Cano and Borucki, 1995b). The soil is generally enriched with dormant spores (Nicholson *et al.*, 2000) and their robustness makes it difficult to argue that they would not persist after deliberate release. One approach might be to construct germination deficient spores but, at best, the germination rate can be reduced to 0.0015% (Mauriello *et al.*, 2007) which is unlikely to satisfy regulatory authorities. Another approach that could be considered is that of thymine starvation since prokaryotes carrying a mutated thymidylate synthase are unable to grow in low concentrations of thymidine or thymine, resulting in cell death. Thymine, also known as 5-methyluracil, is a pyrimidine, required for DNA synthesis. Since thymine (or thymidine) is not readily found in nature other than in the DNA of living cells, this would result in what is known as a 'thymine-less death' (Goulian *et al.*, 1986, Ahmad *et al.*, 1998). A thymine-less death has been documented for *B. subtilis* (Rolfe, 1967). Two genes of *B. subtilis*, *thyA*, and *thyB* encode a thymidylate synthase enzyme that is responsible for the production of thymine. Mutation of both these genes leads to a strain that lacks thymidylate synthase and as a result, the strain becomes a thymine auxotroph. Also, deactivation of *thyA* and *thyB* will enable the strain to become trimethoprim resistant which can be used as a selective marker.

The antibiotic trimethoprim can disrupt the folate pathway by inhibiting the thedihydrofolate reductase enzyme, which leads to the synthesis of purine and pyrimidine being blocked as well as a block in the synthesis of some key amino acids (**Figure 3.2**)

(Neuhard *et al.*, 1978). If both *thy* genes are mutated by insertion of a chimeric gene that encodes for an antigen fused to the *B. subtilis* spore coat protein such as CotB, then the outcome could be a strain of *B. subtilis* that is thymine auxotroph and displays chimeric protein on its spore surface. Therefore, the principles of a thymineless death to exploit *Bacillus* for the introduction of heterologous genes without the introduction of antibiotic genes can be adopted. If these recombinant spores dessiminate into the environment and germinate, they would exhibit a thymineless death due to rapid cessation of metabolism and fail to survive. The method described in this chapter has been validated using a number of examples and solves a problem arising from the need to contain spore GMOs in the environment.

3.2 Aim

The aim was to develop a cloning system that results in *B. subtilis* recombinant spores expressing heterologous protein on their surface without using an antibiotic-resistance gene as a selection marker. Both the *thyA* and *thyB* genes (encoding for thymidylate synthase required for thymine synthesis) of PY79 will be mutated by inserting heterologous genes fused to genes that encode proteins on the PY79 spore coat, resulting in expression and display chimeric proteins on the surface of the spores. The *thyA thyB* insertion mutant strains become thymine dependant entirely (thymine auxotroph). The resulting recombinant strains will be characterised for their growth in different growth media, sporulation, the stability of the double cross-over and their persistence in the mice GI-tract.

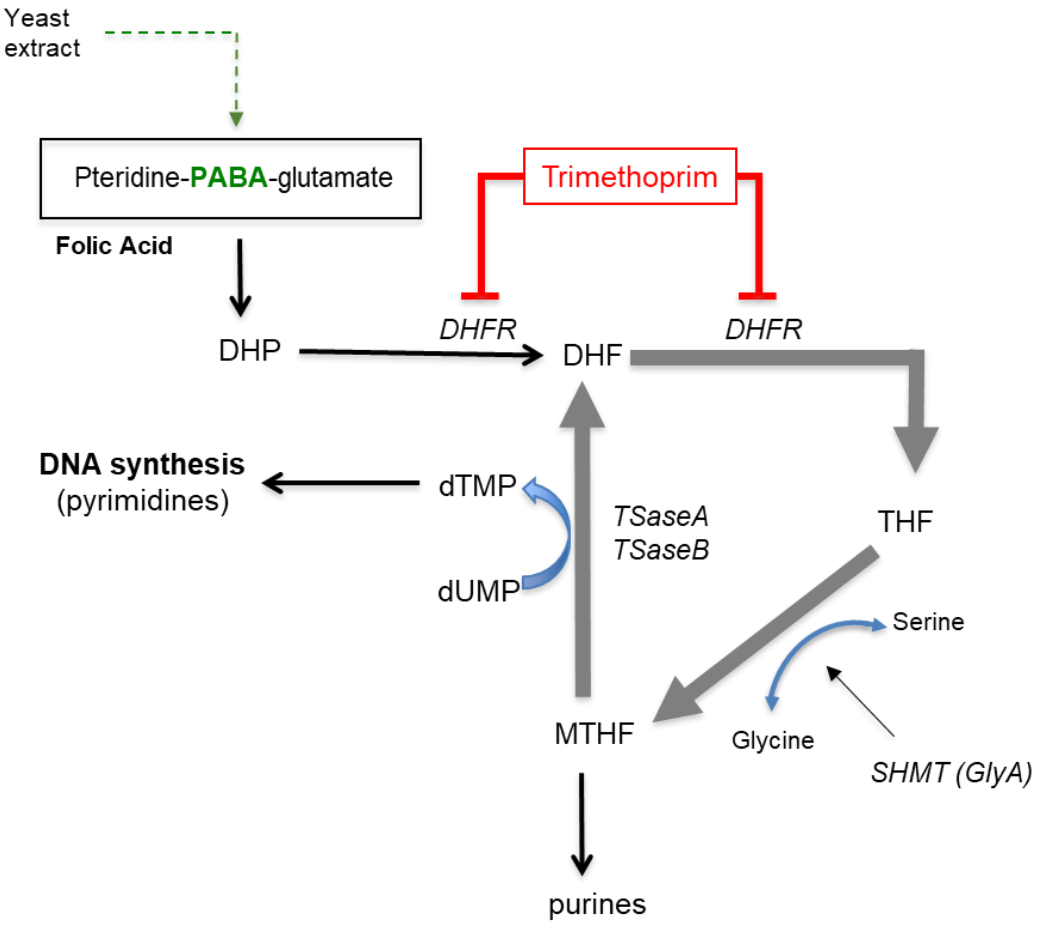

Figure 3.2: The action of trimethoprim on the folate pathway. Trimethoprim inhibits the enzyme dihydrofolate reductase (DHFR) which is required for production of dihydrofolate (DHF) from dihydropteroate (DHP) as well as for synthesis of tetrahydrofolic acid (THF). This prevents synthesis of methylenetetrahydrofolic acid (MTHF) and synthesis of both purines and pyrimidines. In *B. subtilis*, thymidylate synthase A (TSase A) and thymidylate synthase B (TSase B) use MTHF to produce the pyrimidines, thymidine and thymine. Inactivation of both thymidylate synthase enzymes is lethal unless exogenous thymine (or thymidine) is supplied. Also shown is *para*-aminobenzoic acid (PABA) which is a component of folic acid and serine hydroxymethyltransferase (SHMT).

3.3 Results

3.3.1 Construction of *B. subtilis* recombinant strains

To mutate the *thyA* gene by inserting the chimeric gene, purified pThyA plasmids were transformed into competent cells of PY79. The transformants were recognised by their inability to grow at 46^{o}C without thymine supplementation. Typically, transformation frequencies were about 2 x 10^{3}/μg of competent cells with about 15-20% of colonies carrying the correct insertion. After the first mutation, pThyB was introduced into a *thyA* mutant strain via electroporation and trafnsformants were plated on SMM supplemented with CAA, thymine, and trimethoprim, and strains carrying insertions at *thyA* and *thyB* were identified by their failure to grow at both 37^{o}C and 46^{o}C in the absence of thymine. **Figure 3.3** shows the schematic diagram of the two-step, ectopic cloning system.

Using this two-step process, different strains carrying insertions at the *thyA* and *thyB* loci were constructed (**Table 3.1**). These included strains carrying insertions of one or two different proteins on the spore surface by fusion with different spore coat anchors (*cotB* and *cotC*). For each strain constructed in **Table 3.1**, using nucleotide sequence analysis, the integrity of the *thyA* or *thyB* insertion was confirmed.

Neuhard et al. (1978) reported that inactivation of *thy* genes in *B. subtilis* would render the strain resistant to trimethoprim (Neuhard *et al.*, 1978). Thus, the level of resistance of the *thyA* and *thyA thyB* insertional mutants to trimethoprim relative to wild-type PY79 was evaluated (**Table 3.1**). For that purpose, a minimal inhibitory concentrations (MIC) test to determine the lowest amount of trimethoprim needed to inhibit the mutant strains growth was performed. The procedure was performed by a method of serial dilutions of trimethoprim in SOC2 broth supplemented with thymine. As expected, the determined

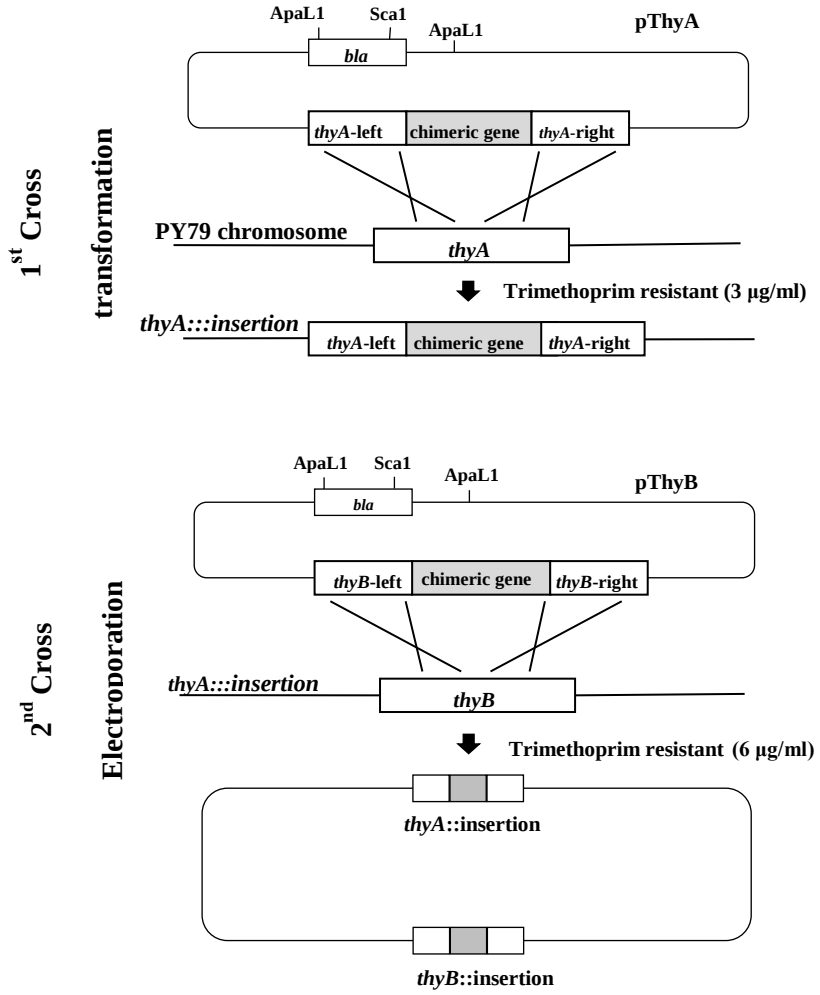

Figure 3.3: Schematic diagram of *thyA thyB* construction. To construct ectopic insertions at the *thyA* and *thyB* loci of *B. subtilis*, two steps are required. In step 1 a pThyA plasmid carrying a chimeric gene is linearised (ApaLI or ScaI digestion) and introduced into cells of a wild-type *B. subtilis* strain (in this case strain PY79) by DNA-mediated transformation. Trimethoprim-resistant transformants are selected on an SMM agar containing trimethoprim (3 µg/ml) and thymine and carry an insertion of homologous *thyA* DNA together with the chimeric gene, by marker replacement, as shown. In the second step, linearised plasmid DNA of a pThyB vector carrying the same or a different chimeric gene is introduced by electroporation into cells of the *thyA* insertion strain created in the 1st step. Selection for trimethoprim resistance is made on the SMM agar supplemented with CAA, trimethoprim (6 µg/ml) and thymine.

Table 3.1: Phenotypes of *B. subtilis* recombinant strains.

Strain	Genotype	37°C[1]		46°C[1]		MIC[2] (μg/ml)
		+ thy	- thy	+ thy	- thy	
PY79	*thyA*$^+$ *thyB*$^+$	+	+	+	+	0.25
SH11	*thyA::cotC-vp26*	+	+	+	-	16
SH12	*thyA::cotC-vp26* *thyB::cotB-vp28*	+	-	+	-	>64
AC01	*thyA::cotB-vp28*	+	+	+	-	16
AC02	*thyA::cotB-vp28* *thyB::cotB-vp28*	+	-	+	-	>64
SH13	*thyA::cotB-tcdA*$_{26-39}$	+	+	+	-	16
SH14	*thyA::cotB-tcdA*$_{26-39}$ *thyB::cotC-tcdA*$_{26-39}$	+	-	+	-	>64
SH15	*thyA::cotB-SA*	+	+	+	-	16
SH16	*thyA::cotB-SA* *thyB::cotB-SA*	+	-	+	-	>64
SH17	*thyA::cotB-amyE*	+	+	+	-	16
SH18	*thyA::cotB-amyE* *thyB::cotB-MCS*[3]	+	-	+	-	>64
SH19	*thyA::cotB-aprE*	+	+	+	-	16
SH20	*thyA::cotB-aprE* *thyB::cotB-MCS*	+	-	+	-	>64

[1] Growth (+) or no growth (-) on SMM agar with or without thymine (50 μg/ml)

[2] Minimal inhibitory concentration (MIC; μg/ml) of trimethoprim determined using a microdilution method

[3] Multiple-cloning site

minimal inhibitory concentration value of trimethoprim for the *thyA* and *thyA thyB* insertional mutants was much higher than the wild-type PY79 with an minimal inhibitory concentration of 16 μg/ml and >64 μg/ml respectively. After the integration of the fusions gene into the *thy* genes was confirmed, the next step was to check if the fusion proteins are expressed and displayed on the spore surface. For that purpose, Western blotting was performed using the recombinant spore coat extracts. When extracted spore coat proteins from purified spores were subjected to Western blotting, immunoreactive bands of the

expected molecular weight of each protein were seen (**Figure 3.4)**. As a further verification, whole-spore ELISA was performed for the confirmation of the presence of chimeric proteins on the spore surface (**Figure 3.5). Figure 3.5A** and **Figure 3.5B** show surface detection of the $TcdA_{26\text{-}39}$ antigen and VP28 respectively by whole-spore ELISA. Production of the $TcdA_{26\text{-}39}$ antigen at both the *thyA* and *thyB* loci (SH14 in **Figure 3.5A**) was higher than the expression at one locus (i.e., *thyA*, SH13). Using polyclonal antibodies to detect VP28, expression levels of strains carrying *cotB-vp28* inserted at *thyA* (AC01) or at both *thyA* and *thyB* (AC02) were examined (**Figure 3.5B**). The ELISA result showed that the expression of the VP28 antigen at both the *thyA* and *thyB* loci was greater than expression at one locus, an indication that having two inserts of the same gene results in higher expression of the protein on the spore surface.

3.3.2 Growth of the *thyA thyB* insertional mutants in different media

a) Growth in minimal media

Both *thy* gene are essential to maintain the folate cycle responsible for synthesis and also critical for cell survival and both pathways involve the synthesis of some key amino acids (Neuhard et al. 1978). Therefore, there is a possibility that mutating one or both *thy* genes would render the growth of these strains in comparison to wild-type, although supplementing the growth media with thymine. For that purpose, the growth of the insertional mutants in a minimal SMM media has been examined. **Figure 3.6** shows examples of strains carrying *cotB-tcdA*$_{26\text{-}39}$ and *cotC-tcdA*$_{26\text{-}39}$ insertion at *thyA* (SH13) or both *thyA* and *thyB* (SH14) loci. The *thyA* insertion strain grew normally with or without thymine supplementation (**Figure 3.6B**) and was indistinguishable from wild-type PY79 growth (**Figure 3.6A**).

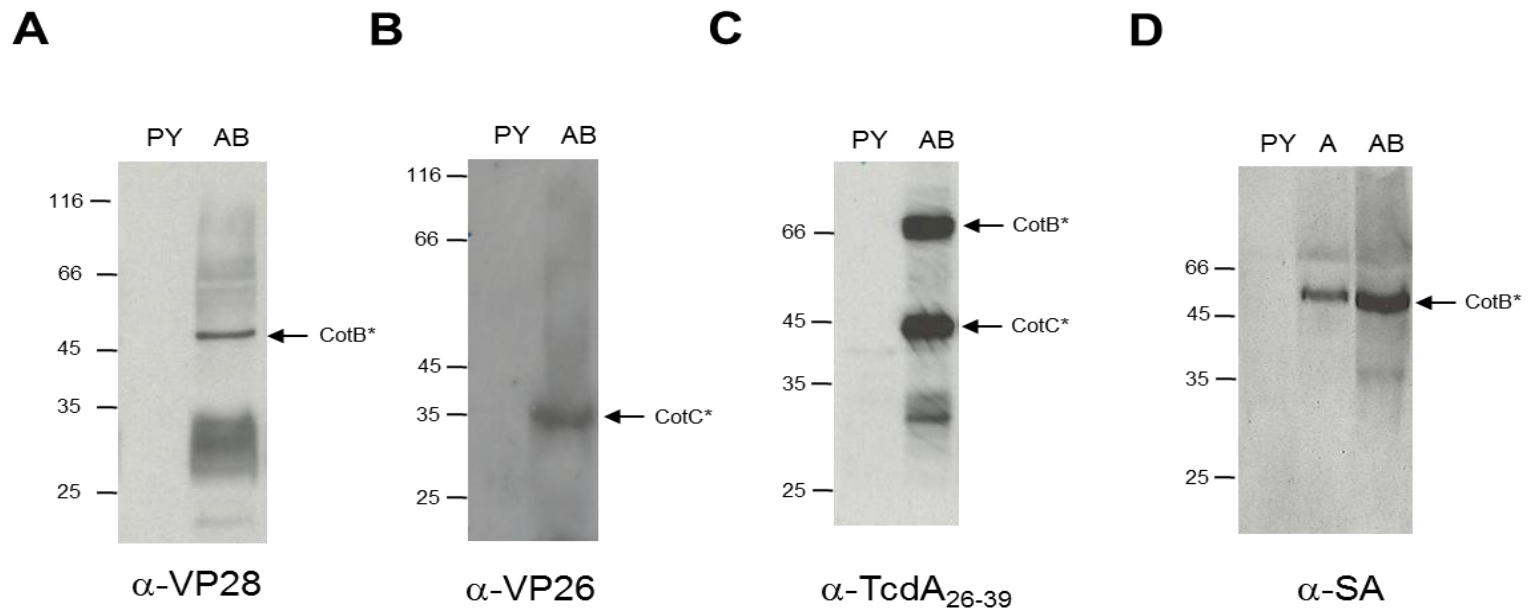

Figure 3.4: Spore coat expression. *B. subtilis* strains carrying insertions at the *thyA* and *thyB* loci were examined by Western blotting of spore coat proteins extracted from preparations of pure spores. Each *panel* shows bands obtained in extracts of wild-type spores (PY79) or spores carrying *thyA* and *thyB* insertions (AB) or in *panel D* spores carrying only a *thyA* insertion (*panel A*). *Panel A* and *B* show analysis of SH12 (*thyA::cotB-vp28 thyB::cotC-vp26)* with anti-VP28 and anti-VP26 antibodies. *Panel C* shows analysis of SH14 (*thyA::cotB-tcdA*$_{26-39}$ *thyB::cotC-tcdA*$_{26-39}$*). Panel D,* shows SH15 (*thyA::cotB-streptavidin (SA)*) and SH16 (*thyA::cotB-SA thyB::cotB-SA).* The size of each band is: CotC-VP26; 31 kDa, CotB-VP28; 63 kDa, CotC-TcdA$_{26-39}$;49 kDa, CotB- TcdA$_{26-39}$; 69 kDa, and CotB-SA; 56 kDa.

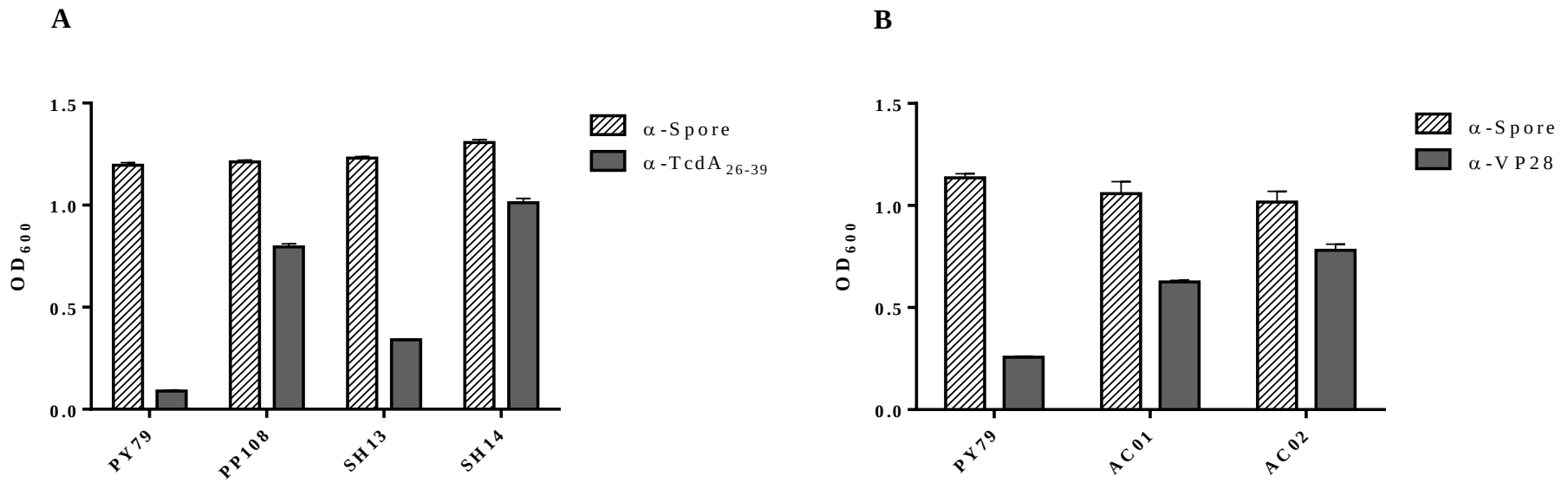

Figure 3.5: Surface expression determined by "Whole Spore ELISA". *Panel A*: Microtiter plates were coated with spores (2 X 10^8/well) of PY79 (*spo*$^+$), PP108 (*amyE::cotC-tcdA*$_{26\text{-}39}$ *thrC::cotB-tcdA*$_{26\text{-}39}$), SH13 (*thyA::cotB-tcdA*$_{26\text{-}39}$) and SH14 (*thyA::cotB-tcdA*$_{26\text{-}39}$*; thyB::cotC-tcdA*$_{26\text{-}39}$) and then probed with either anti-spore (1:1,000) or anti-TcdA$_{26\text{-}39}$ (1:500) rabbit polyclonal antibody. Secondary polyclonal antibodies were 1:5,000 and naive serum was used for comparison, and basal levels were subtracted. *Panel B*: same as for *Panel A* but using spores of PY79, AC01 (*thyA::cotB-vp28*) and AC02 (*thyA::cotB-vp28; thyB::cotB-vp28*) probed with either anti-spore (1:1,000) or anti-VP28 (1:300) rabbit polyclonal antibody. Stripped bars represent the detection of spores and grey bars represent the detection of the chimeric proteins. This experiment was replicated three times.

By contrast, the *thyA thyB* insertion strain was thymine dependent but, in the presence of thymine, had reduced fitness as shown from the lower maximal OD (**Figure 3.6C**). Since the optimal growth of *thyA thyB* insertion mutants by supplementing SMM with thymine was not fully restored, it suggests that other pathway/molecules could have been affected by the absence of a functional thymidylate synthase. Thus, the growth of *thyA thyB* insertional mutants in SMM using additional supplements, to achieve optimal growth, was further investigated.

Mutating the *thy* genes can also result in disruption of glycine and purine synthesis, and since the folate pathway is also connected to the salvage cycle (Ferla and Patrick, 2014), the synthesis of methionine will also be affected (Kwon *et al.*, 2010, Nixon *et al.*, 2014). The purine that is affected by disruption of the folate pathway in *E. coli* was shown to be adenine. It has been previously reported that supplementing the minimal media with CAA and adenine can restore the growth of thymineless *E. coli* to full saturation (Amyes and Smith, 1974). Therefore, the growth of double *thy*-insertional mutants in SMM supplemented with CAA, adenine and thymine with and without trimethoprim was measured (**Figure 3.7**). In the presence of trimethoprim, growth was optimal, reaching a maximal OD_{600} of ~2 in SMM containing either CAA and thymine or CAA, adenine and thymine. The growth was suppressed/slowed in the media supplemented with only thymine and adenine. This indicates that supplementing minimal media with CAA and thymine can fully restore the growth of the *thyA thyB* mutant strains. To further investigate the effect of the double *thy* mutation on amino acid synthesis in the folate and salvage pathway and to check whether by supplementing the depleted amino acids (glycine and methionine) full growth could be restored, the growth of a *thyA thyB* insertion strain (SH14) in an SMM supplemented with thymine and either methionine, serine, glycine or a mixture of these supplements with or without trimethoprim was measured. SH14 in an SMM supplemented with thymine an CAA with or without trimethoprim was used as a control for

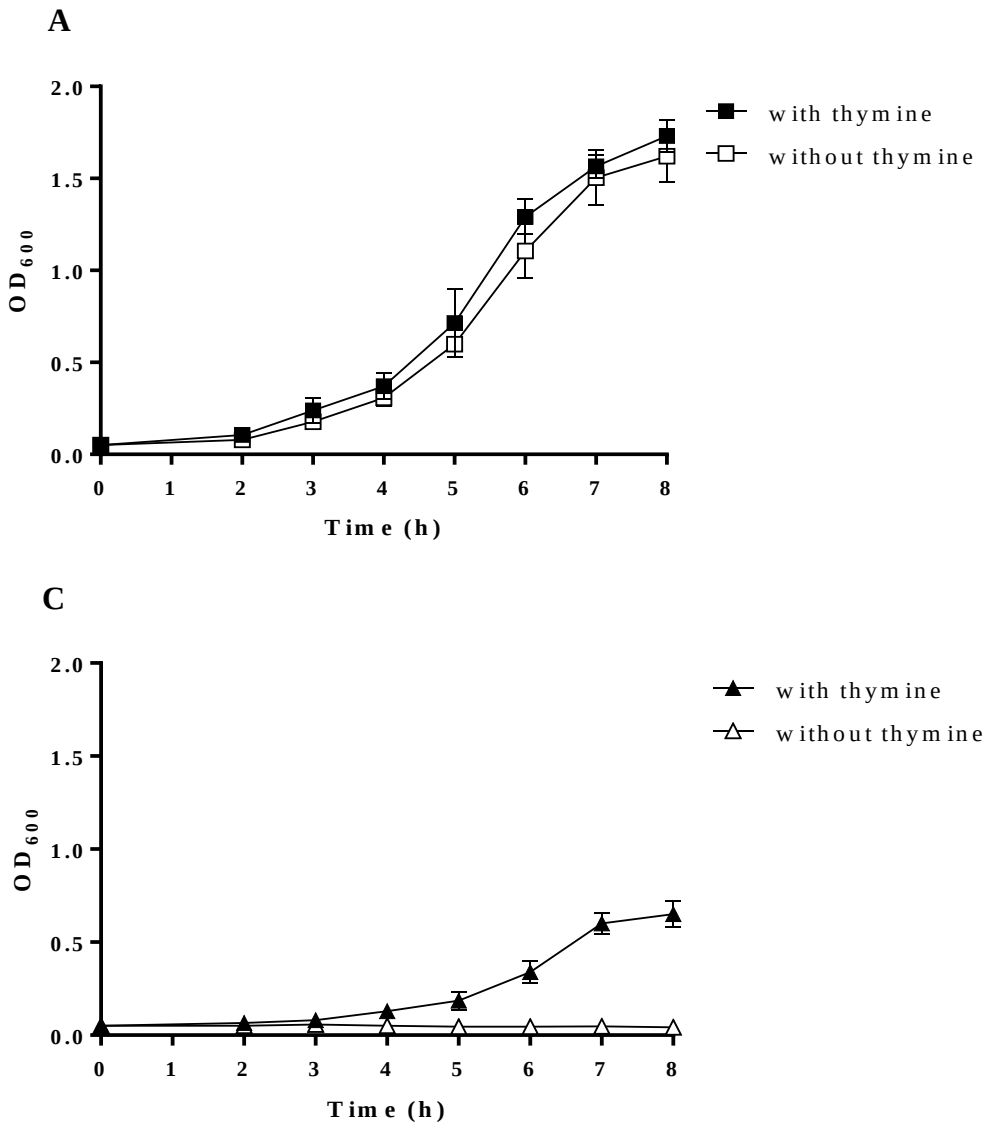

Figure 3.6: Growth of the PY79, *thyA* and *thyA thyB* strains in minimal media. Wild-type PY79 (*panel A*), SH13 *thyA::cotB-tcdA*$_{26\text{-}39}$ (*panel B*) and SH14 *thyA::cotB-tcdA*$_{26\text{-}39}$ *thyB::cotC-tcdA*$_{26\text{-}39}$ (*panel C*) were grown in the SMM media at 37^{o}C with or without thymine supplementation (50 μg/ml). The starting OD_{600} of each culture was 0.05. This experiment was replicated twice.

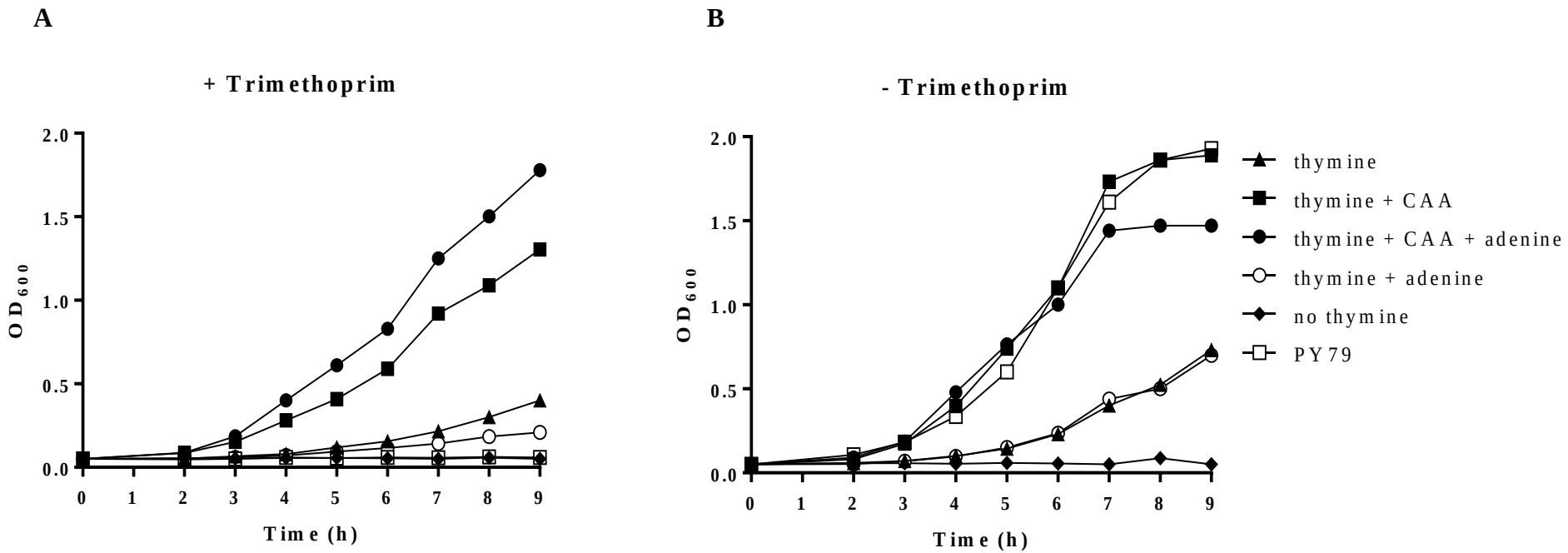

Figure 3.7: Growth of *thyA thyB* mutant in SMM media. Growth at 37ºC of SH14 in an SMM media using different supplements as indicated (thymine 50 µg/ml, 0.2% CAA (w/v), Adenine (20µg/ml)). Media were supplemented with trimethoprim (3µg/ml) in *panel A* and no trimethoprim in *panel B.* As a control *B. subtilis* strain PY79 was also evaluated for growth in SMM with no supplements. ▲: SMM + thymine, ■: SMM + thymine + CAA, ●: SMM + thymine + CAA + adenine, ♦: SMM only, □: PY79 in SMM only, ○: SMM + thymine + adenine. This experiment was replicated twice.

optimum growth. The results indicated that the growth of SH14 in SMM with different supplements other than CAA was the same as SH14 growth which was only supplemented with thymine. This result suggests that the synthesis of other amino acids or molecules required for the optimal growth of the *thyA thyB* might have been affected (**Figure 3.8**).

b) Growth in rich media

The growth of the *thyA* and *thyA thyB* insertional mutant strains at 37^{o}C in an LB and DSM media was assessed, to check if the rich media would affect the growth of the mutant differently than PY79. Without thymine, in both LB and DSM, strains carrying the insertion in both *thyA thyB* showed no growth while the strain carrying the insertion in *thyA* only grew normally (**Figure 3.9B** and **Figure 3.9D**). In the presence of thymine, strains carrying insertions at the *thyA* locus grew normally yet, unexpectedly, strains carrying *thyA thyB* insertions were severely affected. In LB supplemented with thymine, cells of a *thyA thyB* insertion strain showed no growth (**Figure 3.9A**) while in DSM, growth was markedly reduced with a reduced OD (**Figure 3.9C**).

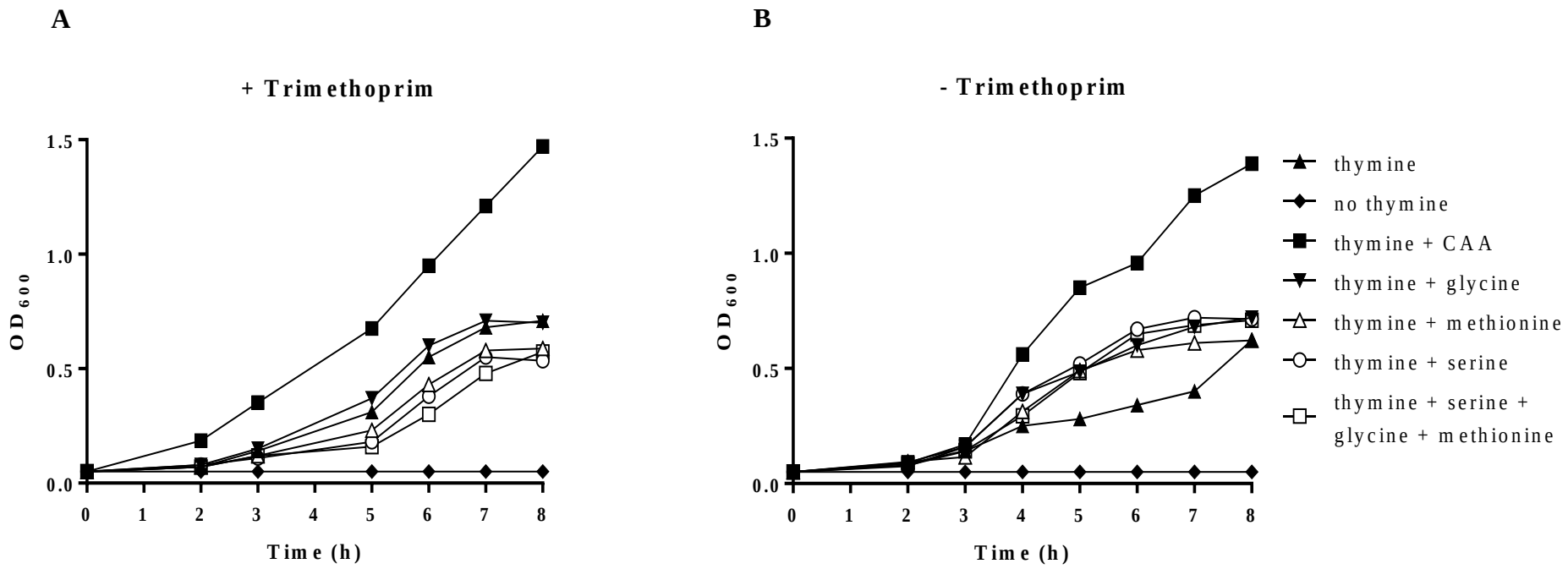

Figure 3.8: Growth of the *thyA thyB* mutant in SMM media with different amino acids. Growth at 37ºC of SH14 in an SMM media with different supplements as indicated (thymine (50 μg/ml), 0.2% CAA (w/v), glycine (50 μg/ml), serine (50 μg/ml) and methionine (50 μg/ml)). Media in *panel A* were supplemented with trimethoprim (6 μg/ml) and *panel B* were without trimethoprim. ▲: SMM + thymine, ■: SMM + thymine + CAA, ▼: SMM + thymine + glycine, Δ: SMM + thymine + methionine, □: SMM + thymine + glycine + methionine + serine, ○: SMM + thymine + serine, ♦: SMM only. This experiment was replicated twice.

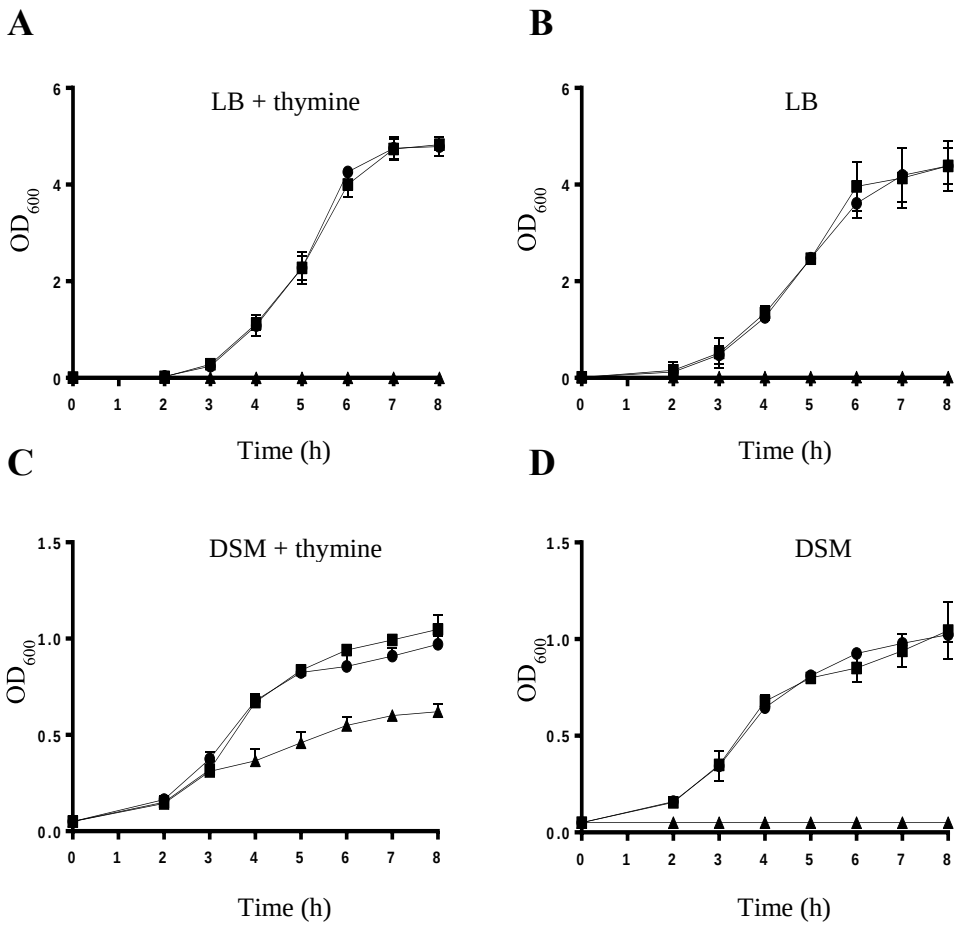

Figure 3.9: Growth of PY79, *thyA* and *thyA thyB* insertion strains in rich media. Growth at 37ºC of PY79 (■), SH13 (●) and SH14 (▲) were measured in: *Panel A*: LB with thymine (50 μg/ml); *panel B*: LB without thymine; *panel C*: DSM with thymine (50 μg/ml) and *panel D*: DSM without thymine. This experiment was replicated twice.

Since the *thyA thyB* insertion mutant strains were not growing in LB supplemented with thymine, it seemed possible that one or more components of LB were inhibiting the growth. Thus, the growth of the strain *thyA thyB* in the LB media supplemented with thymine and with varying levels of yeast extract (YE), a component of LB, was assessed. The optimal growth of the *thyA thyB* was noted in the media containing 0 and 1 mg/ml of yeast extract and yet the growth did decrease in LB containing 2 mg/ml of YE (**Figure 3.10**). The concentration of 3, 4 and 5 mg/ml of yeast extract have completely abolished the growth of

the *thyA thyB* mutant. However, increasing concentration of yeast extract has increased the growth of wild-type PY79. These results indicate that the presence of ≥2 mg/ml of yeast extract in LB inhibited the growth of the strains carrying two *thyA thyB* insertions.

To determine whether tryptone (another component of LB) might have an inhibitory effect on the *thyA thyB*, the use of a growth media other than LB was necessary. Since SOC2 media contain tryptone and no yeast extract, then the growth of the *thyA thyB* mutant with different concentrations of tryptone in SOC2 was measured. Increasing the concentration of tryptone leads to higher growth of the *thyA thyB*, with a similar benefit as for PY79 (**Figure 3.11**). This observation indicates that tryptone had no inhibitory effect on the *thyA thyB* mutant growth.

The inhibitory effect of yeast extract could be due to adenosine, contained in yeast extract, as it has been previously shown that adenosine has a bactericidal effect against thyminless *E. coli* (Kinoshita *et al.*, 1969). Therefore, the effect of adenosine on the *thyA thyB* insertion strain (SH14) was investigated using an agar well diffusion assay (**Figure 3.12**). However, no inhibitory effect caused by the different concentration of adenosine on the *thyA thyB* mutant was observed suggesting that the inhibitory effect is not due to adenosine only. Yeast extract (5 mg/ml) and sterile dH_2O were used as positive and negative control respectively.

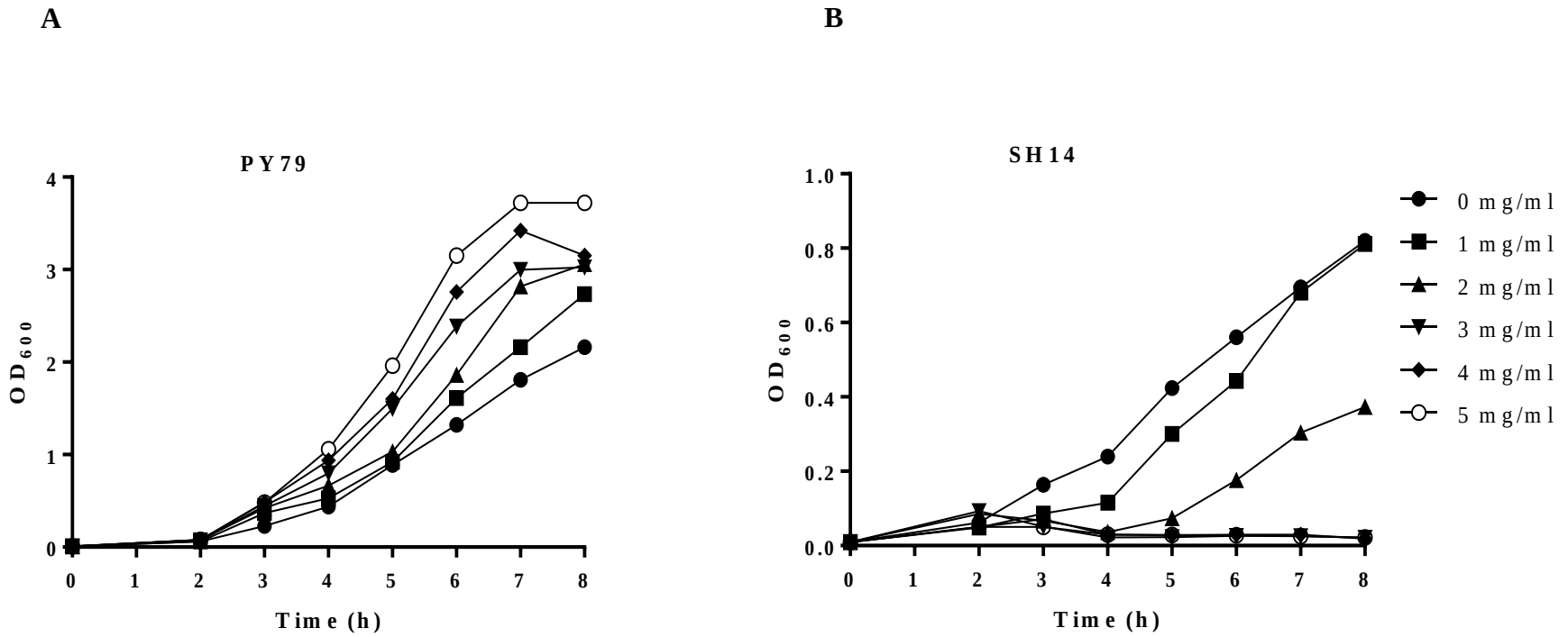

Figure 3.10: Growth of *thyA thyB* insertion strain in rich media with different concentrations of yeast extract. Growth at 37ºC of PY79 (*panel A*), and SH14 (*panel B*) were measured in LB supplemented with thymine (50 μg/ml) with different concentrations of yeast extract, (●) 0 mg/ml, (■) 1 mg/ml, (▲) 2 mg/ml, (▼) 3 mg/ml, (♦) 4 mg/ml and (○) 5 mg/ml. This experiment was replicated twice.

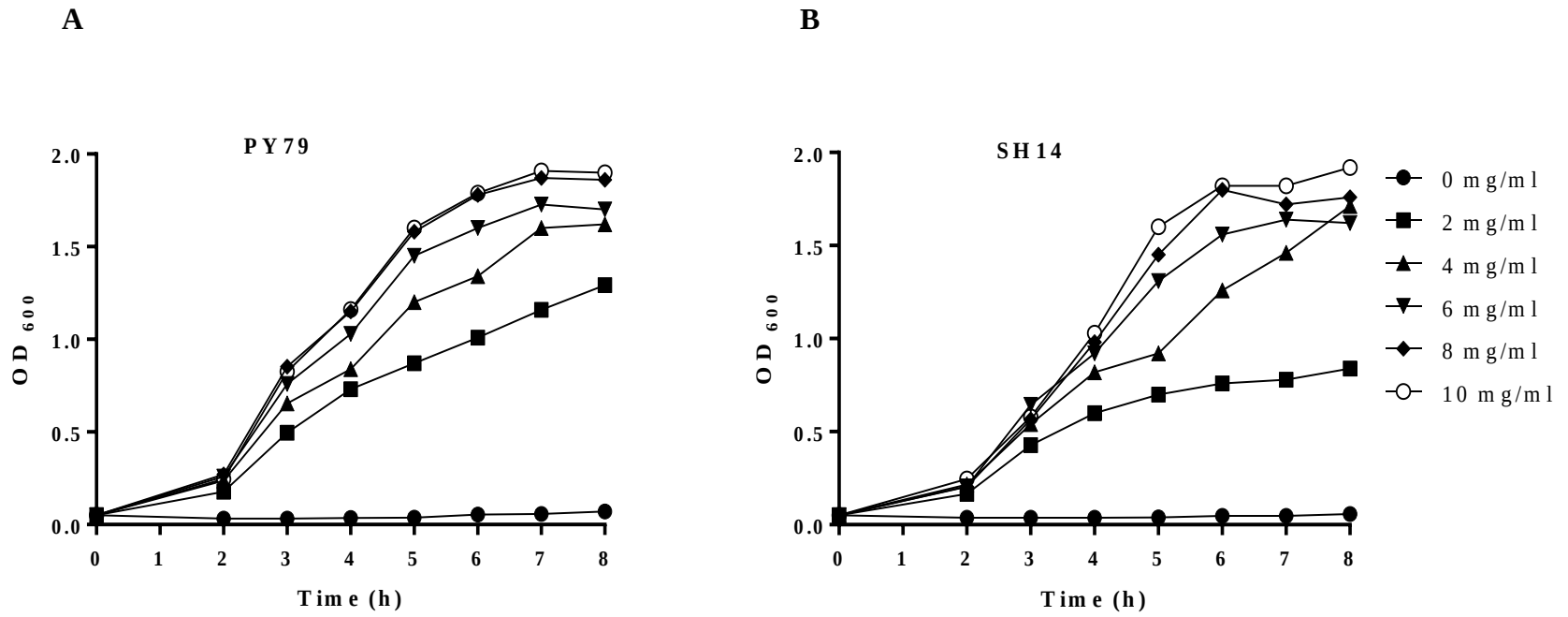

Figure 3.11: Growth of *thyA thyB* insertion strain in rich media with different concentrations of tryptone. Growth at 37ºC of PY79 (*panel A*), and SH14 (*panel B*) were measured in SOC2 supplemented thymine (50 µg/ml) and different concentrations of tryptone, (●) 0 mg/ml, (■) 2 mg/ml, (▲) 4 mg/ml, (▼) 6 mg/ml, (♦) 8 mg/ml and (○) 10 mg/ml. This experiment was replicated twice.

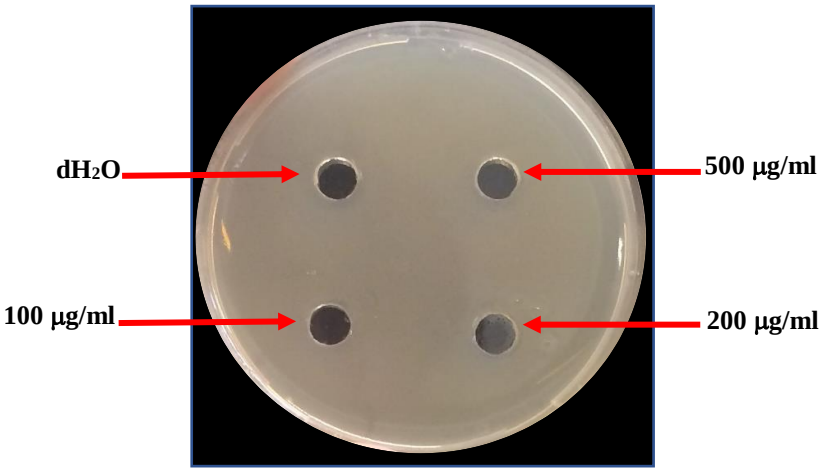

Figure 3.12: Effect of adenosine on the growth of SH14. An SMM agar plate supplemented with thymine and 0.2% (w/v) CAA was flooded with SH14 culture in an SOC2 media and grown for 8h. Wells were made in an SMM agar and into each well, a different concentration of adenosine ranging from 0 to 500 µg/ml was added.

3.3.3 Sporulation of *thyA thyB* mutants

Since one of the goals of the *thy*-insertion cloning system is to produce spores that display chimeric proteins on their spore surfaces, it was important to investigate whether the sporulation of the *thyA thyB* mutants was affected. To check this, sporulation of the *thyA* and *thyA thyB* insertional mutant relative to PY79, in DSM, was measured. In comparison to PY79, the *thyA* and *thyA thyB* mutant had a similar percentage of sporulation, indicating that the mutation of *thyA* and/or *thyA thyB* does not affect sporulation (**Table 3.2**).

3.3.4 Different types of colony after the first genetic cross

Following the first genetic cross (*thyA* insertional mutation), two colony types were apparent on the SMM supplemented with thymine (50 µg/ml) and trimethoprim (3 µg/ml)

Table 3.2: Percentage sporulation of the PY79, *thyA* and *thyA thyB* insertional mutants[1]

Strain	Genotype	Total Count[2]	Heat count[3]	% Sporulation
PY79	*thyA*$^+$ *thyB*$^+$	2.8 x 10^8	2.4 x10^8	85.7
SH11	*thyA::cotC-vp26*	1.9 x 10^8	1.6 x10^8	82
SH12	*thyA::cotC-vp26* *thyB::cotB-vp28*	2 x 10^8	1.5 x 10^8	75
AC01	*thyA::cotB-vp28*	2.6 x 10^8	2.2 x 10^8	85
AC02	*thyA::cotB-vp28* *thyB::cotB-vp28*	1.9 x10^8	1.5 x 10^8	78.6
SH13	*thyA::cotB-tcdA*$_{26-39}$	2.2 x10^8	1.8 x 10^8	85
SH14	*thyA::cotB-tcdA*$_{26-39}$ *thyB::cotC-tcdA*$_{26-39}$	2.5 x10^8	1.9 x10^8	77.2
SH15	*thyA::cotB-SA*	2.8 x 10^8	2.3 x 10^8	82
SH16	*thyA::cotB-SA* *thyB::cotB-SA*	3.1 x 10^8	2.4 x 10^8	77.4
SH17	*thyA::cotB-amyE*	7.4 x 10^8	6.1 x 10^8	82.5
SH18	*thyA::cotB-amyE* *thyB::cotB-MCS*	1.28 x 10^8	9.8 x 10^7	76.5
SH19	*thyA::cotB-aprE*	4.1 x 10^8	3.7 x 10^8	90
SH20	*thyA::cotB-aprE* *thyB::cotB-MCS*	8.1 x 10^7	6.8 x 10^7	83.9

[1] Different strain were grown for 24h in DSM supplemented with thymine (50 μg/ml); after 24h, untreated and heated (65^{o}C, 1h) culture were plated on appropriated plates. This experiment was replicated twice.

[2] Untreated CFU/ml

[3] Heat treated CFU/ml

plates (**Figure 3.13**). Type 1 were large opaque colonies (2-3mm) and carried the correct insertion at the *thyA* locus as confirmed both by PCR and sequencing. Type 2 colonies were translucent, smaller (1mm) and grew slower than type 1. The type 2 colonies, after 96h,

represented approximately 2/3 of all colonies present on the plate. When investigated by PCR, it was confirmed that only type 1 colonies carried the *thyA* insertion. The type 2 colonies, although they showed the correct phynotype as *thyA* insertion mutant, they showed no insert in their *thyA* gene. Therefore, only type 1 colonies were used for the second step.

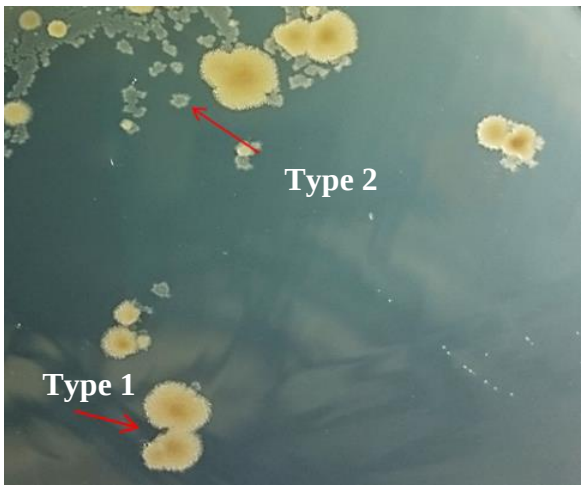

Figure 3.13: Different types of colony after first crossover. The growth of two types of colony on an SMM supplemented with 3μg/ml of trimethoprim and 50 μg/ml of thymine. Type 1 are large opaque colonies, and type 2 are small translucent colonies.

3.3.5 Thymine and thymidine titration

As well as thymine, thymidine could be used for growth of thymineless *E. coli* (Boyle and Jones 1970). To find out the lowest concentration of both thymine and thymidine needed for optimal growth of the *thyA thyB* mutant, an end-point titer test was performed. The growth of PY79 and SH14 in SMM supplemented with 0.2% (w/v) CAA, and various concentrations of thymine and thymidine were evaluated. SH14 in SMM supplemented either with thymine (50 μg/ml) or thymidine (50 μg/ml) was used as a control. Both strains were allowed to grow for 16h at 37ºC in SMM supplemented with CAA and different

concentrations of thymine and thymidine. The results indicated that for optimal growth of SH14, 15 μg/ml of thymine or 20 μg/ml of thymidine was required (**Figure 3.14**).

3.3.6 Elimination of thymine and thymidine from the growth media

Elimination of thymine in minimal media will lead to loss of viability of thyminless *E. coli* (Amyes and Smith, 1974). To test the viability of SH14 after thymine elimination, SH14 was grown to an OD_{600} of 0.5-0.6 in SOC2 supplemented with thymine before eliminating thymine. Thymine was eliminated by centrifugation of the cells and washing the pellet with PBS. At different time points thereafter, 1 ml of cells were removed and plated. The result shows that the CFU count of SH14 after eliminating thymine was significantly reduced compared to the control (+thymine) (**Figure 3.15**). This indicates that elimination of thymine causes a bactericidal effect on the cells of the *thyA thyB* insertional mutant.

3.3.7 Reversion

Although a mutation that occurs by a double cross-over recombinational event is stable there exists the possibility of acquisition of a compensatory suppressor or bypass mutation (Comas *et al.*, 2012). Thus, an experiment to test the stability of the inserts in SH14 was conducted by growing it in DSM supplemented with thymine. Every 24h the growth cultures were sub-cultured into two fresh 20 ml DSM broths, one of which was supplemented with thymine and the other without. At each time point, 1 ml of the culture was removed and plated on a DSM agar with or without thymine. No growth was observed both in the sub-cultures and on DSM plates without thymine supplementation, indicating that the mutations were stable (**Table 3.3**).

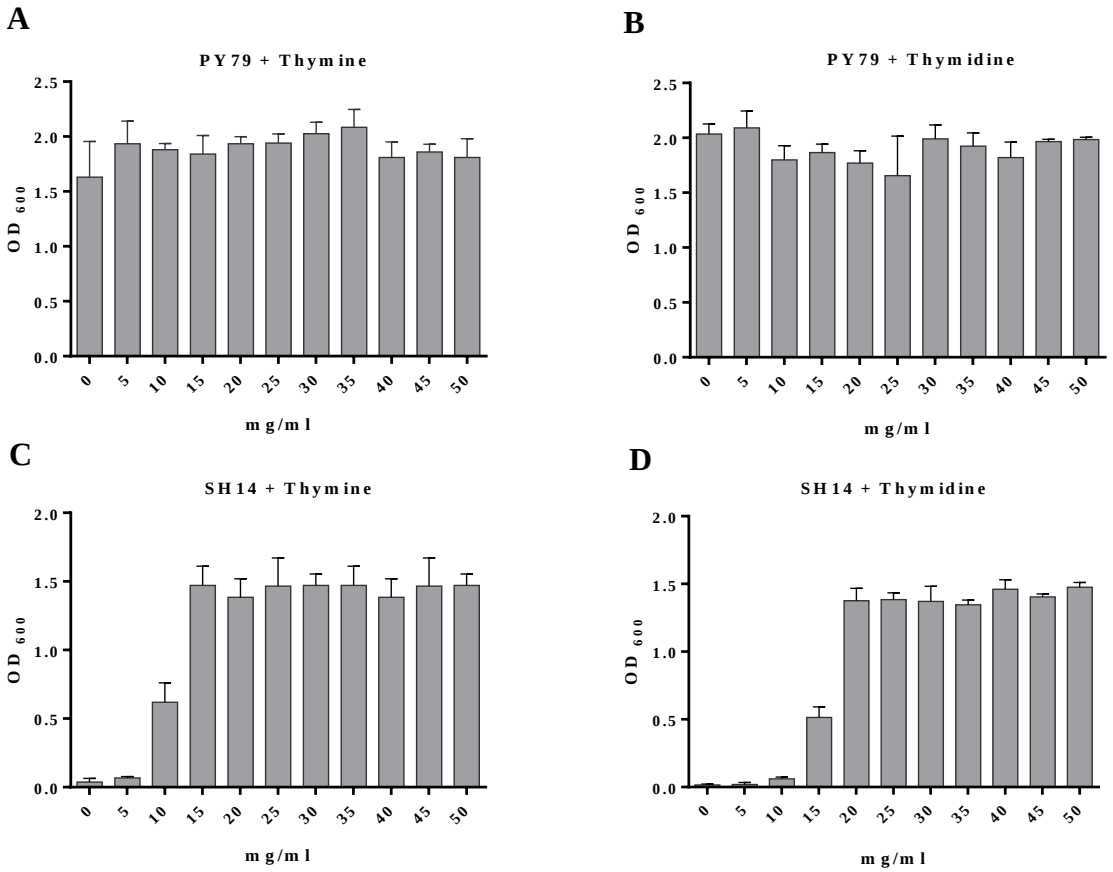

Figure 3.14: Thymine and thymidine titration. *Panel A*, PY79 + thymine; *panel B*, PY79 + thymidine; *panel C*, SH14 + thymine; and *panel D*, SH14 + thymidine. This experiment was replicated three times.

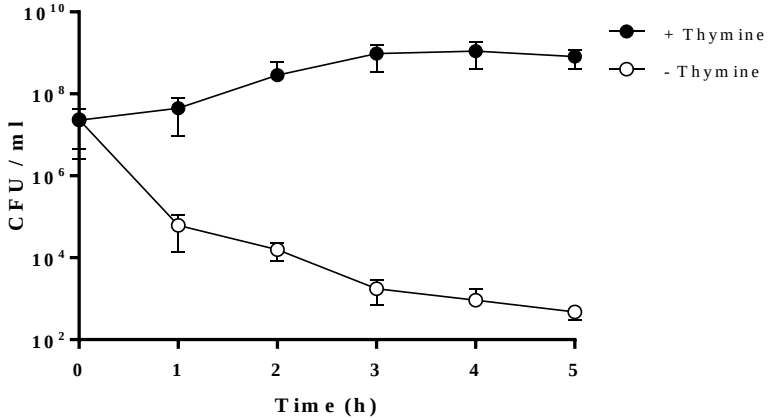

Figure 3.15: Viability of SH14 after thymine elimination. At time point 0 (OD_{600} 0.5-0.6) cells were washed and transferred to a fresh SOC2 media containing no thymine. ●: SOC2 + thymine, and ○: SOC2 only. This experiment was replicated twice.

Table 3.3: Reversion of SH14 upon sub-culture in DSM[1]

Sub-culture	Culture 1 (CFU/ml)		Culture 2 (CFU/ml)	
	DSM + thymine	DSM	DSM + thymine	DSM
1st	2.8×10^8	0	2.1×10^8	0
2nd	1.8×10^8	0	3.4×10^8	0
3rd	1.85×10^8	0	1.73×10^8	0
4th	1.82×10^8	0	1.6×10^8	0
5th	1.54×10^8	0	2.4×10^8	0

[1] Number of bacteria (CFU/ml) every 24h in DSM + thymine and DSM only

3.3.8 *In vitro* germination

Since different proteins (fused to CotB/CotC) will be displayed on the spore surface of PY79 after the double cross-over occurred, it is possible that this might affect germination. Thus, the *thyA* and *thyA thyB* insertional mutants were examined for their germination phenotypes in parallel with the isogenic *spo*$^+$ parent strain, PY79. **Figure 3.16** shows the % decrease of CFU of the PY79, *thyA,* and *thyA thyB* insertion strains after initiation of the germination of purified spores and killing of the germinated spores by heat treatment. This result shows that there was no difference between the mutant strains compared to PY79, which indicates that displaying antigen/proteins on a spore surface would not have any effect on germination.

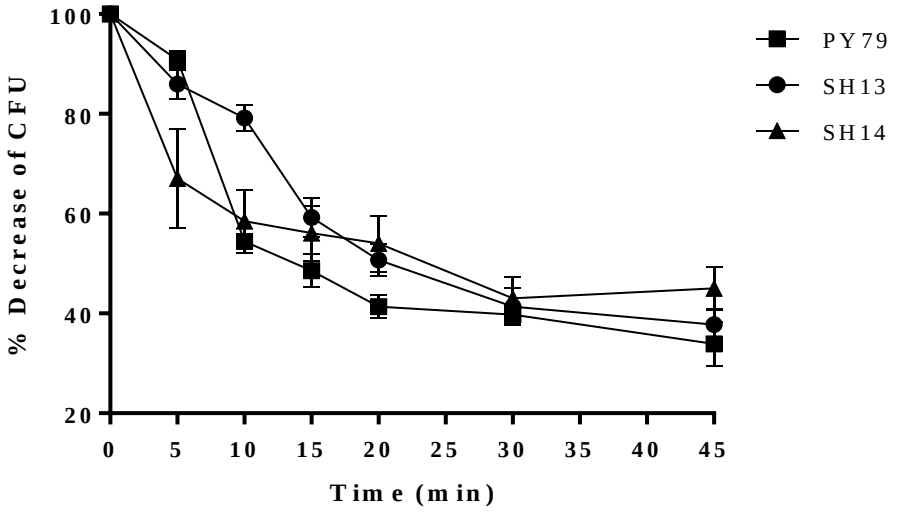

Figure 3.16: Germination of PY79, SH13 and SH14. Percentage decease of CFU was measured at different time points by dividing the CFU of heated spores by the initial volume. ■: PY79, ●: SH13, and ▲: SH14. This experiment was replicated twice.

3.3.9 Persistence of *thyA thyB* mutant spores in GI-tract

The presence of a low concentration of thymidine in the GI-tract of mice might permit limited proliferation of *B. subtilis* carrying the *thyA thyB* mutant alleles. An experiment to address this issue was designed. A single dose of 2 X 10^{10} spores of SH250 (*thyA*$^{+}$ *thyB*$^{+}$ Cm^{R}) or SH14 (*thyA::cotB-tcdA*$_{26\text{-}39}$ *thyA::cotC-tcdA*$_{26\text{-}39}$) spores was given to mice orally. SH250 was made by inserting a chloramphenicol resistance gene into PY79 *amyE* locus. The subsequent shedding of heat-resistant spores in freshly voided faeces was determined. The results showed that, after 240h, the levels of SH250 and spores of SH14 had reached the threshold level of detection (**Figure 3.17**). This result indicates that SH14 spores might colonise in a similar way to SH250 (wild-type) and survive in the GI-tract. To confirm that the spores shed from faeces are PY79 and SH14, PCR was performed on a random selection of 20 colonies at every time point.

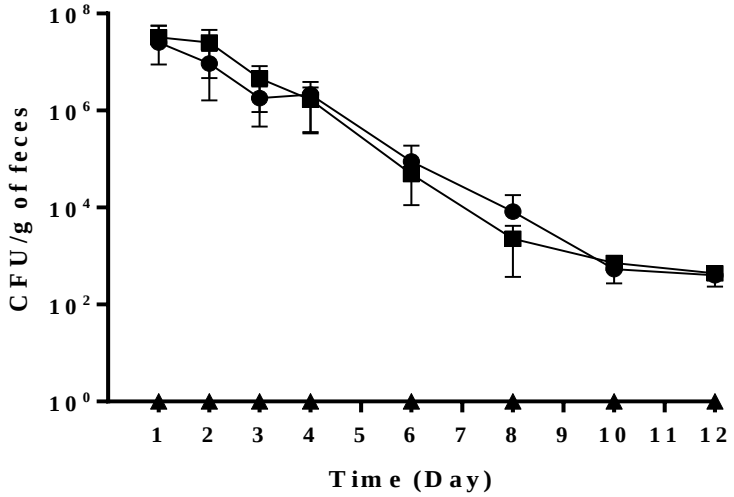

Figure 3.17: Survival of *thyA thyB* insertion mutant in a murine GI-tract. The CFU count of heat treated (65ºC for 1h) spores from mice faeces at different time points. ■: CFU of PY79 plated on DSM supplemented with CAM, ●: CFU of SH14 plated on DSM supplemented with thymine, and ▲: CFU of SH14 plated on DSM only.

3.4 Discussion

This study has provided a new method for making recombinant spores that display proteins on their spore surface without using an antibiotic-resistance gene. Also, should these spores germinate, in environments where thymine (or thymidine) is absent, cells will die since they have a strict dependence on thymine (or thymidine).

3.4.1 Rationale

An absolute requirement for thymine in *B. subtilis* requires two thymidylate synthetases encoded by the unlinked *thyA* and *thyB* genes (Neuhard *et al.*, 1978). These genes are linked to the folate pathway and provide pyrimidines for cell growth. Thymidylate synthase B is thermo-sensitive and retains only ~5-8% activity at a restrictive temperature of 46ºC. Thus, inactivation of the *thyA* locus requires supplementation with thymine (or

thymidine) for growth at 46°C. Thymidylate synthase A is not thermo-sensitive and so inactivation of *thyB* allows cells to grow at an elevated temperature. Inactivation of both *thyA* and *thyB*, however, produces an absolute requirement on thymine for growth at both 37°C and 46°C. As shown by Neuhard (Neuhard *et al.*, 1978) inactivation of *thy* genes produces resistance to the anti-folate drug trimethoprim (or aminopterin) since the need for dihydrofolate reductase, the target for trimethoprim, is dispensed with. However, the level of resistance differs, with insertion at *thyA* producing a lower level of resistance than that found in a *thyA thyB* mutant. This then enables a two-step ectopic cloning system to be considered where, in the first step, a gene is introduced at the *thyA* locus followed, in the second step, by insertion at *thyB.*

To demonstrate proof of concept for ectopic cloning at *thy* loci, a number of heterologous genes whose gene products had previously been expressed on the spore surface were chosen. In each case, the expression had been achieved by chimeric fusion to a *B. subtilis* gene encoding a surface expressed spore coat protein (either CotB or CotC). VP26 and VP28 are capsid proteins of the shrimp white spot syndrome virus and, when displayed on *B. subtilis* spores and incorporated into feed, have been shown to confer protection to shrimps challenged with white spot syndrome virus (Ning *et al.*, 2011, Nguyen *et al.*, 2014, Valdez *et al.*, 2014). $TcdA_{26\text{-}39}$ is a C-terminal domain of *C. difficile* toxin A and when expressed on the spore surface, it has been shown to confer protection from CDI in hamsters (Permpoonpattana *et al.*, 2011a, Hong *et al.*, 2017b). Streptavidin, when expressed on spores, can be conjugated to the monoclonal antibody Cetuximab enabling targeting of colon cancer cells (Nguyen *et al.*, 2013). Finally, the subtilisin E and amylase E enzymes were displayed on the spore surface; they are important enzymes in the industry (Souza, 2010, Li *et al.*, 2013).

The expression of chimeric proteins was confirmed by both Western blotting and ELISA. As expected, expression of the chimeric proteins at both the *thyA* and *thyB* loci (double *thy*-insertion) was greater than expression at one locus. Also the expression of $TcdA_{26\text{-}39}$, in spores of PP108 expressing the same antigen fused with CotB and CotC but inserted at the *thrC* and *amyE* loci respectively, was measured (Permpoonpattana *et al.*, 2011a). Expression levels were somewhat lower, but it should be noted that when using an anti-spore polyclonal antibody to measure levels of spore coat proteins expression levels were correspondingly reduced.

The growth of the *thyA* and *thyA thyB* insertional mutants in minimal media, SMM, was assessed. Surprisingly, the *thyA* insertion strain grew normally with or without thymine and was indistinguishable from wild-type PY79. This illustrates that only the level of expression of thymidylate synthase B via *thyB* is enough for a *thyA* mutant to grow similar to PY79. By contrast, the *thyA thyB* insertion strain was thymine dependent but in the presence of thymine had reduced fitness. Both thymine and thymidine could be used for growth of strains carrying insertions in the *thyA* and *thyB* loci (Boyle and Jones, 1970). However, the concentration of thymine and thymidine needed for the growth of *thyA thyB* were significantly less than the 50 μg/ml reported before (Neuhard *et al.*, 1978) and most probably reflects differences in strain backgrounds used. Cells carrying insertions at the *thyA* and *thyB* loci, although able to grow in media supplemented with thymine, rapidly underwent a massive loss in cell viability when thymine was removed. When growing cells were suspended in a media devoid of thymine, a ~5-log reduction in CFU was observed in just 5h. A similar effect has been shown previously with thymineless *E. coli* (Amyes and Smith, 1974).

3.4.2 Experimental considerations for the use of ectopic insertion at the *thy* loci

a) Growth in rich media

In a rich media, a single *thyA* insertion strain was able to grow without thymine supplementation. Interestingly, the *thyA thyB* mutant failed to restore the normal levels of growth once supplemented with thymine in DSM and limited growth was observed, but in LB it ultimately failed to grow. Since the *thyA thyB* insertion strain could grow, albeit with reduced fitness, in a minimal media supplemented with thymine, a simple explanation for it not to grow in LB or for it to have limited growth in DSM with thymine supplementation is that one or more components of the rich media might inhibit growth potentially by interfering with the folate pathway. A prime candidate was yeast extract that is present in LB and DSM media at 5 mg/ml and 2 mg/ml respectively and absent in an SMM media. Yeast extract has been shown to inhibit thymineless *E. coli* strains where the active bactericidal ingredient has been identified as adenosine (Kinoshita *et al.*, 1969). However different concentrations of adenosine had no inhibitory effect on the growth of the double *thy* mutants meaning that either the inhibitory effect is not due to adenosine or a trace of another element (or elements) from LB together with adenosine is required to inhibit the growth of double *thy*-insertion mutants. Yeast extract concentrations of ≥2 mg/ml inhibited the growth of strains carrying two *thy* (*thyA + thyB*) insertions. Tryptone is also present in LB. However, when the *thyA thyB* mutant strain was grown in SOC2 with a different concentration of tryptone, no inhibitory effect on growth was observed. The inhibitory activity of yeast extract could, therefore, explain why in the LB media the *thyA thyB* double insertion strain failed to grow in the presence of thymine. Similarly, in DSM, where yeast extract is present at a lower concentration, reduced growth was observed.

b) Gene transfer

DNA-mediated transformation of competent cells could be used to introduce plasmid DNA into *B. subtilis* cells. For the introduction of insertions at the *thyB* locus, classical DNA-mediated transformation of competent cells with the pThyB plasmid proved inefficient (with low levels of transformation). Instead, an electroporation method was developed that reliably and reproducibly enabled the introduction of pThyB plasmids at the *thyB* locus in *thyA* strains. An explanation for the low frequency of transformants for the double *thy* mutant by classical DNA-mediated transformation is that the media in which the *thyA* mutant needs to become competent contains yeast extract and, as shown, it can affect the double *thy* mutant's survival. Thus, electroporation using a SOC2 media lacking yeast extract yielded higher frequencies of integration. Following the first genetic cross, two colony types, in equal proportion, were apparent on the SMM agar supplemented with thymine and trimethoprim. Type 1 were large opaque colonies and Type 2 colonies were translucent, smaller and grew slowly. All colonies showed the correct phenotype. When investigated by PCR, about one-third of type 1 colonies were found to carry stable *thyA* insertions while no type 2 colonies carried insertions. One assumption is that these colonies that are able to grow on trimethoprim plates must carry some form of compensatory, yet unstable, mutation/s allowing growth in the presence of the antibiotic. For example, a mutation in *thyA* gene or promotor which could prevent the expression of thymidylate synthase would be an advantage as this allows the colonies to grow in the presence of trimethoprim and the fact that only expression of thymidylate synthase B (*thyB*) is enough for optimal growth.

A second important observation was that, for the second genetic transfer, recombinants could only be selected on SMM minimal media supplemented with thymine, trimethoprim (6 μg/ml) and CAA. If plated directly onto agar lacking CAA, small (<1mm),

slow growing colonies would result, but after reculture, these were found to have lost the *thyB* insertion as determined by colony PCR. Even in the presence of CAA, all colonies were small and only about 20% of colonies that grew on trimethoprim (6 μg/ml) carried a stable *thyB* insertion. Work on *E. coli*, as well as *B. subtilis*, has shown that disruption of the folate pathway can lead to depletion of key amino acids as well as purines and pyrimidines (Amyes and Smith, 1974, Ferla and Patrick, 2014, Stepanek *et al.*, 2016). Trimethoprim-mediated inactivation of thedihydrofolate reductase would deplete intracellular levels of tetrahydrofolic acid, methylenetetrahydrofolic acid as well as dihydrofolate. In turn, this would affect the reversible interconversion of serine and glycine with tetrahydrofolic acid, a vital reaction in the synthesis of purines and catalysed by an serinehydroxymethyltransferase (Ponce-De-Leon and Pizer, 1972, Schirch, 1982). Methyleneterahydrofolic acid is also utilised in the final step of the biosynthetic pathways of cysteine and methionine (Ferla and Patrick, 2014) and disruption of the pathway by the *thyA thyB* alleles could introduce a requirement for methionine.

In thymineless *E. coli*, supplementing CAA and adenine can restore full growth in minimal media in the presence of thymine (Amyes and Smith, 1974, Kwon *et al.*, 2010). Therefore, the ability to grow of the *thyA thyB* mutant in SMM supplemented with CAA and purine (adenine) was assessed. The optimal growth was observed in the SMM containing CAA and thymine, and SMM containing CAA, adenine, and thymine. The growth was markedly reduced in media containing no CAA or carrying only adenine and/or thymine. There was no difference between the growth of *thyA thyB* in SMM containing CAA and thymine with or without adenine. This suggests that supplementing CAA restores the depleted amino acids. Also, supplementing adenine does not have any effect on the growth of the double *thy*-insertion mutant. In the absence of trimethoprim, growth of SH14 remained

weak compared to the wild-type strain PY79 but was i) superior to that in the presence of the antibiotic, ii) growth was restored to normal fitness only in the presence of CAA. Mutating *thy* genes, therefore, disrupt the folate pathway significantly reducing strain fitness and this could not be restored by supplementation with purines or pyrimidines but only with CAA. Since adenine supplementation did not affect the growth of double *thy* mutant strains, then it is possible that supplementing the depleted amino acid by disrupting the folate pathway, as suggested in some publications, would restore the normal level of growth (Amyes and Smith, 1974, Stepanek *et al.*, 2016). In the presence of glycine, methionine, and serine, the normal level of growth wasn't reached suggesting that the production or concentration of other molecules or amino acids might have been affected. Therefore, for construction of strains with double, *thyA thyB,* insertion, in the second step, media such as CAA providing all amino acids are essential. However, once constructed, the use of trimethoprim is no longer required and strains can be cultivated on any media so long as three criteria are met: first, that the media contains thymine or thymidine; second, that yeast extract is absent, and third, that amino acids are provided in the growth media.

c) Choice of one coat protein anchor

For expression of heterologous proteins on the spore surface, the coat proteins CotB and CotC can be used for both mono or divalent expression. Fusion of a chimeric gene to two different spore coat proteins, results in higher display of the chimeric proteins on the spore surface. $TcdA_{26\text{-}39}$ levels were higher in SH14 spores carrying *thyA::cotB-tcdA*$_{26\text{-}39}$ and *thyB::cotC-tcdA*$_{26\text{-}39}$ insertions than in SH13 spores carrying only a *thyA::cotB-tcdA*$_{26\text{-}39}$ insertion. Interestingly, using a fusion of VP28 with CotB and insertion of this chimera at the *thyA* locus alone (strain AC01) or at both the *thyA* and *thyB* loci (strain AC02) lead to higher levels of expression in the latter. This finding requires some consideration since it

must be assumed that each spore would carry a defined number of CotB monomers that could assemble onto the spore surface and simply increasing the number should not lead to higher levels of incorporation in the coat. These strains would, however, carry an intact *cotB* gene (residing at its normal chromosomal locus) so in cells carrying a *thyA::cotB-VP28* insertion (i.e., strain AC01), 50% of displayed CotB proteins should present a wild-type CotB and 50% CotB-VP28. In a double *thyA thyB* insertion strain (AC02) we would predict ~ 66% of displayed CotB proteins would present VP28 and ~33% wild-type CotB. This stoichiometry ratio of CotB-VP28 and CotB would agree with the ELISA detection of VP28. These results raise the possibility that higher surface expression of the proteins fused to CotB or CotC could be achieved by knocking out the wild-type *cotB* or *cotC* genes.

3.4.3 Stability of the *thyA thyB* insertion

An insertion generated by a double-crossover recombinational event should be inherently stable, yet there exists the possibility of acquisition of a compensatory suppressor or bypass mutation. To address this, a straightforward experiment to determine whether, upon repeated culture in the absence of any selective pressure, the thymine dependence could be lost was conducted. This experiment yielded no loss of the thymine dependency showing the insertions were stable and suggesting that the acquisition of compensatory mutations if they were to occur, must be an extremely rare event.

3.4.4 *In vivo* fate of *thyA thyB* insertion mutant spores in the GI-tract

It has been demonstrated that for *E. coli* to colonise the murine GI-tract, synthesis of purines and pyrimidines is necessary (Vogel-Scheel *et al.*, 2010). This implies that the low levels of purines and pyrimidines that might result from digested food or spurious lysis of resident gut microbiota would not be sufficient to permit growth of a *B. subtilis thyA thyB*

mutant. In humans, the intestinal concentration of thymidine is estimated as 0.075 μM and in pigs ~1.0 μM (Steidler *et al.*, 2003).

Spores of SH14 and SH250 (*thyA*$^+$ *thyB*$^+$ Cm^R) were detected in the faeces of mice that were administered with spores orally. The temporal kinetics of shedding was indistinguishable from that of a *spo*$^+$ *thy*$^+$ SH250. After 10 days, the number of SH14 spores being shed in the faeces was (at levels <10^3/g of faeces) at the level of detection. Although it is not possible to determine whether SH14 spores could proliferate in the GI-tract, it was possible to show that in the absence of thymine spores in the faeces could not survive post-germination as no CFU were detected on DSM only.

3.4.5 Comparison of *thy*-insertion cloning system with other existing techniques that do not require an antibiotic-resistance gene

This is not the first report that does not use an antibiotic-resistance gene in the cloning procedures. Iwanicki et al. (2014) designed suicide plasmids without antibiotics resistance genes, and with or without selection markers for ectopic integration which they used to display heterologous proteins on a spore surface (Iwanicki *et al.*, 2014). First, they used plasmids that would integrate into genes such as *thrC*, *trpC*, and *lysA*, disrupting these genes and thus the recombinant strains would be selected on minimal media with and/or without threonine, tryptophan, and lysine respectively. They also introduced a plasmid that harbors *trpC* in addition to fusing a heterologous gene that would be integrated into a non-essential gene, in their experiment *amyE* locus. They introduced this plasmid into a *B. subtilis* 168 laboratory strain that was an auxotroph for tryptophan. Therefore, the prototrophic recombinant strains can be selected by their ability to grow on minimal media without tryptophan supplementation. In another study, a stable gene insertion into the *E. coli*

chromosome was developed by utilising an insertion cassette that contains left and right homologous arms of the chromosomal target locus flaking by *dif* sites, and an chloramphenicol-resistance gene (Bloor and Cranenburgh, 2006). The *dif* site is present in the replication terminus region of the *E. coli* chromosome in which the Xer site-specific recombinase, responsible for resolving a chromosome or plasmid dimer into the monomer, recognises it. This recombination requires XerC and XerD. Once the insertion cassette is introduced into the *E. coli* cell, the right and left homology arm of the inserting cassette target the homologous locus by homologous recombination and the transformants can be recognised by their chloramphenicol-resistance phenotype. Following recombination, the recombinases resolve the two repeats of *dif* sites to one site which results in excising the antibiotic-resistance gene.

Similar to the *thy*-insertion cloning system, the latter techniques also do not require the antibiotic-resistance gene as a selection marker. However, the exciting part of the *thy*-insertion cloning system is that it has a lower chance of survival in the environment should the spores germinate as a result of thymine starvation. The most comparable method to the *thy*-insertion cloning system is the method developed by Iwanicki et al, (2014) in which the recombinant strains become amino acid auxotroph. However, these strains could survive in the environment as many studies have reported that the soil is enriched with amino acids (Sørensen, 1972, Jones *et al.*, 2005, Cao *et al.*, 2016). The *thy*-insertion cloning system could be more favorable for two reasons. First, the recombinant strains, as well as the thymine phenotype, also develop trimethoprim resistance and that makes the selection easier. The second factor is the non-existence of thymine in nature. Does that mean that these strains have absolutely no chance once released into the environment? The answer is no, since there is a possibility that these recombinant spores could be ingested by animals. For example, if

these spores accidentally fall on animal feeds, they can be ingested by animals whereupon they could germinate and replicate in their gut as thymine is present both from the degradation of DNA in the feed such as vegetables or meat, or from the degradation of DNA in lysed microbiota. Thirdly, as well as thymine, these strains also become amino acids auxotroph resulting in further reduction of the ability to survive.

3.5 Conclusion

In conclusion a straightforward method to contain genetically modified bacterial spores was described. This approach follows those described for *Lactobacillus acidophilus* (Fu and Xu, 2000) and *Lactococcus lactis* (Steidler *et al.*, 2003) that rely on the indigenous suicide resulting from a 'thymineless death'. First described in 1954 (Cohen and Barner, 1954), thymine dependence differs from other auxotrophies in that the absence of thymine is bactericidal and so bacteria carrying defects in the thymidylate synthase genes cannot accumulate in the environment. *Bacillus* species carry two thymidylate synthase genes (*thyA* and *thyB*) requiring inactivation of both loci to achieve complete dependence on thymine. A two-step cloning procedure requiring insertional inactivation of first *thyA* and then *thyB* loci that renders recipient cells thymine dependent was demonstrated. This approach does not require the introduction of antibiotic-resistance gene markers for selection but rather the development of increasing levels of resistance to trimethoprim that arise from successive disruption of the folate pathway. Coupled with the temperature sensitive phenotype of the *thyA thyB* recombinants, this method enables both selection and screening of insertions although technically there are a number of constraints that must be considered. It was shown that the absence of thymine is bactericidal and no evidence for reversion or suppression despite the repeated passage of these strains was observed. Of course, the purpose of the *thy* cloning system is to construct *Bacillus* strains able to express proteins for applied purposes,

for example, for expression of heterologous antigens or enzymes. Examples that are used here show that chimeric proteins comprised of a heterologous protein fused to a spore coat protein can be displayed on the spore surface. This has included the delivery of two enzymes (subtilisin E and alpha-amylase), putative vaccine protective antigens as well as streptavidin, although the correct folding of these antigens and enzymes has not been investigated yet. It is clear though that this system could equally be used for expression of proteins in, or secretion from, the vegetative cells.

CHAPTER 4

APPLICATION OF CLONES CONSTRUCTED BY THE *THY*-INSERTION CLONING SYSTEM

4.1 Introduction

Microbial display technology has enabled scientists to express any potential protein on the surface of a microorganism. This technique can be performed in two ways: the first is based on fusing proteins to anchor proteins such as Cot proteins (e.g., CotB) on the spore surface of *B. subtilis*. Microbial display technology is becoming a fundamental tool prevailing over the issues in bioprocesses during harsh industrial processes, environmental protection, and vaccine development (Kim and Schumann, 2009). The typical coat proteins from *B. subtilis* used to display target antigens are CotB, CotC, CotG, and CotZ (Chen *et al.*, 2017). In the second method, proteins can be displayed on the spore surface without the need to fuse them to an anchor protein. The hydrophobic surface layer and negative charge of spores enable the absorption of a protein antigen to its surface. Mucosally immunised mice using appropriate adsorbed spores showed protection and survived when challenged with an anthrax toxin (Huang *et al.*, 2010). However, the adsorption of native proteins to the spore surface can be costly as it requires expression and purification of proteins such as enzymes in their native forms. Immobilisation of proteins on the spore's surface can lead them to be taken orally and thus they can be used as a treatment for diseases (Chen *et al.*, 2017). The ability to express proteins on, especially, *B. subtili*s spores offers a number of industrial

applications. First, and foremost, is their use as mucosal vaccines where antigens can be displayed on the spore surface by fusion to a spore coat protein anchor (Pan *et al.*, 2012). Mucosal vaccination is considered to be a potentially effective treatment or prevention against infections since a large surface of the body is covered by mucosal tissues and many infections are initiated at mucosal sites. Immunisation of recombinant spores by a mucosal route (oral, sub-lingual or nasal) has shown promising results and, in some cases, levels of protection that could be efficacious in humans or animals (Duc *et al.*, 2003, Lee *et al.*, 2003, Permpoonpattana *et al.*, 2011a). In the case of the latter, the ability to incorporate a vaccine in the feed is particularly attractive and, for some animals, arguably, the only way to vaccinate. For example, vaccinating farmed shrimp against the viral pathogen white spot syndrome virus, by recombinant spores expressing the white spot syndrome virus VP28 and VP26 capsid antigens, has been shown to confer protection in them (Fu and Xu, 2000, Ning *et al.*, 2011, Nguyen *et al.*, 2014, Valdez *et al.*, 2014).

A number of *Bacillus* species are considered safe for human consumption and are designated as QPS (Qualified Presumption of Safety as defined by EFSA, European Food Safety Authority) (EFSA 2008) and in the USA some strains carry GRAS (generally regarded as safe as defined by the food and drug administration) status. QPS and GRAS status have supported the use of a number of *Bacillus* strains, including those of *B. subtilis*, as probiotics in both human and animal feeds (Hong *et al.*, 2005). The concept of recombinant probiotics (Amalaradjou and Bhunia, 2013) is, conceptually, a logical next step forward in exploiting the beneficial properties of *Bacillus* (Sorokulova *et al.*, 2008). In addition to vaccines, spores have been shown to facilitate expression of enzymes on the spore surface, for example, the animal feed enzyme phytase (Potot *et al.*, 2010).

The ability to use probiotic bacteria that deliver enzymes negates the need to purify enzymes and could offer significant advantages to industry. Finally, streptavidin, a protein that has a high affinity to biotin through strong non-covalent interaction, has been expressed on *B. subtilis* spores enabling biotinylated monoclonal antibodies to be conjugated to the spore surface to enable the targeting of spores loaded with anti-cancer drugs to cancer cells (Nguyen *et al.*, 2013).

Using a *thy*-insertion cloning system (Chapter 3) several protein antigens and enzymes expressed on a recombinant spore surface were constructed. It is crucial that the proteins expressed on the surface of these recombinant spores are in native form and functional. Therefore, to confirm that the displayed proteins on recombinant spores are active, their functionality needs to be investigated.

4.2 Aim

The aim was to produce and test different recombinant strains that express and display different proteins on their spores for industrial and treatment purposes. Antigen proteins will be expressed on the surface of PY79 spores for the purpose of the mucosal vaccine, and the immunogenicity of the recombinant spores will be tested. Also, streptavidin will be expressed on the PY79 spores where biotinylated antibodies will be conjugated to streptavidin, and this clone will be tested for a strategy to treat CDI. Finally, different enzymes will be expressed on the surface of PY79 spores, and their functionality will be tested.

4.3 Results

4.3.1 Utility of spore display

Using a *thy*-insertion cloning system, different clones encoding different types of proteins were made. Three approaches were used to demonstrate that spores carrying insertions at the *thyA* and *thyB* loci were suitable for applied purposes: 1) as vaccine delivery vehicles, 2) conjugation of proteins/antibodies to the spore surface and 3) to display active enzymes. The functionality and the application of these proteins on the spore surface were investigated.

4.3.2 Vaccine delivery vehicle

Mutating of *thy* genes in *B. subtilis*, PY79, by insertion of chimeric genes allowed the construction of different strains that express chimeric proteins as antigens on their spore surface. Two clones for the purpose of mucosal vaccine have been constructed. The clone that expresses VP26 and VP28 is currently being tested on shrimps in Vietnam. The second clone displays $TcdA_{26\text{-}39}$. The C-terminus of toxin A of *C. difficile* is composed of 38 repeat sequences that encode the receptor-binding domain of TcdA. $TcdA_{26\text{-}39}$ is 14 repeat sequences of 38 repeat of C-terminus of toxin A, also known as 14CDTA, that has been shown to be immunogenic (Ward *et al.*, 1999a). To check whether an expressed antigen on a spore can stimulate an immune response, a clone (SH14 (*thyA::cotB-tcdA*$_{26\text{-}39}$*; thyB::cotC-tcdA*$_{26\text{-}39}$)) that displays $TcdA_{26\text{-}39}$ fused to both CotC and CotB on its spore surface was tested in mice. SH14 is equivalent to strain PP108 that has previously been shown to confer protection to CDI in murine and hamster models of infection (Permpoonpattana *et al.*, 2011a, Hong *et al.*, 2017b). PP108 (*thrC::cotB-TcdA*$_{26\text{-}39}$ *amyE::cotC-TcdA*$_{26\text{-}39}$) is constructed by the insertion of *cotB-TcdA*$_{26\text{-}39}$ and *cotC-TcdA*$_{26\text{-}39}$ into *thrC* and *amyE* genes of PY79 respectively, and using two antibiotic resistance genes (erythromycin and chloramphenicol resistance genes) as selection markers (Permpoonpattana *et al.*, 2011a). Mice were

immunised with spores of SH14, PP108, and PY79 using oral administration. Every batch of spores prepared was confirmed for the presence of $TcdA_{26-39}$ on the recombinant spore surface by Western blotting. After a total of four doses, the titer of $TcdA_{26-39}$-specific IgG (**Figure 4.1A**) and IgA (**Figure 4.1B**) in the serum and faeces respectively were measured using Indirect ELISA (**Figure 4.1**). Compared to control groups (naive and mice dosed with naked PY79 spores) that exhibited no responses, both PP108 and SH14 spores generated high titers of IgG and IgA. This is an indication that SH14 does stimulate an immune response and could be used for preventing the colonisation of *C. difficile* in the gut.

4.3.3 Conjugation of antibody to the spore surface

a) Conjugation of anti-$TcdA_{26-39}$ antibody to SH16

A *Streptavidin* gene was cloned in the PY79 strain and expressed on the spore surface by the *thy*-insertion cloning system. SH16 (*thyA::cotB-SA; thyB:: cotB-SA*) spores were shown, by Western blotting, to express CotB-streptavidin protein on their surface (**Chapter 3 Figure 3.4**). To test the conjugation of the antibody to streptavidin on the spore surface of SH16, first, a rabbit polyclonal $TcdA_{26-39}$-specific antibody was biotinylated. Biotinylated antibodies (anti-$TcdA_{26-39}$) were then incubated with PY79 and SH16 spores for an hour at RT to allow them to bind to streptavidin on the spore surface. Western blotting to confirm that the biotinylated polyclonal antibodies had conjugated to streptavidin on the spore surface was performed. PY79 spores were used as a control. The results indicate that biotinylated anti-$TcdA_{26-39}$ were conjugated to both streptavidin and PY79 spores (**Figure 4.2**). The adsorption of biotinylated antibodies to PY79 spores could be due to a combination of hydrophobic and electrostatic interactions between spores and antibodies (Huang *et al.*, 2010). However, based on the band intensity, more antibodies were bound to SH16 in comparison to PY79.

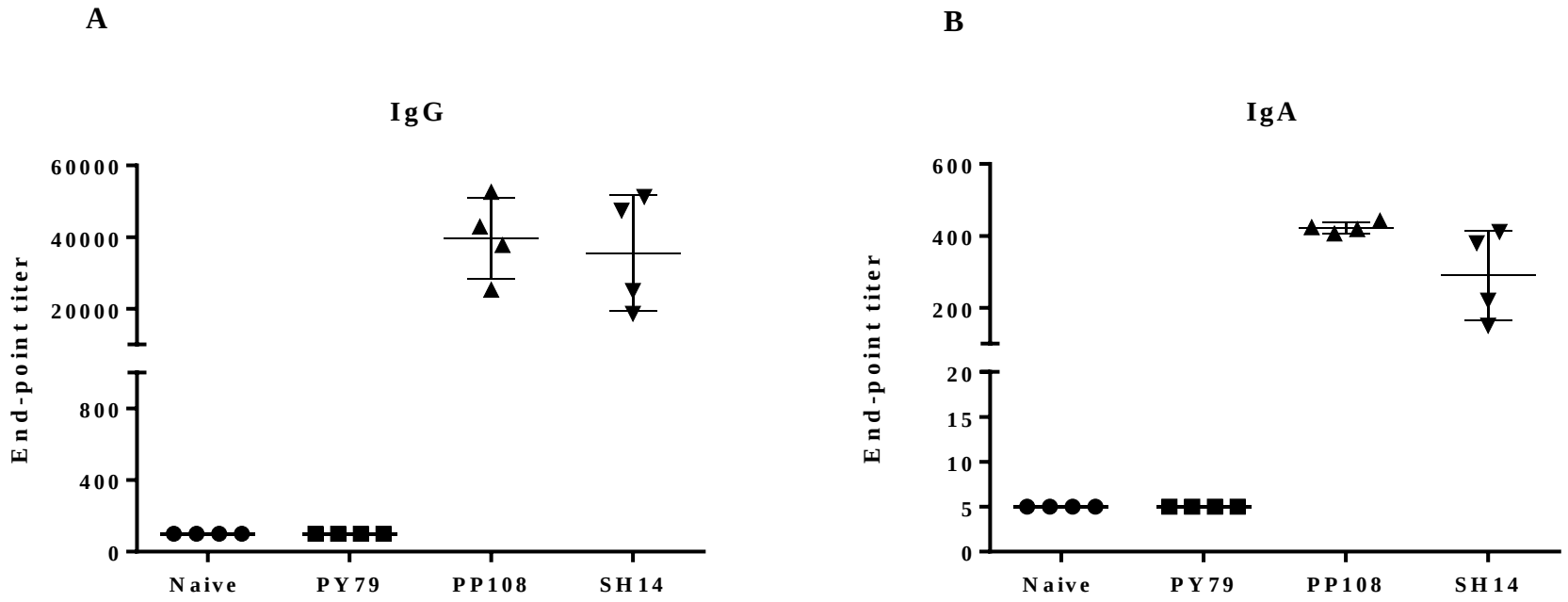

Figure 4.1: Immunogenicity of SH14 spores expressing the *C. difficile* TcdA$_{26\text{-}39}$ antigen. Spores (5 X 10^{10}) of SH14 (*thyA::cotB-tcdA$_{26\text{-}39}$ thyB::cotC-tcdA$_{26\text{-}39}$*), PP108 (*amyE::cotC-tcdA$_{26\text{-}39}$ thrC::cotB-tcdA$_{26\text{-}39}$*) and PY79 (*spo^{+}*) were administered to mice (n=4) by oral gavage on days 1, 14, 35 and 57. Serum IgG and faecal IgA specific to TcdA$_{26\text{-}39}$) was determined by ELISA and endpoint titers are shown. **, $p < 0.005$; ***, $p < 0.0002$. *Panel A* shows the end-point titer of IgG, and *panel B* shows the end-point titer of IgA.

Further verification of an anti-TcdA$_{26-39}$ antibody being displayed on a spore surface was done by both immunofluorescence and whole spore ELISA (**Figure 4.3** and **Figure 4.4**). All these results indicate that biotinylated anti-TcdA$_{26-39}$ can bind to streptavidin and be displayed on a spore surface. Using whole spore ELISA, the expression of CotB- streptavidin in SH16 (double *thy* mutant) was shown to be higher than a single *thy*-insertion mutant SH15 (*thyA::cotB-SA*) (**Figure 4.3**). This indicates that having two insertions of *cotB-streptavidin* results in a higher expression and display of the CotB-streptavidin protein on a spore surface. Moreover, the ELISA result shows that the binding of biotinylated anti-TcdA$_{26-39}$ to PY79 was ~3-fold lower compared to SH15 and ~5-fold lower compared to SH16 (**Figure 4.3**). This again indicates that a low level of antibody does bind to PY79 spores; however, the presence of streptavidin on a spore surface and incubating these spores with biotinylated antibodies results in much higher conjugation. Using immunofluorescence microscopy, further verification of the display of biotinylated antibodies on the spore surface of SH16 was done. The immunofluorescence image confirms the presence of biotinylated antibodies on the spores' surface, and also that only a low level of antibody binds to the PY79 spore (**Figure 4.4B**).

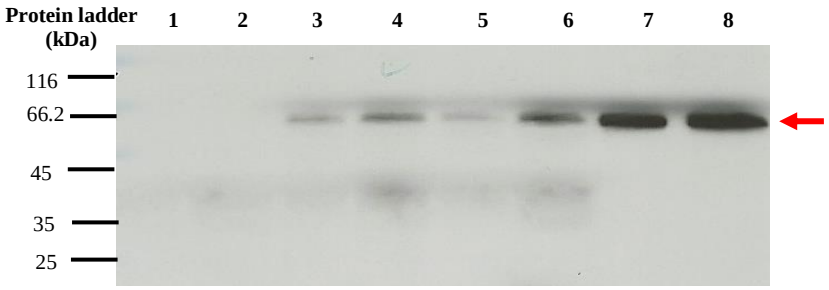

Figure 4.2: Examining the conjugation of anti-TcdA$_{26-39}$ antibody to PY79 and SH16 by Western blotting. Western blotting of the spore coat proteins extracted was preformed from preparations of pure spores and spores conjugated with an anti-TcdA$_{26-39}$ antibody, using an anti-rabbit IgG antibody (1/3,000 dilution). Bands obtained are indicated by a red arrow. *Lanes 1* and *2* are naked PY79 and SH16 spore extracts respectively. *Lanes 3* and *5* are two different batches of PY79 spores incubated with biotinylated anti-TcdA$_{26-39}$. *Lanes 4* and *6* are two different batches of spores of SH16 incubated with biotinylated anti-TcdA$_{26-39}$, and *lanes 7* and *8* are non-biotinylated and biotinylated anti-TcdA$_{26-39}$ antibodies respectively used as controls. The protein loaded per well corresponded to an extraction from 2 X 10^8 spores.

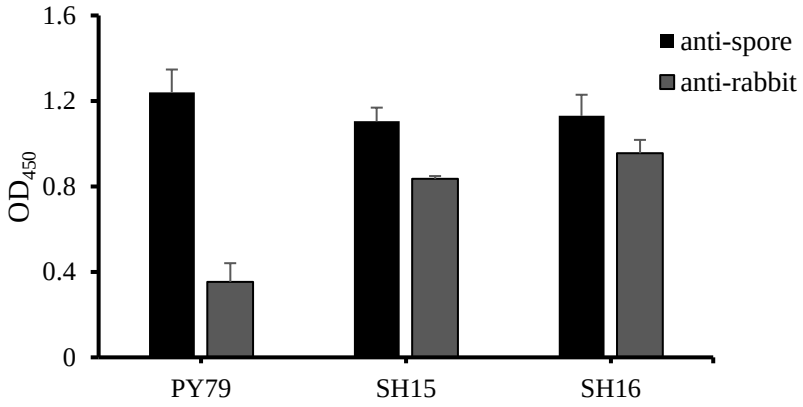

Figure 4.3: Spore surface conjugation determined by "Whole Spore ELISA". Microtiter plates were coated with spores (3 X 10^8/well) of PY79, SH15 (*thyA::cotB-SA*), and SH16 (*thyA::cotB-SA; thyB::cotB-SA*) conjugated with biotinylated TcdA$_{26-39}$ and then probed with anti-rabbit polyclonal antibody (1:6,000 dilution). Naive serum was used for comparison, and basal levels were subtracted. Primary polyclonal anti-spore antibody from a rabbit was used (1:1,000 dilution), to detect the spores, followed by secondary polyclonal anti-rabbit antibody (1:6,000 dilution). This experiment was replicated three times.

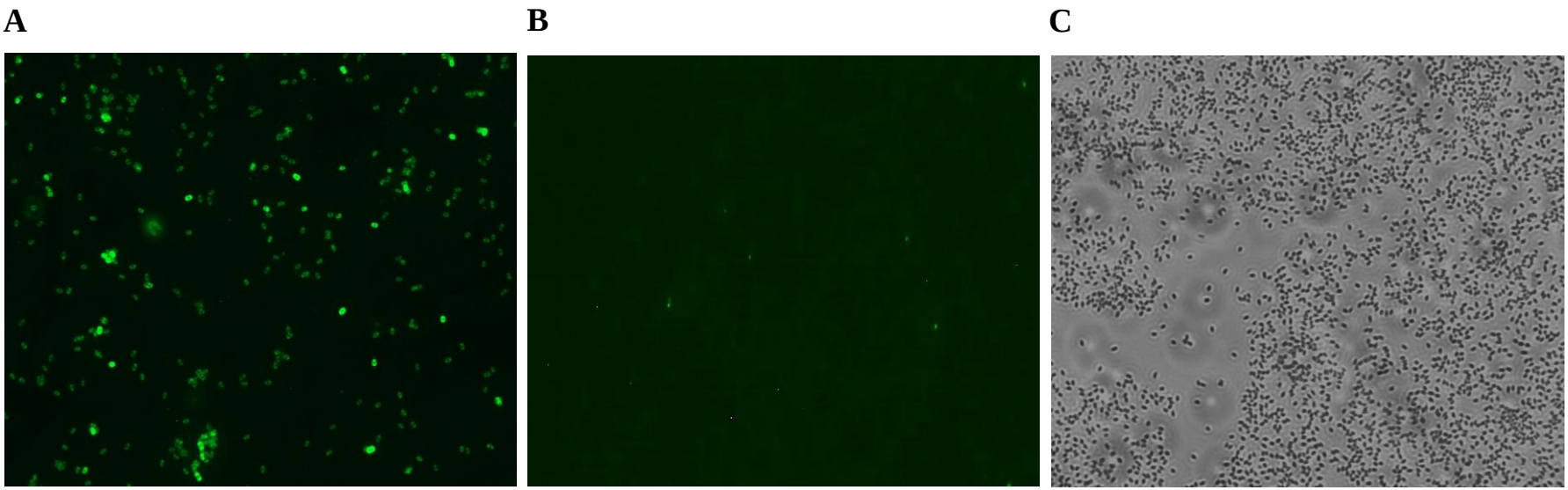

Figure 4.4: Surface display of anti-TcdA$_{26\text{-}39}$ using immunofluorescence imaging of suspensions of SH16 and PY79. The visualisation of conjugation of anti-TcdA$_{26\text{-}39}$ antibodies to SH16 spores using immunofluorescence (*panel A*). As a control (*panel B*) PY79 lacking streptavidin failed to conjugate (although a low level of antibody can still bind to it). *Panel C* is the phase contrast image confirming the presence of PY79 spores.

b) Subtraction of toxin A from C. difficile toxin sample

Both toxin A and toxin B from *C. difficile* are responsible for CDI. These toxins can damage the intestinal epithelium and result in inflammation and diarrhea in an infected person. Subtraction and inactivation of these toxins will prevent CDI (Kuehne *et al.*, 2010). Thus, whether SH16 spores displaying $TcdA_{26\text{-}39}$ IgG could subtract *C. difficile* toxins from a crude cell-free lysate was investigated. To do that, the crude toxin of *C. difficile* R176 (a hypervirulent strain) was mixed with SH16 spores conjugated with anti-$TcdA_{26\text{-}39}$ incubated, and then spores were spun down, and the supernatant was sterilised, after which it was added to HT29 cells. Cytotoxicity was confirmed by examining the HT29 cells after a 24h period under a light microscope. A round morphology indicated the toxicity effect of toxin A on cells (**Figure 4.5**). As shown in **Table 4.1,** incubation of conjugated spores with toxin-containing supernatant for just 5 min reduced toxicity by 90%. PY79 spores also had some ability to bind $TcdA_{26\text{-}39}$ antibodies and were able to provide a modest reduction (10-20%) in toxin activity.

Table 4.1: Determination of reduction in toxicity [1]

Samples	Cytotoxicity (%)
Media only	0
Crude toxin	100
Crude toxin incubated with PY79	80-90
Crude toxin incubated with SH16 conjugates	10

[1] Absorption of toxin to spores was measured on HT29 cells. This experiment was replicated twice.

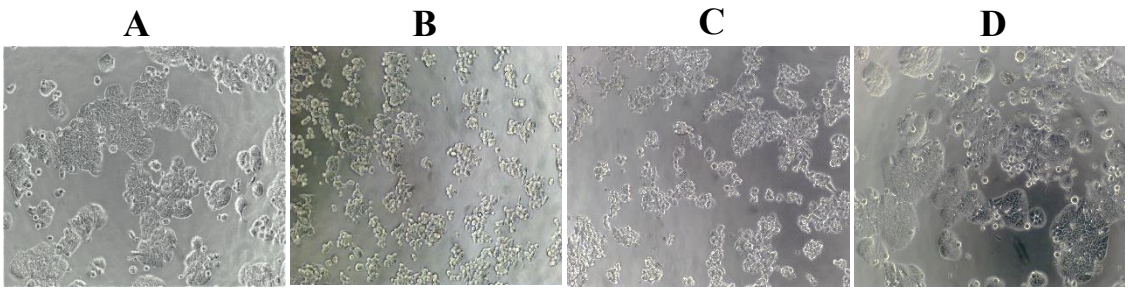

Figure 4.5: HT29 morphology treated with crude toxin. A round morphology indicated the susceptibility of the cells to toxin. *Panel A* shows the untreated HT29 cells; *panel B*, HT29 treated with crude toxin; *panel C*, HT29 cells treated with crude toxin incubated with PY79 spores; *panel D*, HT29 cells treated with crude toxin after 5 min incubated with SH16 conjugates. Images were taken after 24h incubation. This experiment was replicated twice.

4.3.4 Display of active enzymes

Enzymes are commonly included in animal feeds where they improve digestion and nutrition. Proteases and amylases are pertinent examples used here to show that it is possible to display an enzyme that retains activity on the spore surface. Two clones, by inserting chimeric genes into the *thy* genes, were made that express enzymes on their recombinant spore surface. SH18 (*thyA::cotB-amyE; thyB::MSC*) expresses amylase enzyme from *B. amyloliquefaciens*, and SH20 (*thyA::cotB-aprE; thyB::MSC*) expresses subtilisin E enzyme from *B. subtilis* on the spore surface. Both clones only have one copy of the enzyme gene inserted into *thyA* and have the MCS inserted into *thyB*. Insertion of MCS (~60 bp) has disrupted the reading frame and, as a result, stopped the expression of thymidylate synthase, making the strain thymine dependant. It is important that the enzymes fused to CotB have correctly folded on the spore surface to be functional. Therefore, the activity of expressed enzymes on recombinant spores of both clones was tested.

a) Subtilisin E

Subtilisin is a non-specific serine protease that breaks down proteins by cleaving the amide (peptide) bond through a serine residue and the active site. It has many applications such as use in detergents and food processing (Valls *et al.*, 2011, Li *et al.*, 2013). Subtilisin can also break down casein (Rival *et al.*, 2001). To test the functionality of protease enzyme (subtilisin E) displayed on the spores' surface, a simple experiment that proves the proteolytic activity of the enzymes by digesting hide powder remazol blue was performed. Hide powder azure (a blue fibrous substrate) is coupled with a dye remazol brilliant blue. Degrading hide powder azure by proteases will result in releasing dye-bound peptides into a solution which can be measured (Yadav *et al.*, 2010). Naked PY79 spores were used as a control. Using this assay, the proteolytic activity of SH20 spores displaying subtilisin E on their spore surface by digesting the hide powder remazol blue was observed (dark blue colour OD_{600} ~0.5), whereas PY79 had no such effect (OD_{600} ~0.02) (**Figure 4.6**). This indicated that protease enzyme (subtilisin E) displayed on the spore surface is functional.

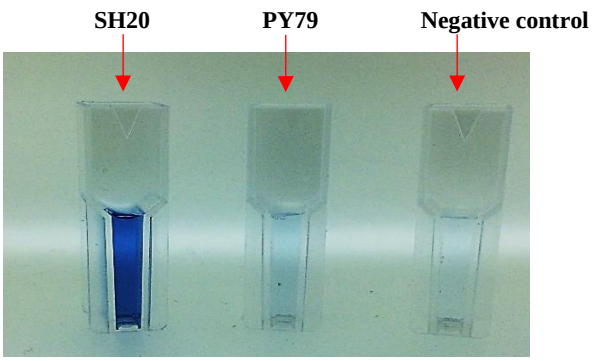

Figure 4.6: Determination of protease activity of PY79 and SH20. Digestion of hide powder remazol blue of PY79 and SH20 spores. Digestion of remazol results in a darker blue colour. SH20 spores showed that they exhibit the protease activity and they resulted in higher blue colour intensity than the negative control.

To verify that the subtilisin enzymes displayed on spores can degrade casein, ~5 X 10^8 spores were spotted on a casein agar supplemented with an antibiotic cocktail (trimethoprim, chloramphenicol, erythromycin) and incubated. PY79 and protease from *Streptomyces griseus* were both used as negative and positive controls respectively. As shown in **Figure 4.7**, spores from SH20 can digest casein whereas the PY79 spores (wild-type) cannot. This, again, indicated that the subtilisin E enzymes displayed on the spore surface are functional and exhibit the casein digestion activity.

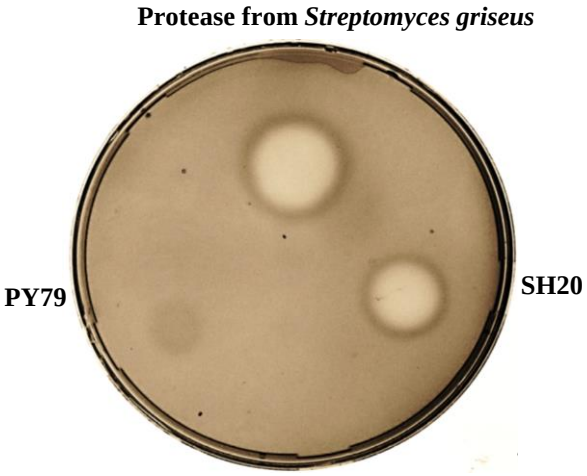

Figure 4.7: Degradation of casein by SH20 spores. Purified spores of PY79 and SH20 were spotted (20 μl) on the casein agar. The zones of inhibition were determined by staining the agar with bromocresol green and incubating. PY79 spores were used as negative control that exhibited no casein digestion activity. Protease from *Streptomyces griseus* was used as a positive control. Both SH20 spores and protease from *Streptomyces griseus* showed the zone degradation. PY79 didn't show any activity for casein degradation.

To determine the unit of activity of casein digestion of SH20 spores, the method described by Folin and Ciocalteu (Folin and Ciocalteu, 1927) was used. Proteases break

down casein into tyrosine, other amino acids, and peptide fragments. The free tyrosine can react with Folin and Ciocalteus Phenol, or Folin's reagent which produces a blue colour chromophore. This can be measured using a spectrophotometer and the absorbance value generated by the activity of the protease can be quantified by comparing it to OD_{660} of a tyrosine standard curve. A tyrosine standard curve was generated by defining the known quantities of tyrosine with the Folin's reagent. **Figure 4.8** shows a standard curve for different concentrations of tyrosine.

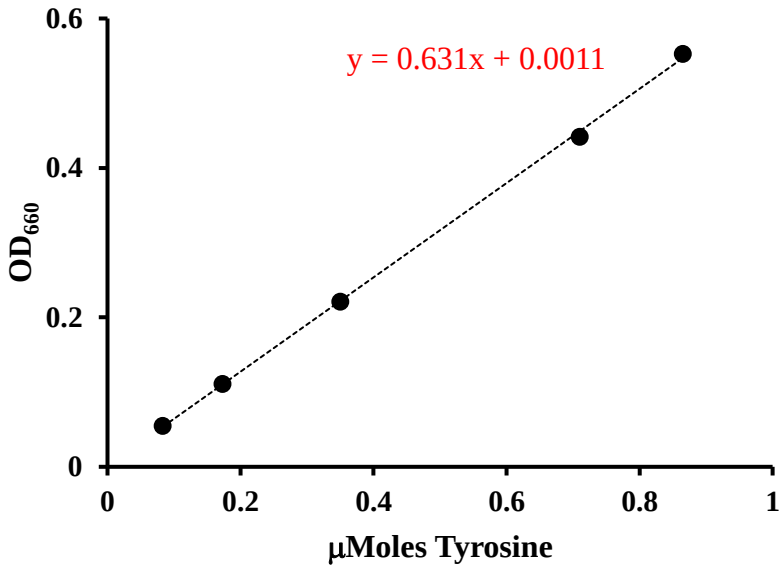

Figure 4.8: Tyrosine standard curve. A tyrosine standard curve is generated by mixing a defined quantity of tyrosine (0.055, 0.111, 0.221, 0.442 and 0.553 µMoles) with Folin's reagent. The absorbance (OD_{660}) generated by each sample was measured using a spectrophotometer. Results are presented on a line graph and the line equation is presented on the graph in red.

Using the line equation generated from the tyrosine standard curve, the amount of

tyrosine released by digesting casein using SH20 spores was determined and the unit of activity (amount of tyrosine (μMoles) released from casein per min) was calculated (**Table 4.2**). The results show that 10^{10} spores of SH20 had ~ 0.13 units of activity per ml for casein digestion whereas PY79 spores showed 0.01 units of activity per ml. This confirms that SH20 recombinant spores exhibit casein digestion activity.

Table 4.2: Determination of the unit of activity for casein digestion[1]

Strain	No. of spores	OD_{660}	Unit of activity/ ml
SH20	10^{10}	0.146	0.127
PY79	10^{10}	0.013	0.01

[1] Using the OD_{660} generated by PY79 and SH20, the amount of the μMoles of tyrosine released by casein digestion was calculated from the tyrosine standard curve and then the unit of activity was determined.

b) Amylase E

Alpha-amylase can break down starch or glycogen into simple sugars, by breaking the internal alpha-1,4 glycoside bonds resulting in the production of oligosaccharides and maltose. A clone (SH18) that expresses amylase enzymes on its recombinant spores was made by the *thy*-insertion cloning system. To confirm whether the amylase expressed on the spore surface is active, different CFU of purified SH18 spores were spotted on starch agar supplemented with an antibiotic cocktail (trimethoprim, chloramphenicol, erythromycin). **Figure 4.9** shows the zone of starch degradation. This result indicates that the amylase E displayed on the spore surface of SH18 is functional. PY79 spores didn't show any zone of degradation meaning that these spores don't naturally have any amylolytic activity.

To determine the unit of activity of SH18 spores in liquid, the method described by Bernfiel (Bernfeld 1955) was used. The maltose released by digesting starch using alpha-amylase can react with the colour reagent (described in method, section 2.21) and, as a result, the OD_{540} can be measured. Therefore, to calculate the amount of maltose released using SH18 spores, a standard curve using different concentrations of maltose reacting with a colour reagent was generated (**Figure 4.10**).

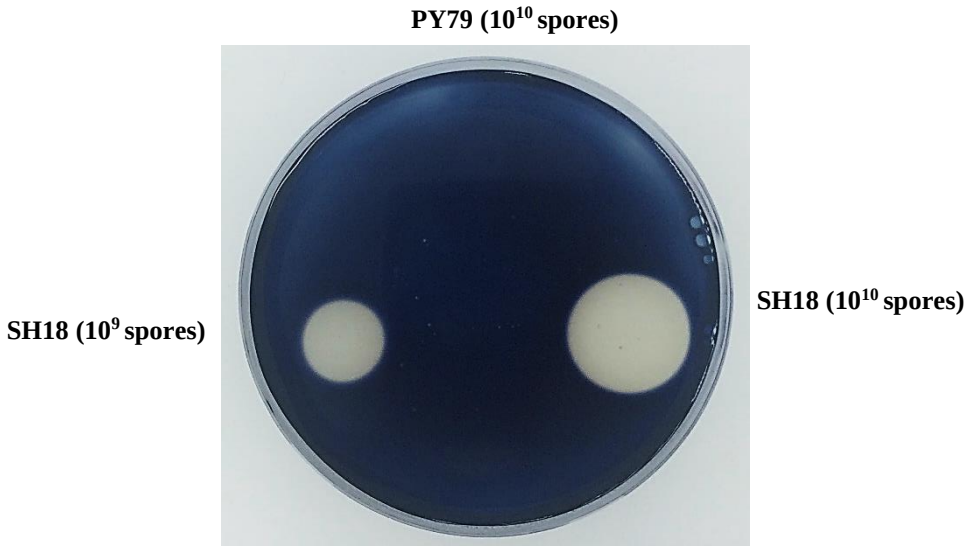

Figure 4.9: Degradation of starch by SH18 spores. Purified spores of PY79 and SH18 were spotted (20 μl) on starch agar and incubated. The agar was stained with Lugol solution to reveal zones of degradation. PY79 spores were used as a control. SH18 spores showed the zone degradation while PY79 didn't show any activity of starch degradation (no zone of clearance).

The amount of maltose released by digesting starch using spores was calculated by obtaining the OD_{540} of the supernatant and comparing it to the maltose standard curve. Finally, the unit of activity was calculated using the equation described in the method

(Section 2). This result indicates that 10^{10} spores of SH20 carried ~3 units of amylase activity with the control PY79 exhibiting almost no activity (**Table 4.3**).

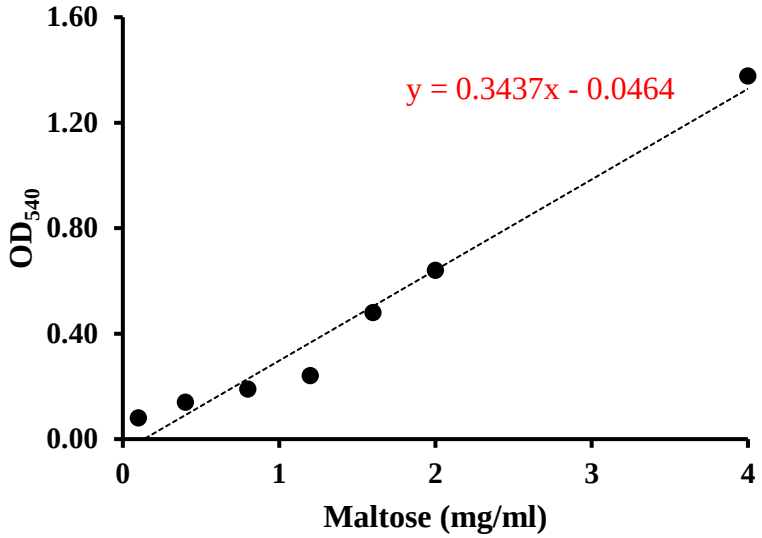

Figure 4.10: Maltose standard curve. A maltose standard curve is generated by mixing a defined quantity of maltose (0.3, 1.2, 2.4, 3.6, 4.8, 6 and 12 mMoles) with a colour reagent (see Method, Chapter 2). The absorbance (OD_{540}) generated by each sample was measured using a spectrophotometer and presented as a line graph. The line equation is marked on the graph in red.

Table 4.3: Determination of the unit of activity for starch digestion[1]

Samples	CFU	OD_{540}	Activity (unit/ml)
SH18	10^{10} spores	0.981	2.99
PY79	10^{10} spores	0.014	0.18

[1] Using the OD_{540} generated by PY79 and SH20, the amount of maltose released by starch digestion was calculated from the maltose standard curve.

4.4 Discussion

The use of *B. subtilis* spores as a vehicle for displaying proteins offers several applications such as for recombinant spore vaccines. Expressing proteins on the spore surface in their native form is very important and the recombinant spores are only applicable if the proteins displayed on them are in their native forms. Here the functionality and some example applications of proteins expressed on *B. subtilis* spores using a *thy*-insertion cloning system were investigated.

4.4.1 Spore display and its use

a) Antigen (recombinant spore vaccine)

Mucosal vaccination showed promising results for inducing immunity and protection against some bacterial pathogens, and currently, there is a great interest in developing these vaccines (Azegami *et al.*, 2014). Being non-invasive, the ease of administration and the fact that large surface areas of the body are covered by mucosal tissues are some of the advantages that mucosal vaccines have. Spores of SH14 expressing the $TcdA_{26\text{-}39}$ antigen of *C. difficile* fused to two spore coat protein anchors, CotB and CotC. Immunised mice with SH14 and PP108 spores delivered through oral administration, compared to control groups (naive and mice dosed with naked PY79 spores) that exhibited no responses, generated a high titer of IgG and IgA and, based on previous work, it would be predicted that these levels of antibodies would be protective (Hong *et al.*, 2017b). Knowing that both clones (SH14, PP108) can produce an immune response, SH14 can be a better vaccine of mucosal, as it does not have any antibiotic-resistant gene genetically inserted into its genome.

b) Enzymes

Enzymes are commonly included in animal feeds where they improve digestion and nutrition (Potot *et al.*, 2010), for instance, phytase, which breaks down the indigestible phytate, a substrate containing phosphorus, which is essential for cell or tissue repair, maintenance, and growth (Boyce *et al.*, 2004). Previous work has shown that fusing phytase to *B. subtilis* spore coat proteins will display the chimeric protein on the spore surface and is functional. These spores can then be mixed with animal feed which will improve their digestion. The low cost and easy preparation of the spores that display enzymes are advantages in comparison to the pure enzyme. Two genes encoding for subtilisin E and amylase were used to show that it is possible to display an enzyme that retains activity on the spore surface. SH18 spores expressing amylase were found to be able to express active amylase on their surface and that 10^{10} spores carried ~3 units of amylase activity. Using a casein agar, it was shown that SH20 spores expressing the alkaline protease, subtilisin E, carried enzymatic activity. In liquid suspensions, it was found that 10^{10} spores of SH20 had ~0.13 units of protease activity. To be able to use these spores for their enzymatic activity in humans or animals feed, first they need to be dried, and the unit of the activity then needs to be calculated (unit of activity/g). Knowing the number of units of activity required for human and/or animals, the right number of dried spores can be calculated.

Displaying enzymes on spore surfaces could be further improved by inserting genes which encode for more stable enzymes, e.g., thermostable alkaline phytase from *Bacillus sp*. MD2 (Tran *et al.*, 2011). The ability to express enzymes on the spore surface can also have therapeutic purposes. Malabsorption syndrome due to pancreatic problems can be treated by taking pancreatic enzymes that are a commercial mixture of protease, lipase, and amylase

(Keller and Layer, 2005). A combination of spores displaying the latter enzymes could be a better potential treatment option for malabsorption syndrome.

SH20 spores which express the alkaline protease were shown to have casein degradation activity, though the unit of activity was not high. Expressing a better enzyme, on the spore surface, with a high unit of activity for casein digestion such as the enzyme subtilisin Carlsberg from *B. licheniformis*, would be more favourable. These spores then could then be used for digesting casein in milk and as a potential probiotic for individuals who are unable to digest casein in milk.

c) Streptavidin

Delivery of drugs to cells using *B. sub*tilis spores is a more efficient system compared to the delivery of drugs on its own. The use of *B. subtilis* spores that express streptavidin for therapeutic drug delivery has been documented (Nguyen *et al.*, 2013). A biotinylating cetuximab antibody bound to streptavidin on killed *B. subtilis* spores could recognise the epidermal growth factor receptor on HT29 colon cancer cells. Paclitaxel, which binds to Cetuximab, could thereby be delivered to cancer cells and kill them by blocking the epidermal growth factor receptor. In this work, the ability of biotinylated polyclonal $TcdA_{26-39}$ antibodies to streptavidin on the surface of SH16 was tested. The conjugation and display of the biotinylated polyclonal $TcdA_{26-39}$ antibodies by various techniques to SH16 spores was shown. Interestingly, PY79 spores also showed some level of conjugation. However, these conjugations could be the result of the adsorption of antibodies to the hydrophobic and negatively charged surface of spores. Using the whole spore ELISA, the conjugation of the polyclonal antibodies to PY79 was about ~5-fold lower compared to SH16. This agrees with the previous report which has shown that protein can be adsorbed to a spore's surface (Huang

et al., 2010). Western blot and ELISA results showed that having two copies of streptavidin, inserted in the genome, will lead to higher expression on the surface of spores. This is an advantage as higher expression of streptavidin results in more antibodies binding to spores, which reduces the overall number of spores that need to be administered.

Using the conjugated spores, we asked whether SH16 spores expressing $TcdA_{26\text{-}39}$ IgG could subtract *C. difficile* toxins from a crude cell-free lysate. Incubation of conjugated spores with toxin-containing lysates for just 5 min reduced toxicity by 90%. Interestingly, PY79 spores also had some ability to bind $TcdA_{26\text{-}39}$ antibodies and were able to provide a modest reduction (10-20%) in toxin activity. Used as an example, this experiment does demonstrate that spores that express and display streptavidin might have a potential for therapeutic purposes, for instance in oral administration of antibodies.

4.5 Conclusion

In conclusion, it was shown that clones made by insertion of a chimeric gene into *thy* genes can be used for various purposes. Immunised mice with SH14 that express $TcdA_{26\text{-}39}$ on the spore surface showed that it could stimulate an immune response. Spores expressing streptavidin can also be used for therapeutic purposes as it was shown that SH16 spores conjugated with anti-$TcdA_{26\text{-}39}$ antibodies could subtract *C. difficile* toxins from a crude cell-free lysate. Finally, two clones which express Subtilisin E and Amylase E showed activity for casein and starch degradation respectively. Proving the proteins expressed and displayed on these clones are functional and the fact that these clones were made with the use of an antibiotic-resistant gene as a selection marker and that they are thymine dependent, indicates that a *thy*-insertion cloning system has excellent potential for environmental and applied purposes.

CHAPTER 5

ARE THE HYPERVIRULENT STRAINS OF *C. DIFFICILE* LESS INFECTIOUS?

5.1 Introduction

5.1.1 Bacterial colonisation

Infection is the presence and invasion of a host by disease-causing organisms such as bacteria, fungi and viruses. For any organism to be able to cause an infection, it must enter the body, colonise and multiply. Colonisation, which is the first step of microbial infection, is defined as the establishment of a pathogen in an area such as skin and intestine (Dani, 2014). Routes of entry for microorganisms into the host are through the digestive tract, the urogenital tract, the conjunctiva and the respiratory tract. The surface area of the urogenital, respiratory and digestive system mucosa is between 300-400 square meters and therefore makes a primary site of contact with microorganisms. These mucous membranes consist of three layers: an epithelial cell layer that secretes mucus, an underlying connective tissue called lamina propria, and a layer of smooth muscle. Each of these layers constitutes frontline barriers that limit the adherence and invasion of both pathogenic or commensal bacteria. Despite this, microorganisms have evolved a wide range of strategies to adhere to, invade a host organ, resist and overcome the multiple host defenses mechanisms at the surface (Ribet and Cossart, 2015).

The adhesion of microorganisms such as bacterial pathogens to eukaryotic cells or tissue surfaces is an important aspect of host colonisation. Colonisation prevents the removal of the pathogen by different immune system clearance mechanisms. The adherence of a bacterium to the host's surfaces, in its simplest form, requires a ligand and a receptor. Usually, such receptors are specific peptide residues or carbohydrates on the surface of eukaryotic cells. The bacterial ligand, which often has a complex molecular structure and is present on the bacterial cell surface, is called an adhesin, which interacts with the host cell receptor (Pizarro-Cerdá and Cossart, 2006, Kline *et al.*, 2009). The common feature about ligands and receptors is that they come in a closely matched pair, with a ligand recognising and binding to one or a few target receptors, and the receptor is specific to one or few ligands. So far, a wide range of adhesins for the surface of bacteria has been identified, and these adhesins recognise different receptors on the host cell surface such as cadherins or integrins, which include transmembrane proteins, collagen, laminin, elastin or fibronectins that are components of the extracellular matrix (Pizarro-Cerdá and Cossart, 2006, Kline *et al.*, 2009, Cossart and Roy, 2010, Chagnot *et al.*, 2012). Pili are a well-characterised representative of the first class of structures involved in the attachment of the bacteria to host cells. Pili are polymeric hair-like appendages that are located on the surface of the bacteria (Kline *et al.*, 2009). The tips of the pili are usually involved in binding to the host cells. For instance, the tip of the pili of the uropathogenic strains of *E. coli* that colonise in the urinary tract and cause kidney infection are constituted by an adhesion factor called PapG, which is involved in attaching glycosphingolipids of the kidney epithelium (Roberts *et al.*, 1994).

5.1.2 *C. difficile* spore structure and proteins

The morphology and structure of *C. difficile* spores are similar to other Gram-positive endospore-forming bacteria such as *B. subtilis*, although it has a notably different outermost

layer as the protein composition of this layer is considerably different compared to other Gram-positive bacteria (**Figure 5.1**) (Paredes-Sabja *et al.*, 2014). Like *B. subtilis*, the coat layer of the *C. difficile* spore is also a proteinaceous layer that is important for protecting the spore from proteolytic enzymes such as trypsin and proteinase K (Escobar-Cortés *et al.*, 2013). The sac-like layer of the *C. difficile* spore, called the exosporium, encasing the coat layer. The exosporium, the outermost layer of the spore, interacts with environmental surfaces and other cells (Bozue *et al.*, 2007a, Chen *et al.*, 2010); and, importantly, it is not impermeable, allowing the passage of small molecules, e.g., amino acids and sugars (Ball *et al.*, 2008). In most strains of *C. difficile*, the exosporium has hair-like projections that interacts directly with the surface of the spore coat layer (Barra-Carrasco *et al.*, 2013, Paredes-Sabja *et al.*, 2014). There is some uncertainty as to the stability of the exosporium as some studies have suggested that this layer is fragile and easily lost (Permpoonpattana *et al.*, 2011b, Permpoonpattana *et al.*, 2013); moreover, several other studies have suggested that the exosporium is relatively stable and is only removed by proteolytic enzymes (e.g., protease or proteinase K) and sonication (Barra-Carrasco *et al.*, 2013, Escobar-Cortés *et al.*, 2013, Pizarro-Guajardo *et al.*, 2014). The exosporium layer is also found in the spore of the *B. cereus* group such as *B. anthracis*. In contrast to the *C. difficile* exosporium, most members of *B. cereus* exhibit the hair-like projection of the exosporium although these do not interact directly with the surface of the coat layer (Pizarro-Guajardo *et al.*, 2016). The exosporium morphology of *C. difficile* is strain dependent. For instance, spores from *C. difficile* 630 (CD630) have a compact exosporium layer whereas spores of R20291 or M120 have similar exosporium structures to *B. anthracis* (Paredes-Sabja *et al.*, 2014). Interestingly all strains of *C. difficile* produce spores with two distinctive thicknesses of exosporium: either thin or thick (Pizarro-Guajardo *et al.*, 2016). A mass spectrometry (MS/MS) analysis of the exosporium of CD630 spores identified 184 proteins. Diaz-Gonzalez et al. (2015)

reported that, out of these 184 identified proteins, six are possibly involved in pathogenicity; six might be involved in spore resistance; seven are characterised as coat and/or exosporium proteins; 13 are uncharacterised; and 146 are cytosolic proteins (Díaz-González *et al.*, 2015).

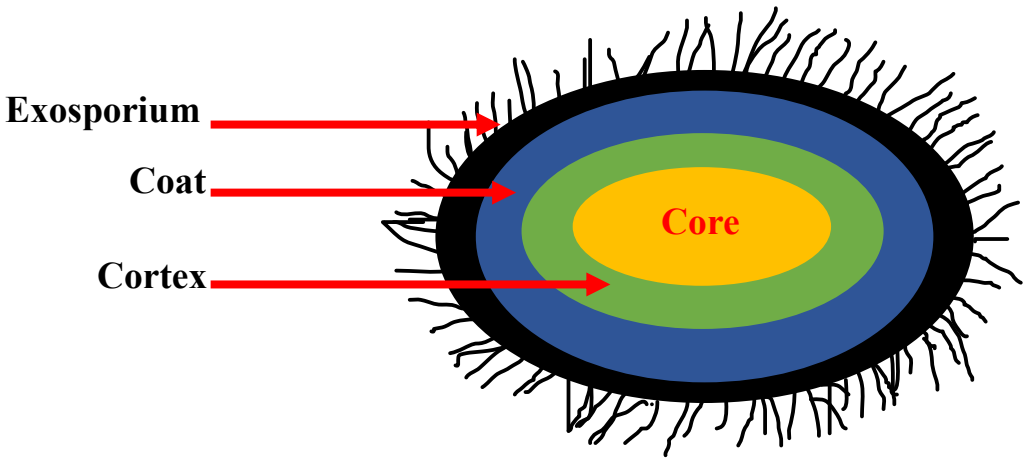

Figure 5.1: Spore structure of *C. difficile*. Similar to *B. subtilis* spores, *C. difficile* spores consist of a core (yellow), cortex (green) and the coat layer (blue). *C. difficile* spores also exhibit an extra layer, exosporium (black), that surrounds the coat layer. The hair-like filaments are believed to be BclA (*Bacillus* collagen-like protein of *anthracis*) proteins.

The CdeC (*C. difficile* exosporium cysteine-rich protein), an exosporium protein, has a unique assembly mechanism, and it plays an essential role in the correct assembly of the exosporium and the coat layer. Based on a Western blot analysis of CD630 and R20291 spores treated with proteinase K (resulting in spores with no exosporium), no immunoreactive band corresponding to CdeC was observed, suggesting that CdeC is localised in the exosporium layer (Paredes-Sabja *et al.*, 2014). Barra-Carrasco et al. (2013) reported that a mutated *cdeC* gene can have various effects on the exosporium. They constructed a Δ*cdec* isogenic knockout mutant of R20291, and, in comparison to the wild-

type R20291, they showed that (a) the exosporium in Δ*cdec* is largely missing, (b) the mutant spores were more sensitive to lysozyme, heat and ethanol treatment in comparison to the wild-type, and (c) the level of adhesion of the mutant spores, despite the absence of the exosporium layer, to intestinal epithelial cell line was higher than the wild-type spores (Barra-Carrasco *et al.*, 2013).

The exosporium of CD630 spores also exhibits three BclA orthologs encoded by *bclA1*, *bclA2*, and *bclA3* genes. The BclA protein, first identified in *B. anthracis*, is a glycoprotein and the primary component of the exosporium in some spore-forming bacteria (Sylvestre *et al.*, 2002). It consists of three domains: an N-terminal domain, a central collagen-like region and a C-terminal domain. Similar to *B. anthracis*, all three BclA proteins of *C. difficile* spores also consist of three domains: the N-terminal domain involved in the localisation of the BclA protein and is anchored to the exosporium layer, a central, collagen-like domain formed by GXX (mostly GPT) repeats; and a C-terminal domain which in *B. anthracis* meditates the trimerization of the BclA monomers (**Figure 5.2**) (Pizarro-Guajardo *et al.*, 2014). The predicted mass for BclA1, BclA2, and BclA3 are 68, 49 and 58 kDa, respectively. However, the Western blot analysis of all three BclA proteins showed a 48-kDa immunoreactive band which suggests the post-translational cleavage and glycosylation of these proteins (Díaz-González *et al.*, 2015). In *C. difficile*, BclA1 and BclA3 are thought to form a stable dimer or trimer complex similar to BclA in *B. anthracis* (Liu *et al.*, 2008), with a high molecular weight of ~120 kDa (Díaz-González *et al.*, 2015).

Phetcharaburanin et al. (2014) constructed *bclA* mutant strains (*bclA1*$^-$, *bclA2*$^-$, and *bclA3*$^-$), derivatives of the wild-type CD630, and investigated the phenotype of each mutant. They found that the spores of *bclA1*$^-$ and *bclA2*$^-$ strains showed substantial aberration on

Figure 5.2: Schematic representation of different types of BclA protein of CD630. All BclA1, BclA2 and BclA3 consist of three regions: The N-terminal region (red), the central collagen-like region (yellow), and the C-terminal (green). The highest sequence similarity of all BclA is in the collagen-like region.

their outermost layer, yet spores from *bclA3*$^{-}$ did not show any apparent defect in their outermost layer compared to wild-type spores. They also reported that BclA proteins have an effect on the hydrophobicity of the spore as all three *bclA* mutants were significantly less hydrophobic than wild-type spores. Moreover, spores of all three *bclA* mutants showed faster germination than the wild-type spores. Therefore, the absence of BclA1 and BclA2 proteins, but not BclA3, impairs the outermost layer of mutant spores. Furthermore, the absence of these genes results in spores with lower hydrophobicity and faster germination rates than the isogenic wild-type spores. Deleting *bclA* in *B. anthracis* also resulted in a reduction of hydrophobicity and an increase in germination rate (Brahmbhatt *et al.*, 2007).

5.1.3 Colonisation of *C. difficile*

C. difficile is the most common cause of hospital-acquired antibiotic-associated diarrhea in the developed countries with high morbidity and mortality (Enoch and Aliyu, 2012). The dormant spore of *C. difficile* is the primary agent of transmission which is due to the strict anaerobic requirement of the bacterium. During antibiotic therapy, the spores can attach to the infected patient gut and colonise the GI-tract where they can germinate to become live cells, outgrowth, proliferate and result in the initiation of CDI (Songer and

Anderson, 2006). This can result in shedding a large number of spores in the faeces (Donskey, 2010). The abundant presence of the three BclA orthologs on the spore surface makes them a prime candidate in the colonisation of *C. difficile* spores and thus Phetcharaburanin et al. (2014) investigated the role of three BclA proteins on host colonisation. They performed an ID_{50} experiment to investigate the colonisation of all three *bclA* mutants in mice. They reported that *bclA1*$^{-}$ mutants had impaired colonisation efficiency and showed that, to infect 50% of mice, the required number of spores was 2-logs higher compared to the wild-type CD630. Moreover, no spores of *bclA1*$^{-}$ were detectable 3 days post infection. They also reported that the ID_{50} of the hypervirulent strain R20291, which has a truncated BclA1 protein was 1 log lower than the *bclA1*$^{-}$ mutant and needed more spores that were required to colonise the mice than for CD630. The truncation of BclA1 in R20291 is the result of an early stop codon caused by a point mutation that reduces the size of the BclA1 to only 48 amino acids. These data suggest that BclA1 plays an important role in the colonisation of *C. difficile*. In *B. anthracis*, however, the BclA protein has not been shown to have a significant role in pathogenicity in mice and guinea pigs (Bozue *et al.*, 2007a), and Brahmbhattet et al. (2007) showed that the LD_{50} (lethal dose, 50%) of a *bclA* mutant strain of *B. anthracis* was similar to the isogenic wild-type strain.

5.2 Aim

The aim was to identify the ribotypes and *bclA* genes in 45 human clinical isolates of *C. difficile.* It is proposed that BclA1 is involved in the colonisation of CD630 with mutant *bclA1* which needs higher CFU to colonise. In this work, the role of various types of BclA1 in different ribotypes will be investigated, and various toxigenic ribotypes strains with different BclA1 size will be tested for their sporulation, *in vitro* cell-cytotoxicity and colonisation. The presence of *bclA2* and *bclA3* in different ribotypes will be investigated.

5.3 Results

5.3.1 Genotypic characterisation of clinical isolates

A total of forty-five clinical isolates of *C. difficile* were obtained from Dr. Scott Curry (University of Pittsburgh Medical Center Presbyterian, Pittsburgh, USA). These strains were analysed for the ribotypes and the presence of *bclA1*, *bclA2,* and *bclA3* genes. PCR ribotyping is a method based on the heterogeneity of 16S-23S intergenic spacer regions of ribosomal RNA and is a preferred method used for genotyping *C. difficile* (Bidet *et al.*, 1999). Six reference strains obtained from different sources were also used in this characterisation (see **Table 5.1)**.

a) Ribotyping

All the strains examined in this study are summarised in **Table 5.1**. From the clinical isolates, eighteen different ribotypes were identified with 42% (nineteen strains) of them being ribotype 027 (R027). Four of the forty-five strains were non-typeable for which the reason was unclear. The remaining strains showed a variety of different types of ribotypes as shown in **Table 5.1**.

b) bclA typing

To check the presence of the three *bclA* genes in the clinical isolates and to identify the type of *bclA1* they have, PCR was performed. For *bclA2* and *bclA3,* only the presence of these genes was confirmed; however, for *bclA1,* the PCR products were sequenced (**Table 5.1**). All strains of ribotype R027 contained truncated *bclA1*. In all strains from R017 and R078, *bclA1* was deleted, and the remaining strains, except R111, showed full-length *bclA1*. A gene that encodes for a *bclA1* with 71 amino acids was detected in R111. Finally, the presence of *bclA2* and *3* was confirmed in all strains (**Table 5.1**).

The different types of *bclA1* genes that were identified previously are genes that encode for a 693-amino acids protein (full-length) and genes that express a 48-amino acids protein (Phetcharaburanin *et al.*, 2014). In this study, a new type of *bclA1* was identified that encodes for a 71-amino acids protein (**Figure 5.3**). Based on the nucleotide analysis, this truncation is the result of a thymine insertion and a thymine substitution (adenine to thymine) at amino acid 48 and 49, respectively, which changes the open reading frame (ORF), and, after 71 amino acids, it ends at a stop codon (**Figure 5.4**).

5.3.2 Identifying the clades of clinical isolates

Multilocus sequence typing is another genotypic method using the sequence of internal fragments of seven house-keeping genes (Griffiths *et al.*, 2010). Each unique combination of these seven alleles is called an allelic profile or sequence type (ST). *C. difficile* strains based on similarity obtained from multilocus sequence typing can be divided into six groups, otherwise known as six clades. Clades one to five represent the toxigenic strains, and clade six represents non-toxigenic strains. Clade two and clade four represent the hypervirulent strains and strains lacking toxin A respectively. To identify which clades the clinical strains belong to, the ribotypes from clinical strains were compared to data available from other publications (Griffiths *et al.*, 2010, McMillen *et al.*, 2016) in which the ribotypes and MLST been compared. Interestingly, once the clades for different ribotypes of the clinical isolates were identified (although there was no strain representing clade three), a pattern based on the length of *bclA1* could be identified (**Table 5.1**).

Table 5.1: Genotypic characterisation of various *C. difficile* clinical strains

Original strain name	Source[1]	Origin	Ribotype[2]	Clade[3]	*bclA1*[4]	*bclA2*	*bclA3*
Reference Strains							
-	LL	Pig	R078	5	Deleted	+	+
-	LL		R010	1	Full length	+	+
630 (ATCC® BAA-1382™)	NF	Human	R012	1	Full length	+	+
VPI10463	NF		R087	1	Full length	+	+
CD196	NF	Human	R027	2	Truncated[48]	+	+
R20291	TL	Human	R027	2	Truncated[48]	+	+
Clinical Strains							
4	SC	Human	R056	1	Full length	+	+
25	SC	Human	R078	5	Deleted	+	+
32	SC	Human	R027	2	Truncated[48]	+	+
38	SC	Human	R038	NI	Full length	+	+

41	SC	Human	R027	2	Truncated[48]	+	+
42	SC	Human	R001/072	1	Full length	+	+
43	SC	Human	R027	2	Truncated[48]	+	+
46	SC	Human	R027	2	Truncated[48]	+	+
83	SC	Human	R027	2	Truncated[48]	+	+
101	SC	Human	R078	5	Deleted	+	+
119	SC	Human	R002	1	Full length	+	+
168	SC	Human	R027	2	Truncated[48]	+	+
193	SC	Human	Nt	NI	Full length	+	+
199	SC	Human	R014	1	Full length	+	+
203	SC	Human	Nt	NI	Full length	+	+
206	SC	Human	R054	NI	Full length	+	+
208	SC	Human	R017	4	Deleted	+	+
222	SC	Human	R027	2	Truncated[48]	+	+
251	SC	Human	R001/072	1	Full length	+	+
335	SC	Human	R027	2	Truncated[48]	+	+
336	SC	Human	R336	NI	Full length	+	+

1385	SC	Human	R027	2	Truncated[48]	+	+
1395	SC	Human	R401	NI	Truncated[48]	+	+
1402	SC	Human	R027	2	Truncated[48]	+	+
1407	SC	Human	R027	2	Truncated[48]	+	+
1411	SC	Human	R027	2	Truncated[48]	+	+
1433	SC	Human	R078	5	Deleted	+	+
1498	SC	Human	R056	1	Full length	+	+
1500	SC	Human	R027	2	Truncated[48]	+	+
1575	SC	Human	Nt	NI	Full length	+	+
1578	SC	Human	R027	2	Truncated[48]	+	+
1634	SC	Human	R005	1	Full length	+	+
1639	SC	Human	R277	NI	Full length	+	+
1647	SC	Human	R017	4	Deleted	+	+
1652	SC	Human	R027	2	Truncated[48]	+	+
1655	SC	Human	Nt	NI	Full length	+	+
1667	SC	Human	R103	1	Full length	+	+
1668	SC	Human	R027	2	Truncated[48]	+	+

1679	SC	Human	R054	NI	Full length	+	+
1682	SC	Human	R075	NI	Truncated[48]	+	+
1685	SC	Human	R027	2	Truncated[48]	+	+
1688	SC	Human	R087	1	Full length	+	+
1693	SC	Human	R111	1	Truncated[71]	+	+
1696	SC	Human	R027	2	Truncated[48]	+	+
1704	SC	Human	R027	2	Truncated[48]	+	+

1 NF, Neil Fairweather, Imperial College, London, UK; TL, Trevor Lawley, Sanger Center, Cambridge, UK; LL, Len Lipman, Univ. Utrecht, Netherlands; SC, Scott Curry, UPMC Presbyterian, Pittsburgh, USA.

2 Nt (non-typeable)

[3] NI (not identified)

4 Full length= 693 amino acids., truncated[48]= 48 amino acids, truncated[71]= 71 amino acids.

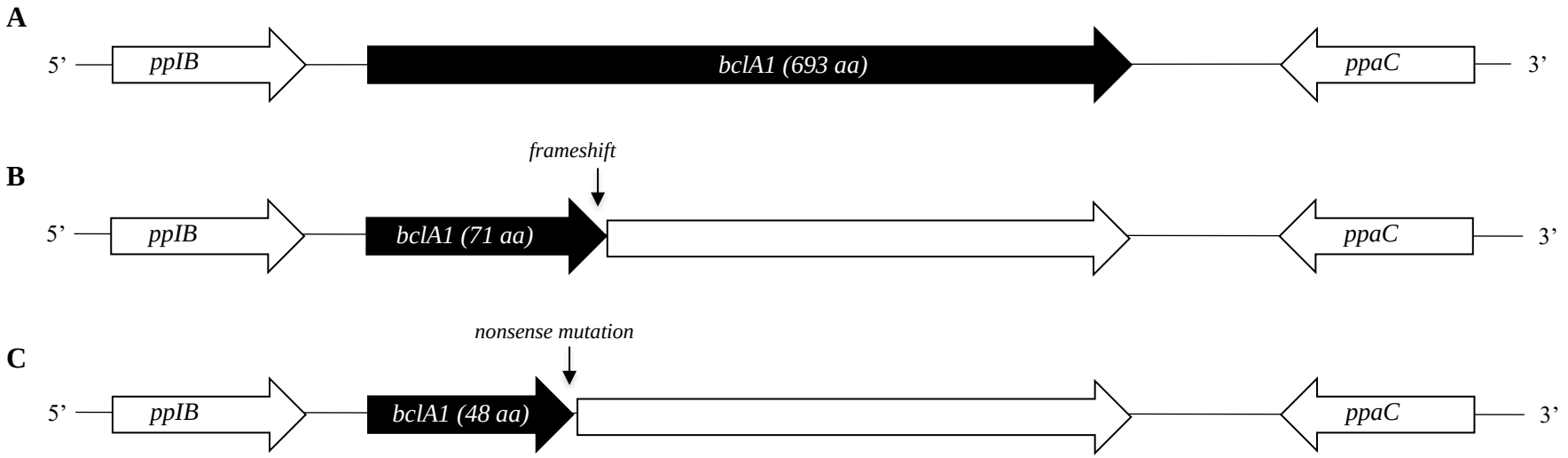

Figure 5.3: Schematic diagram of different types of *bclA1* gene. *Panel A* shows the full length of *bclA1*, *panel B*; 71 codon *bclA1* (*bclAΔ622*) caused by a frameshift mutation, and *panel C* shows the 48 codon *bclA1* (*bclAΔ645*) caused by a nonsense mutation. *ppIB*: peptidylprolyl isomerase B; *ppaC*: manganese-dependent inorganic pyrophosphatase.

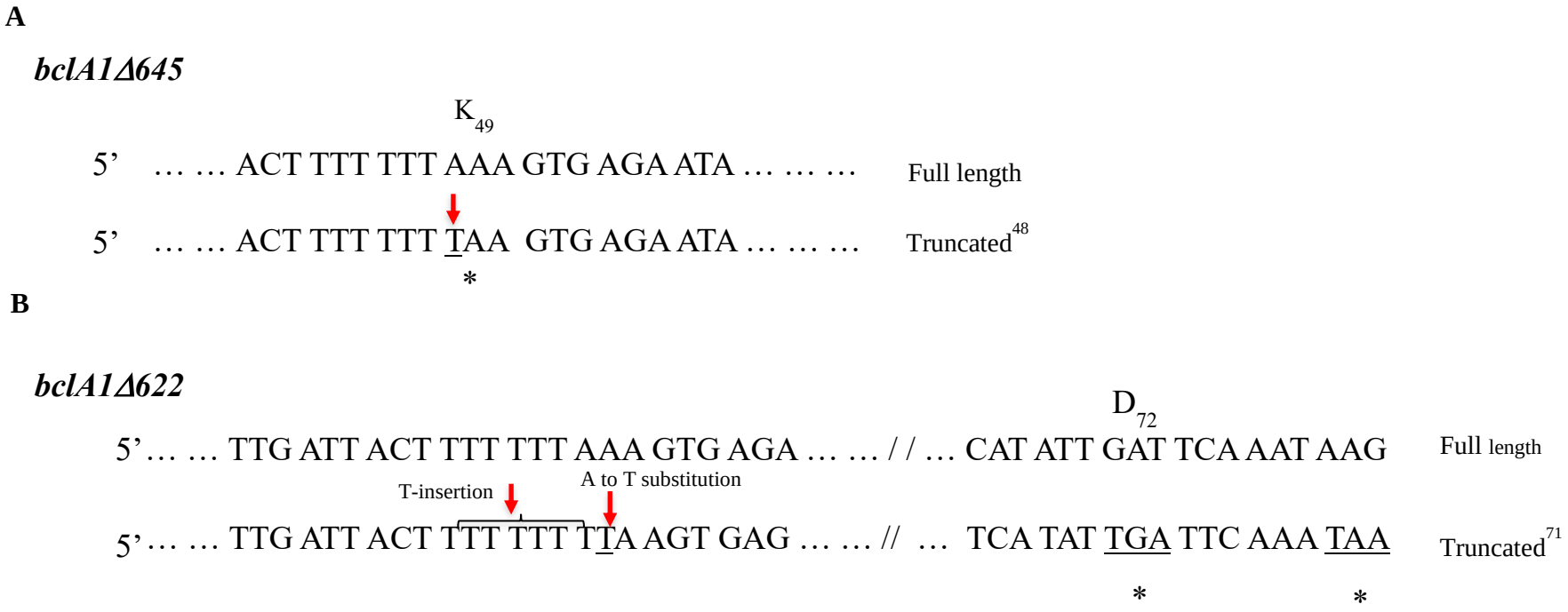

Figure 5.4: Nucleotide analysis of different *bclA1*. *Panel A* shows the aligned nucleotide of full length and truncated *bclA1* encoding for 48 amino acids (truncated[48]). At codon 49 a base substitution changes a lysine (K) into a stop codon. *Panel B* shows the new type of *bclA1* encoding for 71 amino acids (truncated[71]) aligned with full length *bclA1*. In truncated[71] *bclA1*, a thymine-base insertion and also an adenosine (A) to thymine (T) substitution changes the ORF, and, after 71 codons, codon 72 changes from aspartic acid (D) into a stop codon (*).

5.3.3 Pattern of *bclA1* in different clades

The different forms of *bclA1* that were identified from the clinical strains from different clades showed a reduction in size from clades one to five. However, the clinical isolates did not contain any strain that represents clade three. To investigate the pattern, different strains representing each clade were obtained from Dr Michelle Cairns (*C. difficile* ribotyping network [CDRN] London Barts Health). These strains were characterised for the type and the presence of *bclA1, 2* and *3* and finally what kind of *bclA1* they carried. **Table 5.2** shows the characterisation result performed on the reference strains for each clade. Strains in clade one showed full-length *bclA1*. In both clades two and three, truncated *bclA1* was identified. Finally, in clades four and five, *bclA1* gene was absent. The presence of *bclA2* and *3* was confirmed in all strains with one exception: clade three, which showed a partially deleted *bclA2* (**Figure 5.5**). The presence of toxin A and toxin B gene were also confirmed in all strains except clade four in which *tcdA* was deleted (**Table 5.2**). It should be noted that since the pattern of *bclA1* observed was from a small sample size, a larger sample size of different ribotypes, considering the huge number of different *C. difficile* ribotypes, needs to be checked to better understand the pattern of *bclA1* seen within different clades and whether the pattern was seen is correct.

Table 5.2: Genotypic characterisation of reference strains of different clades

Ribotype	Clade	Toxin A&B	*bclA1*	*bclA2*	*bclA3*
106	1	$A^+ B^+$	Full length	+	+
015	1	$A^+ B^+$	Full length	+	+
002	1	$A^+ B^+$	Full length	+	+
176	2	$A^+ B^+$	Truncated[48]	+	+
027	2	$A^+ B^+$	Truncated[48]	+	+
023	3	$A^+ B^+$	Truncated[48]	Partially deleted	+
017	4	$A^- B+$	Deleted	+	+
078	5	$A^+ B^+$	Deleted	+	+
125	5	$A^+ B^+$	Deleted	+	+

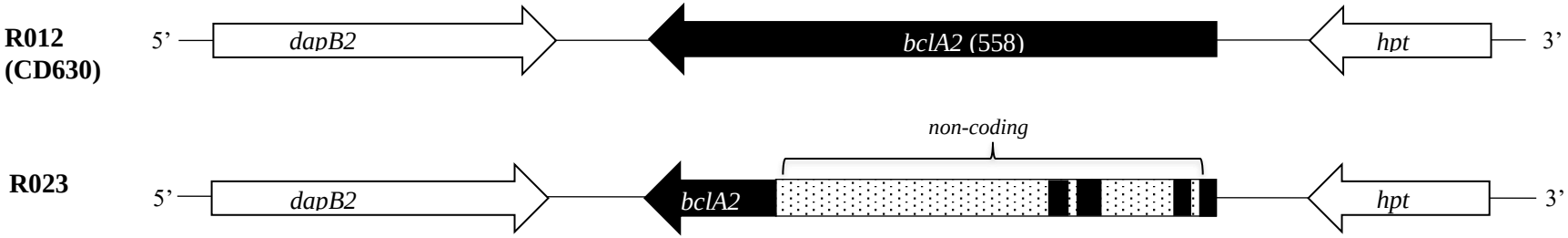

Figure 5.5: Different types of *bclA2*. R012 (CD630) has full-length *bclA*2 whereas, in R023, the *bclA2* gene is partially deleted. The gene *dapB2*: 4-hydroxy-tetrahydrodipicolinate reductase, and *hpt*: hygromycin phosphotransferase.

5.3.4 The role of BclA1 in colonisation of *C. difficile*

The question that needs to be addressed is whether, given the *bclA1* pattern seen in different clades, the infectiousness is reduced from the obtained strains of clade one to clade five. To address this question, an ID_{50} for colonisation: that is, the number (CFU) of spores of a *C. difficile* strain required to infect 50% of animals using colonisation as an indicator of infection was determined. Only one strain from each clade was tested, and CD630 was used as control. Groups (n=4) of mice (C57BL/6) were dosed with spores (using doses of 10^2, 10^3 or 10^4 CFU) from different strains of different clades. Mice were culled after 24h and the CFU, and the level of toxin A and toxin B in their caecum determined. The detection of *C. difficile* CFU indicated successful colonisation. ID_{50} was calculated (Ozanne, 1984). The results (**Figure 5.6**) showed that 10^2 spores of clades one, three, four, and five were required to infect 50% of the mice. However, mice dosed with the strain from clade two needed ~10^3 spores to infect 50% of the mice (**Table 5.3**). The results indicate that the presences of BclA1 on the surface of different non-isogenic strains, is not a necessity for better colonisation. In this study, the presence of toxin A and toxin B in the group infected with 10^3 spores was also confirmed. This result showed that mice infected with R176 (clade two, hypervirulent strain) had the highest level of toxin A and toxin B present in the caecum compared to the group, one of the signs of virulent strains (**Figure 5.7**). Although Griffiths *et al.* (2010) only identified strains in clade 2 as hypervirulent strains, nowadays other strains from different clades are also considered as hypervirulent such as hypervirulent R078 lineage isolates including R078 and R126, and R023 from clade 3 (Valiente *et al.*, 2014, Wu *et al.*, 2016).

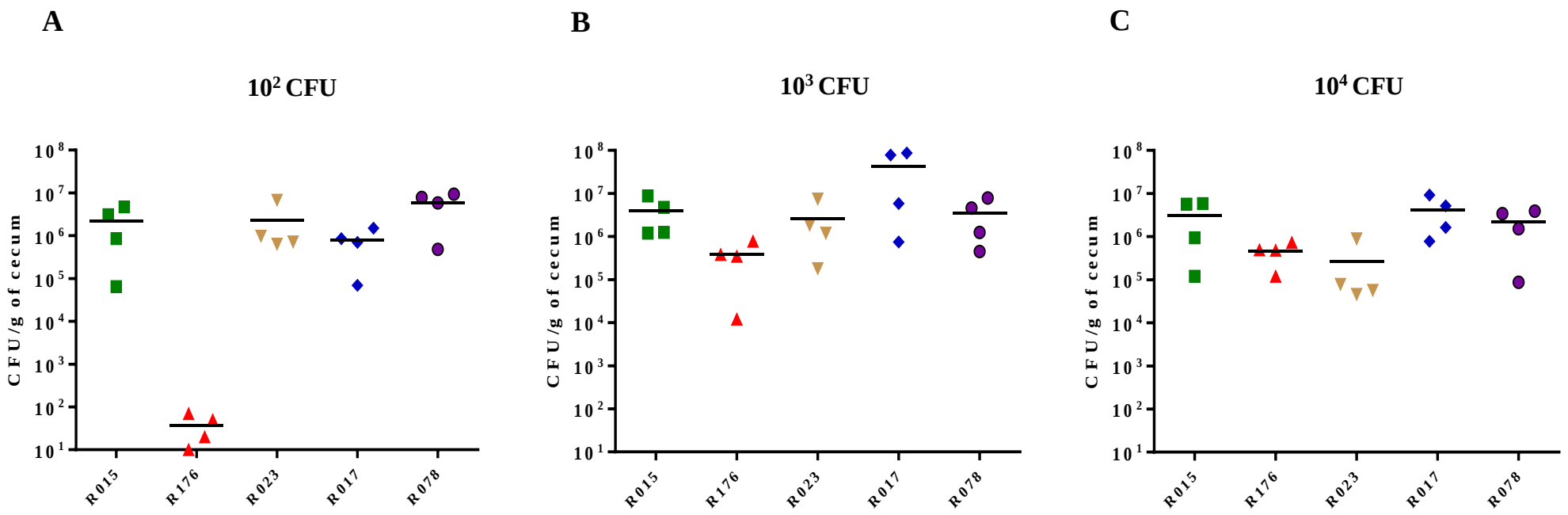

Figure 5.6: CFU determination in infected mice caecum. Groups (n=4) of C57BL/6 (7-8 weeks old, female, Charles River) were administered with two doses of clindamycin (30 mg/kg) at day 1 and day 3 by intra-gastric gavage. At day 8 mice from different groups were dosed with different strains of *C. difficile* spores. Caeca, 24h post-infection, were removed aseptically, heat treated (68^{o}C, 30 min) and the CFU was determined. *Panel A* (10^2 spores), *panel B* (10^3 spores) and *panel C* (10^4 spores) show groups of mice dosed with different amounts of *C. difficile* strains.

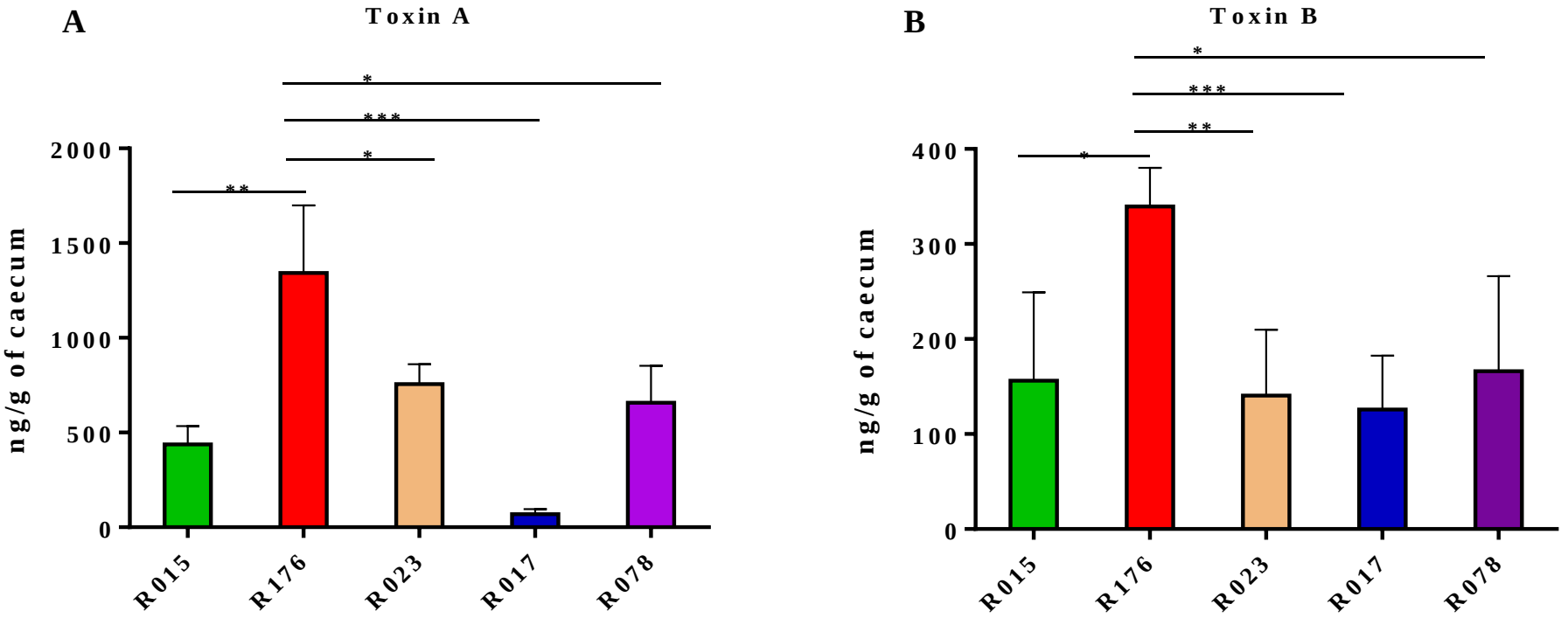

Figure 5.7: Toxin A and toxin B determination of caeca of infected mice. Toxin A and toxin B from groups dosed with 10^3 spores were determined by a capture ELISA method. Both toxin A and toxin B were highest in the group infected with R176 (clade two) which is a hypervirulent strain. *Panel A* shows the result for toxin A, and *panel B* shows the result for toxin B. * ($p = 0.02$), ** ($p = 0.003$), *** ($p < 0.001$).

Table 5.3: Infectivity of spores of different *C. difficile* strains in mice[1]

Clade	Ribotype	ID_{50}[2]
1	R015	1 X 10^2
2	R176 R027[3]	1 X 10^3 1 X 10^3
3	R023	1 X 10^2
4	R017	1 X 10^2
5	R078	1 X 10^2

[1] Groups of mice were first treated with clindamycin and after 5 days mice were dosed once with (10^2,10^3or 10^4/ dose) spores of different *C. difficile* strains. After 24h, mice were culled, caeca were removed, and CFU/g of caeca was determined. Colonisation was defined as animals carrying > 10^3 spores/g of caecum.

[2] Dose of spores required to infect 50% of mice (ID_{50}) was calculated (Ozanne, 1984).

[3] R027 is clade two, and, in a previous study, it also had an ID_{50} of 10^3 (**Phetcharaburanin *et al.***, 2014).

5.3.5 *In vitro* sporulation and cell cytotoxicity of strains from different clades

The sporulation of the five strains for the five clades (**Table 5.3**) was measured in BHIS broth for five days (**Figure 5.8**). CD630 (R012) was used as a control strain. After 24h of incubation, the sporulation of all strains was essentially identical, with approximately 10^3–10^4 spores per ml; however, at day 2 to day 5, the sporulation of R176 per ml was ~1-log higher than other strains. For cytotoxicity, strains were grown in TY broth for 72h, and every 24h 5 ml of each culture was removed, and the level of toxin was measured on HT29 and Vero cells. Again, in this method, CD630 was used as a strain positive for toxin A and toxin B expression. At all time-points, R176 was the most cytotoxic to both Vero and HT29 cells (**Figure 5.9**). Although no cytotoxicity effect is expected from R017, which is a *tcdA*

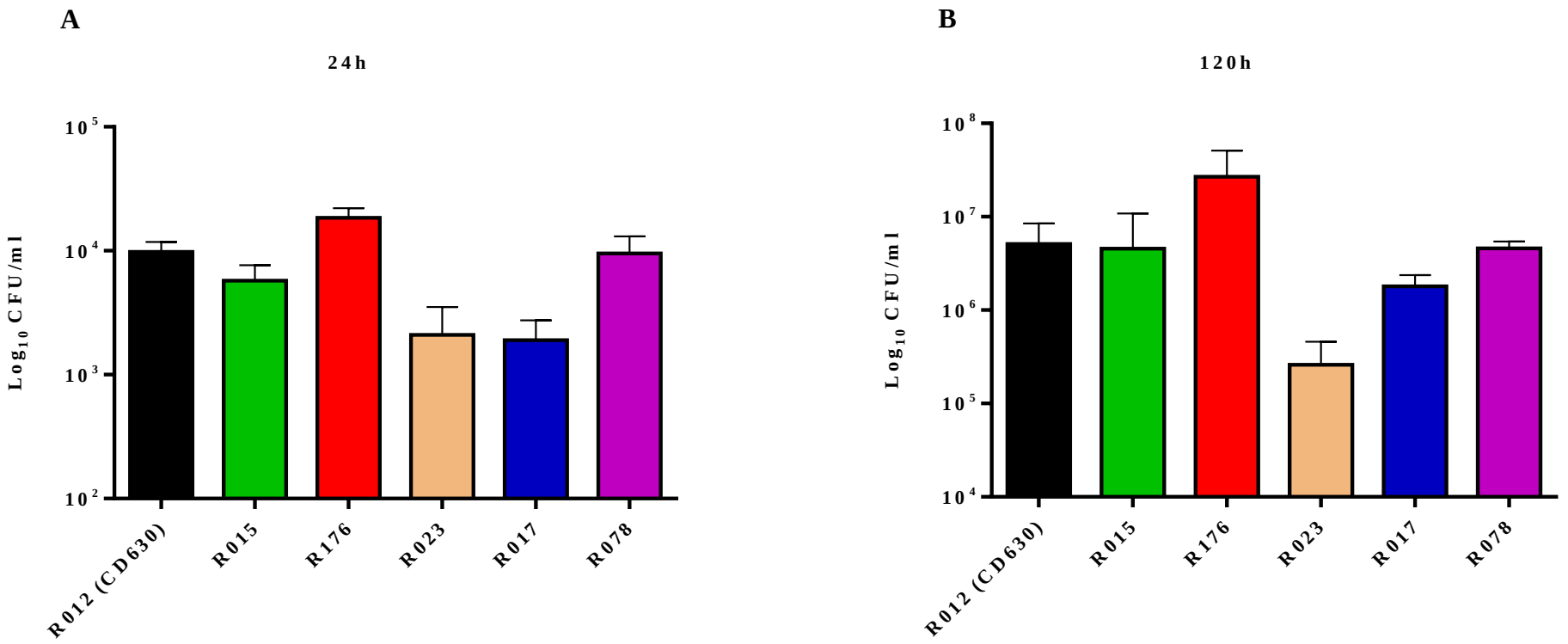

Figure 5.8: Sporulation of clade one to five representative strains. Sporulation of different strains were measured in BHIS broth. At time-points, 24h and 96h, 1 ml of each culture removed, heated to kill vegetative cells, and the CFU/ml was measured. *Panel A*; CFU/ml after 24h, and *panel B* shows the CFU/ml after 96h. There was no significant difference between each group. This experiment was replicated three times.

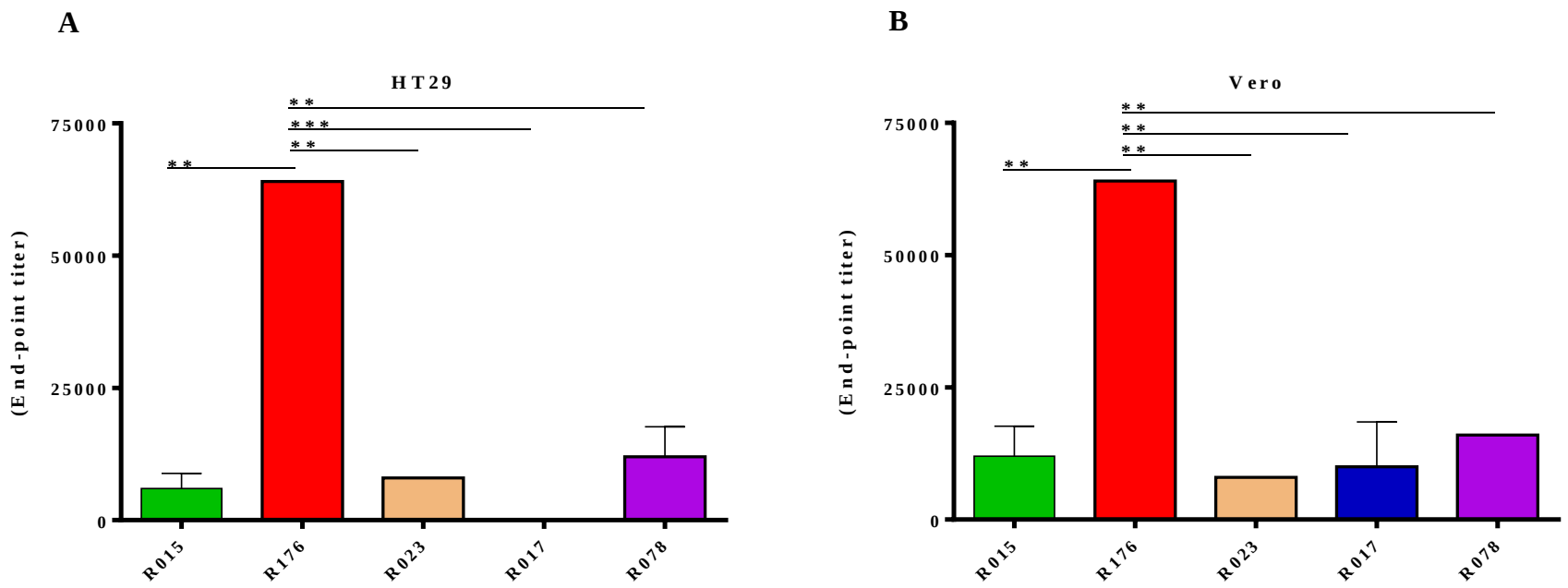

Figure 5.9: Cytotoxicity effect of clades one to five representative strains. Cytotoxicity of different strains were measured on two cell-lines, HT29 and Vero cells. *Panel A* shows the cytotoxic effect on HT29 cells, and *panel B* shows the cytotoxic effect of different strains on Vero cells. ** is a p-value of < 0.005 and *** is a p-value of <0.001. This experiment was replicated twice.

negative strain, it showed a very low cytotoxic effect on the HT29 cell. A similar result for R017 is also shown previously (Carter *et al.*, 2012).

5.4 Discussion

A previous study on initial colonisation showed that BclA1 is important for the initial colonisation in the host gut (Phetcharaburanin *et al.*, 2014). To extend on this, forty-five clinical strains isolated from humans were further studied. The study had two goals. First, it sought to characterise the clinical strains by identifying their ribotypes, as well as to determine the types of *bclA* genes they carry. The second goal was to test the colonisation of different strains with a different type of *bclA1 in vivo*. Identification of ribotypes revealed that the majority of the clinical isolates were R027, reported continuously as strains associated with high incidence and mortality (Kotila *et al.*, 2011, van Beurden *et al.*, 2016, Suzuki *et al.*, 2017). Recently, there have been reports of the emergence of new strains associated with higher incidence: R078, R002, and R106 (Hung *et al.*, 2016, van Dorp *et al.*, 2017). In fact, ~7% of the clinical strains were R078, making them the second highest among the clinical isolates. Next, all strains were checked for the presence or absence of *bclA* genes within the genome of the clinical isolates. Based on nucleotide analysis, all hypervirulent strains, R027, showed truncated[48] *bclA1*. The full-length *bclA1* was present in the majority of remaining strains and deleted in a minority. A new type of *bclA1* was discovered which belongs to R111, encoding for 71 amino acids peptide. The presence of *bclA2* in all strains was confirmed. Finally, the strains were arranged into different clades by comparing the PCR ribotypes to the ST identified in various publications.

Different reference *C. difficile* strains, based on the results from the clinical isolates,

were obtained from clades one to five and were checked for the type of *bclA1* they had. Interestingly, clade one strains had the full-length *bclA1* gene, clades two and three carried the truncated[48] *bclA1* gene, and, finally, in clades four and five, the *bclA1* gene was absent. This was an unusual and intriguing pattern, and this could have helped to illustrate and understand better the role of BclA1 in initial colonisation. The *bclA2* and *bclA3* genes were present in all strains with one exception: R023, which had a partially deleted *bclA2* gene. It is likely that *C. difficile* spores exhibited only one *bclA* gene in the past, and, through evolution, the presence of three *bclA* genes might have been a result of duplication. The higher expression of BclA on the exosporium might have played a significant role, e.g., more successful colonisation. The duplication of the *bclA* gene is also seen in *B. anthracis*, as this species carries *bclA* and *bclB* in its genome (Waller *et al.*, 2005). The presence of three BclA proteins on the spore surface might have been important for the colonisation of the *C. difficile* spore in the past, although Phetcharaburanin et al. (2014) showed that strains of CD630 with mutated *bclA2* or *bclA3* did not have any effect on colonisation. Therefore, this study was designed to investigate the role of BclA1 in the colonisation of different *C. difficile* strains, and we hypothesised that the colonisation, based on the BclA1 length, of clade one should be the highest, clades two and three should have reduced colonisation compared to clade one strain, and the least infective strains must be from clades four and five. However, the *in vivo* results were not as expected. In all mice from different clades, except for clade two, only 100 spores were needed to fully colonise the gut. It should be noted that the study on the role of BclA1 in initial colonisation was made by two isogenic strains—wild-type and BclA1 mutant—and, in this study, non-isogenic strains were used. A point to consider here is that deletion of *bclA1* as shown by Phetcharaburanin et al. (2014) results in a defect including sheet-like materials on the outermost layer. This also suggests that BclA could also play a role in exosporium and coat assembly. In *B. anthracis*, the BclB protein helps to maintain

the integrity of the exosporium (Thompson and Stewart, 2008). The defect of the outermost layer of *C. difficile* spores might result in a loss or reduction of the abundance of different proteins that might also play a role in colonisation, such as CotE, a spore coat protein, recently shown as being involved in binding to mucus and *C. difficile* with mutated *cotE* strains requiring higher spores to infect mice (Hong *et al.*, 2017a).

The most exciting finding of this study was that the strains from clade two (R176), a clade that is genetically similar to the hypervirulent strains, exhibited a lower ability to colonise than the other strains. Comparable colonisation patterns were seen previously from the strain R20291 (Phetcharaburanin *et al.*, 2014). This reduced colonisation ability of the hypervirulent strain observed in this study and reported previously raises the question as to whether the hypervirulent strains of clade 2 are less infective. Infectious dose varies significantly within different pathogenic species, and it is recognised as influencing the severity and dynamics of the disease (Regoes *et al.*, 2002, Li and Handel, 2014). It has been reported that the virulence of a pathogen is typically dose-dependent (Read *et al.*, 1999, Ebert *et al.*, 2000), although studies of the infectious hematopoietic necrosis virus have shown that the most dominant strains with high emergence showed increased virulence over time, yet the infectivity was unchanged (Breyta *et al.*, 2016). The seasonal influenza virus is another example as it is very common and exhibits low virulence, whereas the pandemic virus H1N1 is highly virulent, but it is of rare occurrence. A point to consider here is that mice were given a dose of clindamycin at day 1 and day 3, and although the animals were infected by *C. difficile* five days after second clindamycin dose, it is possible that a trace of clindamycin is still left in the gut. R20291 from the same clade as R176 has been shown to be clindamycin-sensitive previously (Kelly *et al.*, 2016). It could be possible that R176 is also sensitive to clindamycin and therefore once the spores germinate, clindamycin would

affect the growth and survival of this strain. For this reason, further *in vitro* experiments to test the sensitivity of the strains, especially R176, to clindamycin requires to be carried out.

Hypervirulent strains of *C. difficile* are generally known for their high sporulation and toxin production (Hopkins and Wilson, 2017), and, in this work, both toxin A and toxin B extracted from the caecum of the infected animal were significantly higher in the R176 group. This also agrees with the *in vitro* cytotoxicity effect of 176 on both HT29 and Vero cell lines. However, the CFU results for the extracted spores were similar to those for the groups. It should be noted that the mice were culled 24 h post-infection and a longer time may be required, as the highest concentrations of spores are usually detected on day 2 and/or 3 post-infection (Erikstrup *et al.*, 2015). This result also agrees with the *in vitro* sporulation of R176 as a similar spore count to that for the other strains was observed after 24 h. Although R176 showed higher sporulation in comparison to the other strains tested in this study, it cannot be the only reason as to why this strain disseminates faster and have a high incident; as it has been shown by Burns *et al.* (2011) that neither the total sporulation capacity nor the sporulation rate of different type hypervirulent R027 was higher than non-R027 strains. Therefore the hypervirulent strains such as R027 must have developed a different mechanism that helps them to disseminate faster and colonise better than the other non-R027 strains.

It is unclear why clades two and three present truncated BclA1 and in clade four and five BclA1 is absence. It is possible that the orthologs of the BclA family of proteins were necessary for some purposes in the past, and, as different *C. difficile* strains evolved, it seems that BclA1 is not as important; in fact, losing this protein might even favour these strains. For instance, BclA1 could be recognised by macrophages and internalised by phagocytosis;

thus, to avoid phagocytosis BclA1 has been truncated or deleted for the spores of clades two to five. In *B. anthracis*, BclA has been shown to be important for the attachment to macrophages, as the interaction between the spores and macrophages is necessary for the development of anthrax infection (Gu *et al.*, 2012). Yet other reports state that *B. anthracis* with deleted BclA was as infective as the isogenic wild-type and remained entirely virulent (Bozue *et al.*, 2007a, Brahmbhatt *et al.*, 2007). Apart from higher sporulation, there could be other factors that help for better dissemination of hypervirulent spores such as the ability for faster host invasion or more improved resistance to host defence mechanisms or antimicrobials. However, it is clear that BclA1 in a non-isogenic strain is not involved in initial colonisation.

5.5 Conclusion

In conclusion, the role of BclA1 in colonisation was investigated. Forty-five clinical strains were characterised for their ribotype, toxinotype and the presence of three *bclA* genes. Based on nucleotide analysis, the type of *bclA1* has been identified. Strains from different clades with different *bclA1* were tested for their ability to colonise the mice gut. All strains except the strain from clade two showed the need for 100 spores to colonise the gut. However, the hypervirulent strain R176 needed 1,000 spores to colonise the mice. Although the hypervirulent strains need a higher number of spores to colonise, the occurrence of CDI caused by hypervirulent such as R20291 (R027) is the highest. R176 showed higher toxin expression and sporulation than other tested strains. However high sporulation is not the only reason for the faster dissemination of this strain. Therefore, the hypervirulent strains must have developed a different mechanism, helping them to disseminate faster and occur more than non-hypervirulent strains.

CHAPTER 6

GENERAL DISCUSSION

6.1 Concerns over GMOs

In this study, a technique has been developed where proteins could successfully be expressed on the spore surface of *B. subtilis*. However, the clones constructed by this technique will be regarded as GMOs, which is one of the most controversial areas of science. The subject of GMOs has prompted considerable debate among scientists and the public. On the one hand, many believe that genetic engineering is necessary for the continued success of human experiments. It helps to improve the quality and production of plants, for example, and also improves human health. For instance, hypovitaminosis A or vitamin A deficiency, which is a lack of vitamin A in the blood, causes ~500,000 children each year in developing countries to go blind (Sommer *et al.*, 1981, De Luca, 1987). A treatment has been developed that contains enough vitamin A to stop the blindness and keep children healthier; it uses a genetically engineered strain of rice, currently known as golden rice (Ye *et al.*, 2000). In addition, in 2008, a group from the U.K. were able to introduce two genes isolated from *antirrhinum* plants, commonly known as snapdragons, into a tomato to produce a purple tomato that has an increased level of anthocyanins, and it was proven that the purple tomato tested in mice had anti-cancer properties (Butelli *et al.*, 2008). On the other hand, GMOs have met with substantial public opposition. Many people believe that GMOs are unsafe to use, since not enough safety tests have been done that can prove that GMOs do not affect human health and damage the environment. The public also believes that scientists do not

have enough understanding of the risks that can be introduced to human health by GMOs (Lucht, 2015). According to a report prepared by the Law Centre of IUCN, the World Conservation Union (2004), there are numerous possible environmental risks that can occur due to GMOs. For instance, introducing GMOs to an environment increases the possibility of interbreeding with wild-type organisms, leading to the disappearance of the novel traits in the wild-type unless it confers a selective advantage to the recipient. GMOs can have a competitive advantage over the wild-type organism if they are constructed in such a way that they can grow faster. This may allow them to become invasive, and if they spread into new habitats, they can cause economic and ecological damage. Furthermore, the effect of a change in an organism may extend well beyond an ecosystem. It is also impossible to eliminate the GMOs once they have spread into the environment. Once the GMOs spread, they may horizontally transfer the acquired foreign gene to other microorganisms, which may confer a novel trait in a different organism that could have adverse effects on human and animal health and the environment, e.g., the emergence of new diseases or enhanced pathogenicity. For example, horizontally transferring an antibiotic-resistance gene from GMOs into a pathogen would potentially compromise animal and human therapy (Bennett *et al.*, 2004). It is also possible that the introduced genes could be inserted into different genes, resulting in a novel gene. This could result in disrupting the endogenous gene, which could cause an unintended and unpredictable effect. Sometimes, gene transfer from GMOs to other organisms could have a long-term impact. It would take a thousand generations for a recipient organism to become the dominant form in a population, despite the relatively strong selection pressure (Nielsen and Townsend, 2004). While genetic engineering is used in many medical applications such as GM insulin, which is widely accepted, the debates really heat up when it comes to GM foods. Many animal studies have been designed and performed to understand the effect of GM food on animal health. Some studies have shown

that GM foods cause significant immune dysregulation, such as upregulation of cytokines associated with inflammation, allergy, and asthma (Finamore *et al.*, 2008, Kroghsbo *et al.*, 2008). Some animal studies also show altered function and structure of the liver, such as cellular changes that could accelerate ageing and accumulation of reactive oxygen species, as well as altered carbohydrate and lipid metabolism (Kılıç and Akay, 2008, Malatesta *et al.*, 2008). Another study reported that GM corn could affect fertility, as mice that were fed GM corn showed a significant decrease in both litter weight and offspring of mice over time (Cyran *et al.*, 2008). In addition, 400 genes that are known to control cell signalling, insulin regulation, cholesterol synthesis, and protein synthesis and modification expressed differently in mice fed GM corn.

Despite all public fears, protests, and articles on the risks of GMOs, there are already GMOs that have been approved to be safe and are currently being produced. For example, approved by the Food and Drug Administration (FDA) in the USA, genetically engineered planted soybeans and planted maize account for 94% and 93% of these crops (Perry *et al.*, 2016). The most extensive area for growing GM crops is in the USA, followed by Brazil and Argentina. 96% of the total produced cotton in these areas is GM (Wong and Chan, 2016). Although these GM crops are approved to be safe for human use and consumption, there may still be potential damage to the environment, including contamination of wild species, deuteriation of soil, water pollution, gene flow, and a reduction of biodiversity, which could be significant environmental concerns. Furthermore, GM techniques are used in developing various commercial and therapeutic strategies for both humans and animals. Farrar et al. (2005) developed a novel delivery system for biologically active molecules by genetically engineering a commensal bacterium, *Bacteroides ovatus* (Farrar *et al.*, 2005). Using this strategy, the production of the immunotherapeutic agent *in situ* can be controlled and

regulated by dietary factors, and the production of the biologically active molecule could result in the development of long-term immunotherapies for inflammatory gut diseases. In another study, a strain o*f Lactobacillus plantarum* that has been engineered to express a *Mycobacterium tuberculosis* fusion antigen on the bacterial cell wall was used as a potential vaccine strategy against tuberculosis (Kuczkowska et al., 2017). The nasal and oral administration of the recombinant *Lactobacillus plantarum* resulted in induction of specific immune responses in mice and in tuberculosis-positive humans, it has evoked proliferative antigen-specific T-cell responses in white blood cells. Moreover, a potential oral vaccine against *Helicobacter pylori* was developed by expressing a urease subunit of the animal pathogen *Helicobacter acinonychis*, which is recognised as a major antigen of *Helicobacter pylori* that induces an immune response and protection against infection (Hinc *et al.*, 2010b). one of the issues with GM vaccines or even GM crops is in the cloning procedure, where an antibiotic-resistance gene is used that serves as a selection marker for selecting transgenic cells. These genes can be passed to other species in the environment or human gut, giving rise to resistant or multi-resistant pathogens (Bennett *et al.*, 2004, Nicolia *et al.*, 2014). This is a serious issue, as the number of antibiotics is limited to pathogenic infections. There is also an increasing interest in using spores such as spores of *B. subtilis* and using them as a delivery vehicle. The problem with GM spores is the use of antibiotic-resistance gene in the cloning procedure and also their ability to survive indefinitely. If a spore-based vaccine is administered orally, they can germinate in the gut, re-sporulate, survive in the faeces and thus contaminate the environment. The GM spores can germinate if the condition is favourable in the environment and there is the possibility of passing the antibiotic-resistance gene to the same or other bacterial species. Another possibility is that the GM bacteria may become dominant, while wild-type may be gradually lost.

The possibility of the risks involved in GMOs raises several questions: are GMOs something that we should be worried about? Is there enough evidence that GMOs have negative consequences? Does society just have a negative attitude towards GMOs? There are many other dangers to humans that do not get as much attention as GMOs, e.g., plastic pollution and radioactive waste. The cloning system that has been developed in this study can be an excellent solution for the use of bacterial cells (such as *E. coli* or other non-spore-forming bacteria) or spores as a delivery vehicle for both commercial and therapeutic purposes for two reasons: i) this cloning method does not require an antibiotic-resistance gene as a selection marker and thus stops the concerns over passing on this gene within species and the related consequences; ii) The lack of expression of thymidylate synthase makes the strain become thymine dependent, and thus, the spores, should they spread in the environment, would have a low chance of survival, which would thus eliminate the concern over cross breading or any adverse effects on the environment.

6.2 Therapeutic and commercial advantages of clones constructed by *thy*-insertion cloning system

The species (*B. subtilis* spore) that has been chosen for microbial display in this study has some advantages over other species. There are reports on other species such as *Lactococcus lactis*, *Lactobacillus brevis*, and *E. coli* cells that are/can be used for microbial display by inserting a chimeric gene into their *thy* gene and making the clones thymine dependent (Åvall-Jääskeläinen *et al.*, 2002, Steidler *et al.*, 2003, Park *et al.*, 2013). However, firstly, these species are non-spore forming and thus are more sensitive to harsh environmental factors in comparison to robust spores. Secondly, these species have only one *thy* gene, meaning that only one copy of the chimeric gene can be inserted into their genome

without disrupting other genes, and according to this study, a higher number of chimeric proteins is expressed when two copies of a chimeric gene are inserted into the genome.

In this study, it was also shown that various types of protein could be displayed on the surface that can be used both for therapeutic or industrial purposes with various advantages. Enzymes such as xylanase and phytase have gained a lot of interest from commercial industries because of the potential for reducing phytate in animal feed and foods for humans (Konietzny and Greiner, 2004). Expression and purification of the phytase enzyme could be costly. Moreover, expressing enzymes on the surface of cells or spores can enhance the stability of enzymes (Gribenko *et al.*, 2009). Potot et al. (2010) expressed phytase enzyme on the surface of the *B. subtilis* spore by fusing it to coat proteins. If these spores are used commercially for an animal feed, then a high number of GM spores will be shed in the faeces of animals that eat the food, leading to higher exposure to GM spores in the environment. However, if phytase is displayed on a spore surface using the *thy*-insertion cloning system, then the concern over exposing to environment to GM spores will be significantly reduced.

Stabilising protein on the spore surface could also be used to optimise some of the therapeutic procedures that are currently used. For instance, monoclonal antibodies neutralising toxins of *C. difficile* resulted in a significant reduction of CDI relapse (Lowy *et al.*, 2010, Navalkele and Chopra, 2018). In this study, stabilising anti-*C. difficile* toxin A on the spore surface resulted in denaturing the toxin and reducing its cytotoxicity. It is likely that denaturing the toxin by anti-toxin antibodies optimises the efficiency and treatment of CDI relapse further, as stabilising the protein on the cell or spore surface results in enhanced stability and leads to better delivery to the target (Gribenko *et al.*, 2009, Nguyen *et al.*, 2013).

Thus, the strategy introduced in this study for denaturing toxins may be a better potential way to treat CDI.

PP108 is a spore vaccine that has been shown to induce an immune response in hamsters and thus avoid the colonisation of *C. difficile* and prevent CDI (Hong *et al.*, 2017b). Two different antibiotic-resistance genes have been used in the cloning procedure to produce PP108. However, although these spore-based vaccines could be a potential treatment for CDI, the antibiotic-resistance gene could be a risk to the environment, and potentially, the chimeric genes could also be being passed to other species. A better version of this vaccine has been constructed using the *thy*-insertion cloning system, and it has been proven that it induces an immune response similar to PP108, making it a better and safer vaccine for treating CDI. Moreover, the spore-based vaccine produced by the *thy*-insertion cloning system, as well as eliminating concern over the environment, can also be safer for humans in comparison to other vaccine strategies. For example, live attenuated vaccines that are created by reducing the virulence factor of a microbe and are still viable but no longer cause disease have been amongst the most successful interventions in medical history. There are many different types of live attenuated mucosal vaccines, e.g., Dukoral, which targets *Vibrio cholerae*, or Vivotif, which targets *Salmonella typhi* (Azegami *et al.*, 2014). However, there is a possibility that the weakened pathogen reverts to virulence through componentry mutations elsewhere in the genome, back mutation of the attenuating mutation, and recombination (Hanley, 2011).

6.3 Role of BclA proteins

Depside the considerable work on the BclA protein of *B. anthracis* the exact role of BclA is not explicit, and some results in different studies contradict each other. Binding of

B. anthracis spores to phagocytic cells and being internalised seems to be an essential step in the infection, as *B. anthracis* possibly uses macrophages as a vehicle, thereby disseminating in the host (Gu *et al.*, 2012). It has been shown that CD14, an extracellular protein that is anchored to the membrane, can recognise and bind to rhamnose residues of BclA and function as a co-receptor for spores attaching integrin Mac-1. This will promote inside-out activation of the Mac-1 (CD11b/CD18) by involving TLR2 signalling, thereby enhancing spore uptake by phagocytic cells, i.e., macrophages (Oliva *et al.*, 2009). Mice lacking Mac-1 or CD14 showed higher resistance to subcutaneous infection with *B. anthracis* spores (Oliva *et al.*, 2008). Moreover, Buzue et al. (2007) showed that spores with deleted BclA are internalised by macrophages and have the same virulence to the same extent as the wild-type with BclA intact, suggesting that other proteins on the spore surface (exosporium) are recognised by phagocyte receptors. Another possibility for the role of BclA is that it helps to direct the spores toward phagocytic cells, as *B. anthracis* spores with deleted BclA present higher adherence to fibroblasts and endothelial and epithelial cells but not to macrophages (Bozue *et al.*, 2007b). BclA can also play a significant role in protecting the *B. anthracis* spores (Gu *et al.*, 2012, Wang *et al.*, 2016). BclA is involved in the activation of the classical complement pathway, a primary mechanism for spore phagocytosis. Deposition of C3b, a protein of the complement system and promotor of spore phagocytosis, is dependent on C1b recruited by BclA. Spore phagocytosis by mice macrophages resulted in significant reduction in spores with deleted BclA, and it appeared that overall survival of internalised spores by complement opsonisation was better than spores phagocytes by other mechanisms (Gu *et al.*, 2012).

C. difficile does not require being internalised by macrophages for infection, and thus, the three BclA proteins may have different roles. An in-depth investigation of the role

of the three BclA orthologues was conducted by Phetcharaburanin et al. (2014), who showed that only BclA1 is involved in initial colonisation of *C. difficile* spores. The result in this study, except for hypervirulent strains, showed that non-isogenic strains with differences in BclA1 length or where BclA1 is absent showed similar colonisation ability as CD630, which exhibited the full-length BclA1. This may then disprove that BclA1 is involved in colonisation and increases the infectivity in non-isogenic strains. It is likely that BclA1 proteins play a role on the integrity of the exosporium and correct orientation, localisation, and stability of proteins that may have a role in colonisation. It is also possible that in the past, similarly to *B. anthracis*, it used BclA for protection. A point to consider here is that CD630 and VPI 10463 (a non-virulence and high-toxin-producing strain) with full BclA1 are not as infective as hypervirulent R20291, although they have a lower requirement for the number of spores to colonise. This brings up the possibility that other molecules or mechanisms may be involved that increase the infectivity of spores. Alternatively, losing BclA1 may favour the spores from defence mechanisms, as in *B. anthracis*, BclA is shown to be important to induce the complement system and meditate phagocytosis (Bozue *et al.*, 2007a, Oliva *et al.*, 2009, Gu *et al.*, 2012). Moreover, most strains with high prevalence seem to be those either with truncated or deleted BclA. For instance, R017, in which the BclA1 is deleted, is reported to have the highest incidence in Asia. In addition, R027 and R078 with truncated and deleted BclA1 respectively, caused a significant outbreak in Europe and North America (Collins *et al.*, 2013).

Both in this study and others (Merrigan *et al.*, 2010, Vohra and Poxton, 2011), it has been found that hypervirulent strains have enhanced sporulation. Increasing the sporulation of hypervirulent strains could possibly result in faster dissemination of the spores and could be linked to the massive outbreak of these strains. The most predominant strain of *C. difficile*

in Hong Kong, R002, has been associated with enhanced sporulation (Cheng *et al.*, 2011). Enhancing the sporulation could result in faster sporadic aerial dissemination, and this may be an explanation of the high prevalence of newly emerging strains, including R106 in the UK (Sundram *et al.*, 2009), R018 and R078 in Italy, and R018 in Japan and Korea (Baldan *et al.*, 2015).

It is not clear at this stage precisely what the role of BclA proteins on the surface of *C. difficile* spores is, with such high abundance and having three orthologues. Possibly, the best strategy to understand the role of these proteins is creating isogenic double or triple mutant BclA genes and investigating their ability for colonisation, their protection against macrophages, the integrity of exosporium, and the abundance of the proteins on the spore surface in different non-isogenic strains, though this could be costly. Replacing the full-length BclA1 gene in strains such as R027and R078 with truncated and deleted BclA1 respectively could also be another strategy to better understand the role of BclA1. It should be noted that in non-isogenic strains, BclA2 and BclA3 may also play roles. Thus, constructing single, double, and triple BclA mutants in different, non-isogenic strains and performing various tests is necessary and can be very costly if ID_{50} is performed.

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Appendices A

thyA and *thyB* sequences cloned in pThyA and pThyB

Plasmid	Sequence[1]
pThyA	CAGATCATATAAGGAATGAACCGCTGCCAAATATCATAAAAAAGTTGTTAAT GATCAAATGAATGAAATTAGAGAGAATTTATTTTAAAGAAAGCCCAATTGCA CATGGACAAATGACAATTGATATTGAGGTAAAATACAATGCATTAAATCAGA AAAAGCTATTGGAGGATTTAATGTGTTTAGACAATTTCCAATTTGGTATACAC AAACACCTGACTATTTGAATTTTTATGTACCGCAATATCAAACCATTTCGTAT AATCCTCAACAATGTTATCAACGGTGTATGTACCAAACTGGCGGTAACTATGA GCTATGTGACAGACTATGTTATGGAGAAATACAGGTGTAAAAGAGGGGGATT AACTCCTCTTTAAACACACAGTGAGTGGAATAAGATCCTCACTTTATCTGCAA GTGCTTAGTATTTGCGATAATATTGCATTCGTAATAAATTATGCTTAGCAACT GAAAATGAAAGAAGGATATGAATAGTC**ATG**ACGCAATTCGATAAACAATAC AATTCAATTATAAAGGATATTATCAATAATGGAATCTCAGACGAAGAGTTTG ATGTAAGAACCAAGTGGGACTCAGATGGAACACCGGCACATACTCTAAGTGT AATCAGTAAGCAAATGAGATTCGACAACTCAGAGGTTCCGATTTTAACGACA AAAAAGGTTGCCTGGAAAACAGCCATTAAAGAGTTGCTCTGGATTTGGCAGC TGAAATCTAATGATGTTAATGATTTAAACATGATGGGCGTCCATATTTGGGAT CAGTGGAAACAAGAAGACGGAACCATCGGACATGCATATGGATTTCAGCTGG GGAAGAAAAACAGAAGTCTAAATGGAGAAAAAGTGGATCAGGTAGACTATC TTCTTCATCAAT*AAGCTTGCATGCCTGCAGGTCGACTCTAGAGGATCCCCCGGGG* *GTACCGAGCTCGAATTC*TGAAGAACAATCCATCTTCACGCAGACACATTACAAT GCTGTGGAATCCTGATGAATTAGACGCAATGGCCTTAACGCCATGTGTATACG AGACACAATGGTACGTTAAACATGGGAAACTCCACCTTGAGGTAAGAGCACG GAGCAATGATATGGCATTGGGAAATCCATTCAATGTATTCCAGTATAATGTGT TGCAGCGCATGATTGCTCAAGTGACTGGTTATGAGCTTGGTGAATATATCTTT AACATTGGGGATTGCCATGTGTACACACGTCATATAGACAATTTGAAAATTCA AATGGAAAGAGAACAGTTTGAAGCACCTGAACTATGGATCAATCCTGAAGTG AAAGATTTTTATGACTTTACCATTGATGATTTCAAGTTAATCAACTATAAACA TGGGGACAAGCTTTTATTTGAGGTAGCGGTTTAATGCTGCCTTTTTATTGTGCA GTGAATAGATAGCAGGTATCCTAATTTCATTAAGCAATCTGGAAGATGAATA AAAATTGAAGGACAAACACGTATAATACATAAAAAAGATTAACTCTACAGTT AATCTTTTTTATTCAGAAGAAAATATCCTAACTTTGAAACTAAATACAAAGTA AAAGCAATCATTACAGTTCTAGATATTACAATTCCATGAATAGCTAGATCATA TCCAGCAGGTATCAACGCATTTGTATTACACATAAAATATATAGATATTAGAA GTGCTACAATAACTAAAATCATTCCAAAAAGACTTGTTTTTTCATATTTCATA CCAATTTCCACCCTTATTAAAGTTAGGTTTAAACAAAAGAGCTGAAGAAACG AACTATGACCAGTATGCTCCAAGGAAAACCGCCAGACAATGCTGGCGGCTTT TTGCTGCTTCGTTTATTTATTAACAGAGATCGTAACGTTATTTCCTGCAACTGA AACCTTTGCGAAATCC-3’
pThyB	CCAAATCTGCCGCTCAGTGTTTGCATGGAGAATGTAGAAAAAGTCCTGAACA AACGTGAAATTATTCATGCTGTTTTGACAGGCCTTGCACTCGATCAGCTTGCA GAACAGAAACTTCTCCCCGAACCGCTGCAGCACCTTGTTGAAACGGATGAAC CGCTTTACGGCATAGATGAAATTATCCCGCTTTCAATCGTTAATGTGTACGGG TCGATCGGTTTGACCAATTTCGGTTATTTGGATAAAGAGAAGATTGGAATTAT TAAGGAACTTGATGAAAGTCCAGACGGTATTCACACCTTTTTGGATGATATTG TGGCAGCTCTTGCTGCAGCAGCGGCGAGCAGAATTGCACATACGCATCAGGA

	TCTGCAAGATGAAGAAAAAGAACAGGATGAAAAGCCTGTCGTCAGCTGACTA TAAAAAAATCATTTCTGGGTTCAGAAATGATTTTTTATTGTGTTACACTACTA GAAGACTACTTTTAAAGGATGAAAAAA**ATG**AAACAGTATAAGGATTTCTGCA GACATGTTTTAGAGCATGGTGAGAAAAAGGGAGACCGGACTGGGACCGGAA CAATCAGCACTTTCGGATATCAAATGAGATTTAATTTACGGGAAGGCTTTCCG ATGCTCACCACTAAAAAACTCCACTTTAAATCAATTGCGCATGAACTGCTGTG GTTCTTAAAAGGAGATACGAATGTACGCTATCTGCAGGAAAACGGAGTGCGA ATCTGGAATGAGTGGGCTGATGAAAACGGTGAACTTGGACCTGTATATGGCT CCCAATGGCGTTCTTGGCGGGGAGCTGATGGAGAAACCATTGATCAAATTTCC CGTCTTATTGAAGATATTAAAACAAATCCGAACTCCAGACGCTTAATCGTCAG CGCCTGGA*AAGCTTGCATGCCTGCAGGTCGACTCTAGAGGATCCCCCGGGGGTAC* *CGAGCTCGAATTCTG*ATGTTGGTGAAATTGATAAAATGGCGTTGCCGCCGTGCC ATTGCCTGTTCCAATTCTATGTGTCTGACGGCAAGCTGTCCTGTCAGCTGTATC AGCGCTCTGCCGATGTTTTCTTAGGTGTGCCGTTTAATATTGCATCTTATGCCC TCCTAACCATGATCATTGCTCATGTGACTGGGCTTGAACCGGGCGAGTTCATC CATACGTTTGGTGATGTTCATATTTACCAAAATCATATTGAACAAGTCAATTT GCAGCTGGAAAGAGATGTTAGACCGCTTCCGCAGCTTCGTTTCGCCAGAAAG GTTGATTCTATTTTTAACTTTGCATTTGAGGACTTTATCATCGAGGATTATGAT CCGCATCCTCATATAAAAGGGGCGGTCAGCGTATGATTTCATTCATTTTTGCG ATGGATGCCAACAGGCTTATCGGCAAAGACAATGATTTGCCGTGGCATTTGCC CAATGATCTTGCATACTTTAAGAAAATAACATCGGGCCATTCAATCATTATGG GCCGGAAAACATTTGAATCGATCGGACGTCCGCTTCCAAATCGGAAAAATAT TGTCGTTACCTCAGCGCCGGATTCAGAATTTCAGGGATGCACGGTTGTCAGTT CATTAAAGGATGTACTGGACATTTGTTCAGGCCCTGAAGAATGCTTTGTGATC GGAGGGGCTCAGCTCTATACGGACCTGTTCCCTTATGCGGACAGACTGTATAT GACGAAAATTCATCACGAGTTTGAGGGTGACCGTCACTTTCCTGAATTTGATG AATCCAATTGGAAGCTGGTTTCTTCTGAGCAGGGGACCAAAGACGAAAAAAA CCCGTATGATTACGAATTTCTAATGTATGAAAAAAAGAAATCTTCTAAAGCGG GAGGATTTTAATTGGTTCGCTACAGCCTTCTAGTGGTTTATATTGTGTATATGC TGTTAAAAAATATGAAACAATTATTTAATCAAACAATGCTCGATCCCCGTCTG TCATACAAAAAACAGATGGCTCTTGTGTACGAACAGCCAAAGGCGTTTTTAG AAGGCTGTATCGGCATCTCCGGTTCAGTTGTGACGATCCATCAGCCAGA-3’

[1] The *thy* ORF (5’-3’) is shown in grey shading with the start codon in bold and flanking DNA (unshaded). The MCS is in italics. Primer annealing sites (forward and reverse) used to verify insertion are underlined

Appendices B

Amino acid sequences of the fusion genes[1]

CotC-VP26

MGYYKKYKEEYYTVKKTYYKKYYEYDKKDYDCDYDKKYDDYDKKYYDHDKKDYDYVVEYKK
HKKHYKLMEFGNLTNLDVAIIAILSIAIIALIVIMVIMIVFNTRVGRSVVANYDQMMRVPIQRRAKV
MSIRGERSYNTPLGKVAMKNGLSDKDMKDVSADLVISTVTAPRTDPAGTGAENSNMTLKILNNTG
VDLLINDITVRPTVIAGNIKGNTMSNTYFSSKDIKSSSSKITLIDVCSKFEDGAAFEATMNIGFTSKNVI
DIKDEIKKK

CotB-VP28

MSKRRMKYHSNNEISYYNFLHSMKDKIVTVYRGGPESKKGKLTAVKSDYIALQAEKKIIYYQLEHV
KSITEDTNNSTTTIETEEMLDADDFHSLIGHLINQSVQFNQGGPESKKGRLVWLGDDYAALNTNEDG
VVYFNIHHIKSISKHEPDLKIEEQTPVGVLEADDLSEVFKSLTHKWVSINRGGPEAIEGILVDNADGH
YTIVKNQEVLRIYPFHIKSISLGPKGSYKKEDQKNEQNQEDNNDKDSNSFISSKSYSSSKSSKRSLKSS
DDQSSKLMDLSFTLSVVSAILAITAVIAVFIVIFRYHNTVTKTIETHTGNIETNMDENLRIPVTAEVGS
GYFKMTDVSFDSDTLGKIKIRNGKSDAQMKEEDADLVITPVEGRALEVTVGQNLTFEGTFKVWNNT
SRKINITGMQMVPKINPSKAFVGSSNTSSFTPVSIDEDEVGTFVCGTTFGAPIAATAGGNLFDMYVHV
TYSGTETE

CotB-TcdA$_{26\text{-}39}$

MSKRRMKYHSNNEISYYNFLHSMKDKIVTVYRGGPESKKGKLTAVKSDYIALQAEKKIIYYQLEHV
KSITEDTNNSTTTIETEEMLDADDFHSLIGHLINQSVQFNQGGPESKKGRLVWLGDDYAALNTNEDG
VVYFNIHHIKSISKHEPDLKIEEQTPVGVLEADDLSEVFKSLTHKWVSINRGGPEAIEGILVDNADGH
YTIVKNQEVLRIYPFHIKSISLGPKGSYKKEDQKNEQNQEDNNDKDSNSFISSKSYSSSKSSKRSLKSS
DDQSSKLASTGYTSINGKHFYFNTDGIMQIGVFKGPNGFEYFAPANTHNNNIEGQAILYQNKFLTLN
GKKYYFGSDSKAVTGLRTIDGKKYYFNTNTAVAVTGWQTINGKKYYFNTNTSIASTGYTIISGKHFY
FNTDGIMQIGVFKGPDGFEYFAPANTDANNIEGQAIRYQNRFLYLHDNIYYFGNNSKAATGWVTID
GNRYYFEPNTAMGANGYKTIDNKNFYFRNGLPQIGVFKGSNGFEYFAPANTDANNIEGQAIRYQNR
FLHLLGKIYYFGNNSKAVTGWQTINGKVYYFMPDTAMAAAGGLFEIDGVIYFFGVDGVKAP

CotC-TcdA$_{26\text{-}39}$

MGYYKKYKEEYYTVKKTYYKKYYEYDKKDYDCDYDKKYDDYDKKYYDHDKKDYDYVVEYKK
HKKHYKLASTGYTSINGKHFYFNTDGIMQIGVFKGPNGFEYFAPANTHNNNIEGQAILYQNKFLTLN
GKKYYFGSDSKAVTGLRTIDGKKYYFNTNTAVAVTGWQTINGKKYYFNTNTSIASTGYTIISGKHFY
FNTDGIMQIGVFKGPDGFEYFAPANTDANNIEGQAIRYQNRFLYLHDNIYYFGNNSKAATGWVTID
GNRYYFEPNTAMGANGYKTIDNKNFYFRNGLPQIGVFKGSNGFEYFAPANTDANNIEGQAIRYQNR
FLHLLGKIYYFGNNSKAVTGWQTINGKVYYFMPDTAMAAAGGLFEIDGVIYFFGVDGVKAP

CotB-SA

MSKRRMKYHSNNEISYYNFLHSMKDKIVTVYRGGPESKKGKLTAVKSDYIALQAEKKIIYYQLEHV
KSITEDTNNSTTTIETEEMLDADDFHSLIGHLINQSVQFNQGGPESKKGRLVWLGDDYAALNTNEDG
VVYFNIHHIKSISKHEPDLKIEEQTPVGVLEADDLSEVFKSLTHKWVSINRGGPEAIEGILVDNADGH
YTIVKNQEVLRIYPFHIKSISLGPKGSYKKEDQKNEQNQEDNNDKDSNSFISSKSYSSSKSSKRSLKSS
DDQSSIQDPSKDSKAQVSAAEAGITGTWYNQLGSTFIVTAGADGALTGTYESAVGNAESRYVLTGR
YDSAPATDGSGTALGWTVAWKNNYRNAHSATTWSGQYVGGAEARINTQWLLTSGTTEANAWKS
TLVGHDTFTKVKPSAASIDAAKKAGVNNGNPLDAVQQ

CotB-subtilisin E

MSKRRMKYHSNNEISYYNFLHSMKDKIVTVYRGGPESKKGKLTAVKSDYIALQAEKKIIYYQLEHV
KSITEDTNNSTTTIETEEMLDADDFHSLIGHLINQSVQFNQGGPESKKGRLVWLGDDYAALNTNEDG
VVYFNIHHIKSISKHEPDLKIEEQTPVGVLEADDLSEVFKSLTHKWVSINRGGPEAIEGILVDNADGH
YTIVKNQEVLRIYPFHIKSISLGPKGSYKKEDQKNEQNQEDNNDKDSNSFISSKSYSSSKSSKRSLKSS
DDQSS**KL**AQSVPYGISQIKAPALHSQGYTGSNVKVAVIDSGIDSSHPDLNVRGGASFVPSETNPYQD
GSSHGTHVAGTIAALNNSIGVLGVAPSASLYAVKVLDSTGSGQYSWIINGIEWAISNNMDVINMSLG
GPTGSTALKTVVDKAVSSGIVVAAAAGNEGSSGSSSTVGYPAKYPSTIAVGAVNSSNQRASFSSAGS
ELDVMAPGVSIQSTLPGGTYGAYNGTSMATPHVAGAAALILSKHPTWTNAQVRDRLESTATYLGNS
FYYGKGLINVQAAAQ

CotB-amylase E

MSKRRMKYHSNNEISYYNFLHSMKDKIVTVYRGGPESKKGKLTAVKSDYIALQAEKKIIYYQLEHV
KSITEDTNNSTTTIETEEMLDADDFHSLIGHLINQSVQFNQGGPESKKGRLVWLGDDYAALNTNEDG
VVYFNIHHIKSISKHEPDLKIEEQTPVGVLEADDLSEVFKSLTHKWVSINRGGPEAIEGILVDNADGH
YTIVKNQEVLRIYPFHIKSISLGPKGSYKKEDQKNEQNQEDNNDKDSNSFISSKSYSSSKSSKRSLKSS
DDQSS**KL**ETANKSNEVAASSVKNGTILHAWNWSFNTLTQNMKEIRDAGYAAIQTSPINQVKEGNQ
GDKSMRNWYWLYQPTSYQIGNRYLGTEQEFKDMCAAAEKYGLKVIVDAVINHTTSDYAAISDEIK
RIPNWTHGNTQIKNWSDRWDVTQNSLLGLYDWNTQNTEVQTYLKGFLERALNDGADGFRYDAAK
HIELPDDGNYGSRFWPNITNTSAEFQYGEILQDSASRDTAYANYMNVTASNYGHSIRSALKNRNLSV
SNISHYASDVSADKLVTWVESHDTYANDEEESTWMSDDDIRLGWAVIGSRSGSTPLFFSRPEGGGN
GVRFPGKSQIGDRGSALFKDQAITAVNQFHNVMAGQPEELSNPNGNNQIFMNQRGSKGVVLANAG
SSSVTVNTSTKLPDGRYDNRAGAGSFQVANGKLTGTINARSAAVLYSDDIGNAPQVFLENYQTGAV
HSFNDQLTVTLRANAKTTKAVYQINNGQQTAFKDGDRLTIGKGDPIGTTYNIRLTGTNGEGAERTQ
EYTFVKKDPAQTNIIGYQNPDHWGQVNAYIYKHDGGGAIELTGSWPGKAMTKNANGIYTLTLPAN
ADTANAKVIFNNGSAQVPGQNQPGFDYVQNGLYNNSGLNGYLPH

[1] The fusion is made of the anchoring motif (Black), heterologous protein (Red) and a HinDIII site (Blue and Bold) except for CotB_SA.