

T.R.
GEBZE TECHNICAL UNIVERSITY
GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

**MOLECULAR CHARACTERIZATION OF PEPTIDYL-
PROLYL-CIS/TRANS-ISOMERASES (PPIASES) AND
IDENTIFICATION OF THEIR INTERACTING PARTNERS IN
NOSOCOMIAL PATHOGEN CLOSTRIDIUM DIFFICILE**

MUSTAFA SAFA KARAGÖZ
A THESIS SUBMITTED FOR THE DEGREE OF
MASTER OF SCIENCE
DEPARTMENT OF MOLECULAR BIOLOGY AND GENETICS

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**PEPTİDİL-PROLİL-CİS/TRANS-
İZOMERAZLARININ (PPİAZLARIN)
NOZOKOMİYAL PATOJEN CLOSTRIDIUM
DIFFICILE'DE MOLEKÜLER
KARAKTERİZASYONU VE ETKİLEŞİM
ORTAKLARININ TANIMLANMASI**

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YÜKSEK LİSANS TEZİ
MOLEKÜLER BİYOLOJİ VE GENETİK ANABİLİM DALI

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SUMMARY

Clostridium difficile is a toxin producing enteric pathogen leading to often hospital acquired infections worldwide. It causes the disease *Clostridium difficile* infection (CDI). The phenotype of the toxin caused CDI ranges from mild diarrhea to toxic megacolon and can result in death.

Peptidyl-prolyl-*cis/trans*-isomerases (PPIases) are ubiquitous proteins with protein folding and chaperoning activities. As such they, on the one hand, are part of the very general protein maturation pathway. On the other hand, they can be involved in very specific protein-protein interactions supporting unique phenotypes related to important physiological or pathogenic features. In Gram-positive bacteria the most analysed PPIases are the surface exposed and membrane-associated parvulins PrsA of *Bacillus subtilis* and its homologs in *Listeria monocytogenes*, streptococci and staphylococci. These are post-translocational protein folding enzymes that have been connected to several phenotypes including antibiotic resistance, hemolysis or invasion. In *B. subtilis* PrsA is even essential for cell viability. In contrast, *C. difficile* PPIases are completely unexplored regarding their biochemical and biological properties.

We analysed the cyclophilin-type PPIase PpiB of *C. difficile* and found that this enzyme could be associated with the cysteine metabolism. Besides, PpiB can repress the toxic effect of the pathogen, which should be studied more detailed. Furthermore, we found that PpiB interacts with other proteins but the interaction partners need further analysis to understand the role of PpiB in various cellular mechanisms like protein folding, toxin production and secretion, DNA interaction and gene regulation.

Key Words: *Clostridium difficile*, PPIases, PpiB, *Clostridium Difficile* Infection (CDI)

ÖZET

Clostridium difficile, dünya genelinde hastane kaynaklı enfeksiyonlara yol açan, toksin üreten bir patojendir ve *Clostridium difficile* enfeksiyonu (CDE) hastalığına neden olur. Toksinin neden olduğu CDE'nin fenotipi hafif ishalden toksik megakolona kadar değişir ve bu hastalık ölümle sonuçlanabilir.

Peptidil-prolil-*cis/trans*-izomerazlar (PPİazlar), protein katlama ve şaperon aktivitelerine sahip proteinlerdir. Bu şekilde, bir yandan, genel protein olgunlaşma aşamalarının bir parçasıdır. Öte yandan, önemli fizyolojik veya patojenik özelliklerle ilgili fenotipleri destekleyen çok spesifik protein-protein etkileşimlerine dahil olabilirler. Gram-pozitif bakterilerde en çok analiz edilen PPİazlar, *Bacillus subtilis*'e ait yüzeyde ve membrana bağlı parvulin PrsA ve bunun *Listeria monocytogenes*, streptokoklar ve stafilokoklardaki homologlarıdır. Bu mikroorganizmalarda antibiyotik direnci, hemoliz veya invazyon gibi çeşitli fenotiplerle alakalı post translokasyonel protein katlama enzimleridir. Hatta *B. subtilis*'te PrsA hücre canlılığı için bile gereklidir. Bununla beraber *C. difficile* PPİazları, biyokimyasal ve biyolojik açıdan araştırılmamış olup bu patojenin fizyolojisi ve virülansına katkıları bilinmemektedir.

Bu çalışma dahilinde *C. difficile*'de siklofilin tipi PPİaz PpiB'yi analiz ettik ve bu enzimin sistein metabolizması. Ayrıca PpiB bu patojenin toksik etkisinin baskılanmasıyla alakalı olabilir ve bu konu daha detaylı çalışılması gerekir. Dahası, PpiB'nin bazı proteinlerle etkileşebileceğini gösterdik fakat olası etkileşim ortaklarının PpiB'nin protein katlanması, toksin üretimi ve salınımını, DNA etkileşimi ve gen regülasyonu gibi çeşitli hücresel mekanizmalardaki rolünü anlamak için bu etkileşim ortaklarının daha fazla analize ihtiyaç duyduğu sonucuna vardık.

Anahtar Kelimeler: *Clostridium difficile*, PPİazlar, PpiB, *Clostridium Difficile* Enfeksiyonu (CDI)

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LIST of ABBREVIATIONS and ACRONYMS

<u>Abbreviations</u> <u>and Acronyms</u>	<u>Explanations</u>
μL	: Microliter
mL	: Milliliter
v	: Volume
g	: Gram
l	: Liter
nm	: Nanometer
mM	: Millimolar
mA	: Milliampere
H ₂ O	: Water
dH ₂ O	: Distilled water
ddH ₂ O	: Double distilled water
CO ₂	: Carbon-dioxide
H ₂	: Hydrogen
N ₂	: Nitrogen
-Cl	: Chloride
HCl	: Hydrochloric acid
NaCl	: Sodium chloride
MgCl ₂	: Magnesium chloride
Na ₂ S ₂ O ₃	: Sodium thiosulphate
AgNO ₃	: Silver nitrate
Na ₂ CO ₃	: Sodium carbonate
Bis	: Bisacrylamide
Acryl	: Acrylamide
Tris	: Tris(hydroxymethyl)aminomethane
TCA	: Taurocholic acid
DCA	: Deoxycholic acid
LCA	: Lithocholic acid
UDP-glucose	: Uridine diphosphate glucose
GTPase	: Guanosine triphosphatase
EDTA	: Ethylenediaminetetraacetic acid

EtOH	: Ethanol
TBS	: Tris Buffered Saline
PBS	: Phosphate Buffered Saline
FBS	: Fetal Bovine Serum
SDS	: Sodium dodecyl sulfate
APS	: Ammonium persulfate
PFA	: Paraformaldehyde
MS	: Mass spectrometry
MIC	: Minimal inhibitory concentration
DAPI	: 4',6-Diamidino-2-Phenylindole
DMEM	: Dulbecco's Modified Eagle Medium
TEMED	: Tetramethyl ethylenediamine
DMSO	: Dimethyl sulfoxide
TYG	: Tryptone- Yeast Extract-Glucose
TY	: Tryptone- Yeast Extract
BHIS	: Brain-Heart-Infusion supplemented with salt
OD	: Optical Density
DNA	: Deoxyribonucleic acid
RNA	: Ribonucleic acid
mRNA	: messenger RNA
mTOR	: Target of rapamycin
Rac1	: Ras-related C3 botulinum toxin substrate 1
Cdc42	: Cell Division Cycle 42
RhoA	: Ras homolog gene family, member A
PaLoc	: Pathogenicity Locus
FMT	: Fecal Microbiome Treatment
ER	: Endoplasmic Reticulum
CsA	: Cyclosporin A
Ess1	: A parvulin of <i>Saccharomyces cerevisiae</i>
Pin1	: A parvulin of <i>Homo sapiens</i>
RRM	: RNA-binding domain
TPR	: Tetratricopeptide repeat
FK1	: Active FKBP isomerase domain

FK2	: Inactive FKBP isomerase domain
CBD	: Calmodulin binding domain
WW	: Protein-interaction domain
Sp	: <i>Schizosaccharomyces pombe</i>
At	: <i>Arabidopsis thaliana</i>
Sc	: <i>Saccharomyces cerevisiae</i>
Hs	: <i>Homo sapiens</i>
Mip	: FKBP12 homolog of <i>Legionella pneumophila</i>
FKBP	: FK506 binding protein
PrsA	: A parvulin of <i>Bacillus subtilis</i>
CypA	: Cyclophilin A
MAPK	: Mitogen-Activated Protein Kinase
COX-2	: Cyclooxygenase 2
NF- κ B	: Nuclear Factor 'kappa-light-chain-enhancer' of activated B-cells
TNF	: Tumor Necrosis Factor
IL	: Interleukin
Spo0A	: Master regulator of sporulation
PpiB	: Peptidyl-Prolyl- <i>cis/trans</i> -Isomerase B
Tcd	: <i>Clostridium difficile</i> toxin
CDT	: Cytotoxic distending toxins
CDI	: <i>Clostridium difficile</i> infection
PPIase	: Peptidyl-Prolyl- <i>cis/trans</i> -Isomerase
USA	: United States of America
NIH	: National Institute of Health

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1. INTRODUCTION

Clostridium difficile is a rod shaped, anaerobic Gram-positive bacterium that forms very resilient endospores. The species shares several common features with other members of the *Clostridium* genus, however, due to substantial differences regarding metabolism and sporulation it recently has been reclassified as *Clostridioides difficile* [1]. Nevertheless, these are toxin producing pathogen bacteria, which lead to hospital acquired infections worldwide [2]. This nosocomial pathogen causes the disease *C. difficile* infection (CDI) which strongly correlates with administration of antibiotics in health care facilities. The symptoms of the toxin caused CDI varies from mild diarrhea to toxic megacolon which ends fatally if not operated surgical [3]. Moreover, the incidence of CDI has been rising mainly in industrialized countries like the USA, Germany, Spain etc. [4-6] Thus, this type of infection is one of the most common and cost-effective health care-associated infections with approximately 29,000 deaths by half a million cases of CDI in the United States. The bacteria are transmitted via the orofecal route, they colonize the colon and can form spores under unfavourable conditions. However, the intestinal microbiome seems to prevent the infection of *C. difficile* and in cases such as antibiotic treatment or chemotherapy the intestinal microbiome is disturbed and *C. difficile* can colonize, proliferate and infect the host [7]. But in some cases, human digestion of *C. difficile* spores does not consequence in symptomatic infection [7,8]. In case the conditions are not favourable, exemplarily healthy intestinal microbiota, the asymptomatic carriers have the bacteria as spores in their gut [7]. Thus, the major cause of hospital-acquired diarrhea, CDI, occurs via the germination of spores in the human gut, under favourable conditions [7]. The exotoxins of *Clostridium difficile*, TcdA and TcdB, lead to the deterioration of colon and initiate the symptoms of CDI [8].

Peptidyl-prolyl-*cis/trans*-isomerases (PPIases) are ubiquitous proteins with protein folding and chaperone activities. As such they, on the one hand, are part of the very general protein maturation pathway. On the other hand, they can be involved in very specific protein-protein interactions supporting unique phenotypes related to important physiological or pathogenic features. In Gram-positive bacteria the most analysed PPIases are the surface exposed and membrane-associated parvulins PrsA

of *Bacillus subtilis* and its homologs in *Listeria monocytogenes*, streptococci and staphylococci [9-12]. These are post-translocational protein folding enzymes that have been connected to several phenotypes including antibiotic resistance, hemolysis or invasion [10, 11]. In *B. subtilis* PrsA is even essential for cell viability [13]. In contrast, *C. difficile* PPIases are completely unexplored regarding their biochemical and biological properties except the recent identification of two PrsA-homologs on the cell membrane in a lipoproteomic approach [14]. Despite the considerably high sequence homology between PPIase homologs, unique functions likely depend on the species analysed.

Several surface associated or secreted factors have been described in the context of virulence in addition to the main virulence factors, the two glycosylating toxins TcdA and TcdB. Among those are the flagellum, proteases, surface layer proteins and adhesins of the extracellular matrix [15-18], all of which need to be translocated and folded correctly in order to perform their *in-situ*-functions. PPIases are an important part of quality control mechanisms during protein export in bacteria and by that also influence the correct localization of translocated substrates. Hence, their analysis is central to understanding how *C. difficile* responds to its environment at the interface of its interactions, including its cell membrane, cell wall, surface layer and spore cortex. The mechanisms, mediating the release of the large glycosylating toxins and other virulence factors into the environment, are to a great part unknown. Here, intra- and extracellular PPIases may act as facilitators during export and folding. In accordance with this, clostridial binary toxins, including CDT of *C. difficile*, are dependent on several host PPIases for their efficient translocation into the host cytosol and toxicity [19, 20].

Another interesting, but in case of microbial PPIases not yet addressed issue is their active involvement in gene expression via DNA-interaction including the recruitment of further transcriptional regulators. This has recently been shown for the eukaryotic nuclear PPIase FKBP25, which can bind to DNA via its N-terminal helix-turn-helix motive and remains a novel angle in the analysis of bacterial cytosolic PPIases [21].

In this master thesis the cytosolic cyclophilin, a class of PPIases, PpiB was analysed. Reviewing cyclophilins *in silico*, lead us to see that cyclophilins exist in a number of different bacteria species, but the functions are rarely known in bacteria

[22]. In *E. coli* PpiB is responsible for the regulation of motility and biofilm formation [23]. Whereas, PpiB affects these functions negatively, plays also role in bacterial cell division in this organism [24]. This cyclophilin has been analysed in *Staphylococcus aureus*, also a Gram-positive pathogen, seems to affect the activity of secreted protein, and has no effect on bacterial growth in rich medium but under starvation condition the growth is affected by this cyclophilin in these organisms [22]. Yet, the host cyclophilin A is shown to play a role in the uptake mechanism of clostridial toxins [20]. Mammalian cyclophilin CypA is also shown to play in cytokinetic abscission [25]. Thus, the cyclophilin in general takes place in different cellular processes, however, the function of the PpiB in *C. difficile* was unidentified until now and we wanted to characterize this enzyme at molecular level in this organism.

1.1. The Purpose, Contribution and Content of Thesis

The study aims to characterize the cytosolic cyclophilin PpiB in the pathogen *C. difficile*. These analyses will help us to understand the infection mechanisms of this organism and lead us to find some diagnostics and/or therapeutics against CDI, on the one hand. On the other hand, the well-conserved PPIase family can be understood better with our findings, which will help to have greater idea about the effects of these enzymes in other organisms. Thus, it is worth to study this enzyme considering the possible functions, of PpiB in *C. difficile*, which are depicted in the Figure 1.1.

In this thesis, we claim that the PpiB plays a role in the L-cysteine tolerance in *C. difficile*, has a role in the secretion and/or production of CDI causing exotoxins and finally that this enzyme interacts with other proteins.

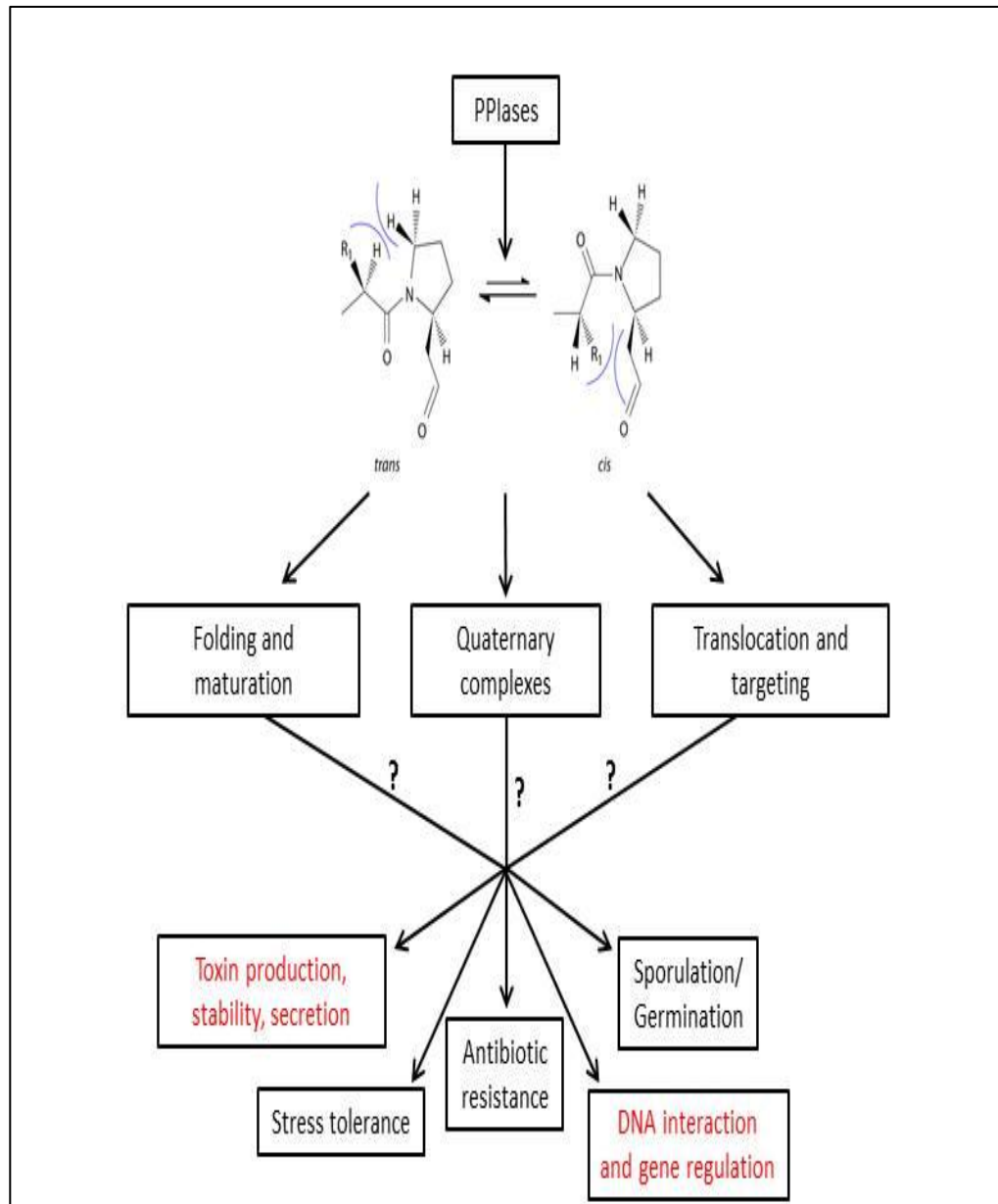


Figure 1.1: Peptidyl-prolyl-cis/trans-isomerization as modulator of protein transport, folding and formation of protein complexes at the cross roads of physiology and virulence (illustrated by Assoc. Prof. Dr. Can Murat Ünal).

2. LITERATURE REVIEW

2.1. *Clostridium difficile*

C. difficile was first isolated from intestinal flora of healthy infants in 1935. In 1978 this bacterium was recognized as the causative agent for pseudomembranous colitis and enterocolitis, following the introduction of clindamycin [26].

Two large glycosylating toxins produced by *C. difficile*, TcdA and TcdB, cause the disease whereas non-toxigenic strains, are considered asymptomatic colonizers [27]. The symptoms can range from asymptomatic colonization to abdominal pain, fever, mild diarrhea and leucocytosis. Most commonly, the disease is recognized with pseudomembranous colitis, which is identified by the destruction of large intestinal tissues that is a result of a strong inflammation. At the cellular level, the activation of inflammatory pathways with MAPK, COX-2 and NF- κ B and cause the secretion of proinflammatory cytokines such as TNF- α , IL-1 and IL-8 [28]. The progression of disease may lead to a phenotype called toxic megacolon syndrome that is lethal in the absence of operative response [29]. Although the medical treatment of CDI relies on antibiotics such as vancomycin or metronidazole, the recurrence rates are considerably high [30].

Due to the anaerobic life style of the bacteria, they are mostly found as spores that are formed by endosporeulation, which is a very complex process regulated by Spo0A [31]. The regulator Spo0A is activated by yet unknown histidine kinases. The signals activating these kinases are not known in detail. However, they set off a cascade of sigma factors to start the process in mother cell and in the forespore, hierarchically [31]. The spores are the infectious form because they can resist unfavourable conditions including the passage through the stomach. Germination in the intestine is initiated mainly by primary bile acids like taurocholic acid (TCA) and glycine [32]. In healthy individuals, members of the gut microbiome enzymatically transform host derived primary bile acids into secondary bile acids like lithocholic acid (LCA) or deoxycholic acid (DCA) which in turn suppress the growth of vegetative *C. difficile*. In the large intestine, an environment with enormous degree of competition, disturbing the microbiome, e.g. by antibiotic treatment, leads to the accumulation of primary bile acids and a decrease in the concentration of secondary

bile acids. This most likely results in higher germination rates and less suppression of vegetative outgrowth of *C. difficile*. [33].

The two main virulence factors of *C. difficile* are the enterotoxins TcdA and TcdB that use UDP-glucose and glycosylate host GTPases such as Rac1, Cdc42 or RhoA in host cells [Figure 2.1]. The inactivation of these essential regulators through glycosylation leads to actin depolymerization affecting the maintenance of cytoskeleton and the contacts of the cells with other cells and/or extracellular matrix [34]. It leads to inflammation and recruitment of immune cells after the destruction of epithelial barrier initiated by the activity of toxins [2].

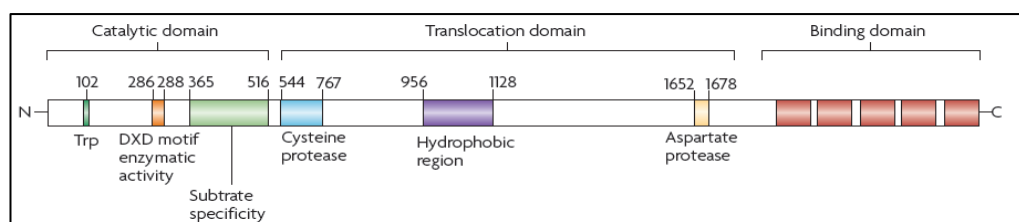


Figure 2.1: The structure of TcdA. TcdA and TcdB are highly similar and have three main domains: N-terminal catalytic domain performing the glycosylation of host GTPases, a central translocation domain that facilitates the transfer of the catalytic domain from endosomes into the host cell cytosol, and a C-terminal binding domain that binds to various and not fully identified host cell receptors.

TcdA and TcdB are 50% identical to each other and on the amino acid level they share 70% similarity, and are secreted into the supernatant [36]. In the C-terminus they have a cell binding domain, the toxic effect is orchestrated via the enzymatically active domain at the N-terminus and the translocation domain which is between the N- and C-terminus is a hydrophobic translocation domain [Figure 2.1]. Toxins bind to multiple receptors of host enterocytes via yet unknown mechanisms and they are taken up via clathrin-mediated endocytosis [37]. The endosome maturation acidifies the content, so the toxins go through conformational change and the toxins can go through the endosomes via the translocation domain of the protein [38]. Despite the high similarity between TcdA and TcdB, TcdB is 10 times more effective than TcdA in human colon [39]. Also, the receptors recognizing the two toxins differ from each other [40]. In the genome the toxins and their regulatory genes are located in a 19 kb long pathogenicity locus (PaLoc) (Figure 2.2).

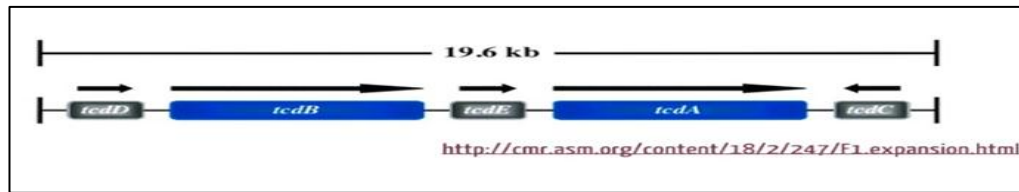


Figure 2.2: Structure of the PaLoc (pathogenicity locus) in *C. difficile*. *tcdD* is a positive regulator of the *tcdB* and *tcdA*, whereas *tcdC* is the negative regulator of these toxins. And *tcdE* encodes for a holin-like protein that is believed to be involved in the secretion of the toxins.

TcdD (TcdR) is an alternative sigma factor that drives the transcription of *tcdA* and *tcdB*, whereas the TcdC is a negative regulator and represses the toxin production [41]. Therefore, the absence of TcdC correlates with hypervirulence in *C. difficile* strains [42]. After germination, *C. difficile* is able to produce these toxins and cause disease. A CDI is typically treated with antibiotics such as metronidazole and vancomycin [43]. In case of recurrence vancomycin is the antibiotic of choice. Recently, fidaxomicin has been used which is more effective than vancomycin in the reoccurrence, and it damages the microflora less than vancomycin [44]. The indigenous microflora is important in CDI and therefore fecal microbiota treatment (FMT) has been also used. In a study the FMT and vancomycin treatment have been compared with each other and FMT was more effective than vancomycin. The overall cure rate of this treatment was about 94%, whereas the vancomycin used group was cured to 31% [45]. Although it has not been used so much in Europe compared to USA, the FMT method is a promising curing method [46]. Another, interesting treatment alternative is to use antibiotically active secondary bile acids [47]. There is a practical treatment of this on a patient in the literature, a hard case, in which the CDI is reoccurred after antibiotics treatment, FMT and antibiotics treatment again. But treatment with secondary bile acid resulted with suppression of the vegetation from *C. difficile* [48]. TcdA and TcdB produced by the bacteria leads to CDI, and these toxins are produced by vegetative bacteria. Therefore, the secondary bile acids can be used as a treatment method.

2.2. Peptidyl-prolyl-*cis/trans*-isomerases (PPIases)

Peptidyl-prolyl-*cis/trans*-isomerases (PPIases) are specific enzymes

functioning in the folding of proteins that were first discovered in 1984 by Fischer et al. These enzymes catalyse the rotation of the peptide bond anticipating a proline which unlike all other peptide bonds does not have an intrinsic preference for the *trans* conformation due to steric hindrance of the adjacent α -carbon [49]. These enzymes accelerate the *cis/trans* isomerisation by the factor of 10^3 to 10^6 , whereas in some cases the spontaneous isomerisation is presumed to be in much lower rates due to the steric hindrance [50]. The catalytic activity of this enzyme is driven via the energy coming from conformational changes in the substrate [51]. This folding i.e. isomerisation activity of PPIases affects the conformation of nascent proteins and mature proteins, hence the activity and interaction of the proteins with others [52]. Considering the correlation of the topology and the function of proteins, this kind of enzymes are very important in terms of cellular activity of biological organisms. PPIases are conserved amongst prokaryotes, archaea and eukaryotes, and can be grouped in three distinct protein families, namely cyclophilins, parvulins and FKBP (FK506 binding proteins), by their sequence and structural homology [53] (Figure 2.3).

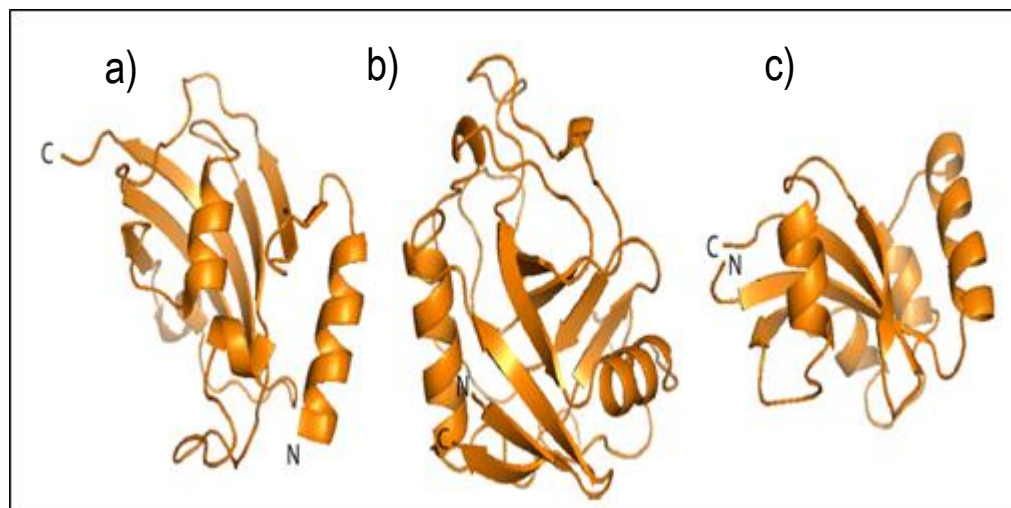


Figure 2.3: Representative structures of the three classes of PPIases, a) FK506-binding proteins (FKBP), b) cyclophilins and c) parvulins. (taken from the Reference number 53).

These different families have specific catalytic domains and substrate specificity which can be also analysed by the structures. The complexity and the localization of the proteins vary amongst the organisms. For instance, in humans two parvulins, 16 FKBP and 18 cyclophilins are distributed among all cellular

compartments such as nucleus, mitochondria, ER. In plants PPIases can also be found in the chloroplasts, whereas in *E. coli* there are two parvulins, two cyclophilins and three FKBP's localizing in the membrane and cytoplasm [49]. In bacteria, it is worth to divide PPIases as in soluble or membrane-bound enzymes. The soluble ones can be found in the supernatant due to secretion or localization in the cytosol or periplasm in Gram-negative bacteria. FKBP's and cyclophilins have either multidomain PPIase activities or single domain depending on the organisms. Although some parvulins have multidomain PPIases, in most of the cases they have just one domain catalytically active [54]. Multidomain FKBP's and cyclophilins can interact with other proteins and with their other parts of the proteins [55]. These domains and variety in different organisms are depicted in Figure 2.4. As you can follow from the structure, the PPIase domains are small, however there are some additional domains such as tetratricopeptide repeats (TPR) that mediate protein-protein interactions [56]. In PPIases WD40 domains, RRM (RNA recognition motif), zinc finger domains etc. facilitating interactions with lots of partners and function in various processes can be found. The plasticity that can be achieved by modularity was nicely shown by a recent study where a fusion of *E. coli* chaperone domain of FKBP homolog, SlyD, with an unrelated PPIase domain resulted in the change of the targeting residue [57]: so, the human FKBP12 was targeting, in that case, lysine, alanine and glutamine like *E. coli*, rather than targeting leucine or phenylalanine. Accordingly, it can be concluded that the modular effects of different domains in PPIases influence their functions.

FKBP's and cyclophilins are also referred to as immunophilins due to the mediation of the effects from immunosuppressive drugs rapamycin, FK506 and cyclosporin A (CsA) [58]. In humans, although these drugs inhibit the PPIase catalytic activity of the corresponding enzymes, the immunosuppressive effects come from the extra function gained by the immunophilin-drug complexes. For instance, the cyclosporin A-cyclophilin A complex or the FKBP12-FK506 complex prevent the phosphatase activity of calcineurin and promote T-cell activation. Also, rapamycin, another inhibitor of FKBP's mediates the inhibition of mTOR (target of rapamycin) kinase by forming a complex with FKBP12, and preventing the signalling in T-cells, which is activated in response to cytokine stimulation [58]. Juglones are considered as inhibitors of parvulins, such as Ess1 and Pin1 -these

regulate cell cycle in eukaryotic cells-, used often in mammalian cell culture due to their cell cycle blocker effects [58].

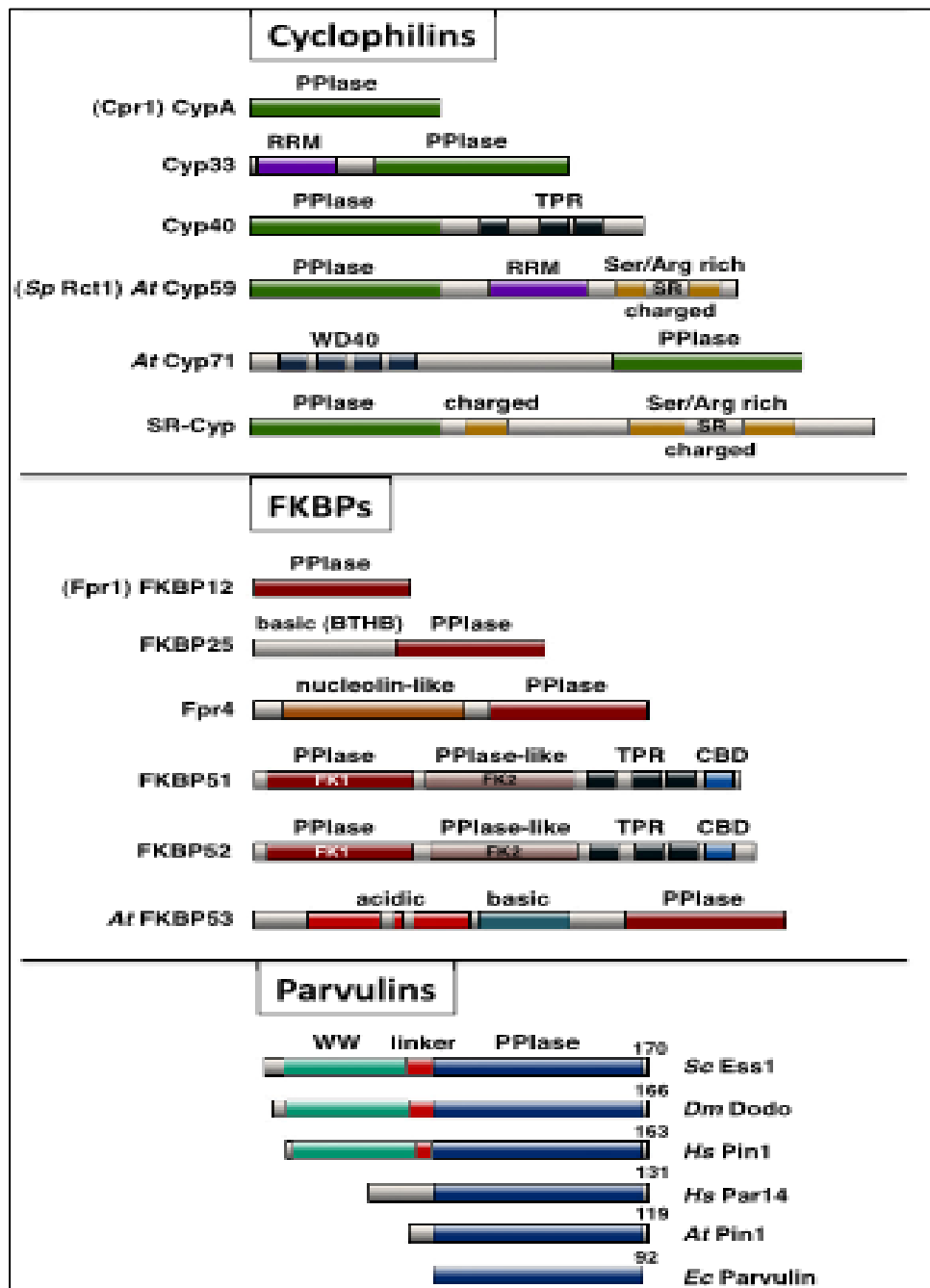


Figure 2.4: “Alignment and domain structure divided into major families. If not indicated, the protein is from yeast or mammals.” (paraphrased and taken from S.D. Hanes / Biochimica et Biophysica Acta 1850 (2015) 2017–2034.).

PPIases affect the folding of proteins also refolding of Rnase T, but in many instances the enzymatic activity has no or little impact on the *in vivo* function folding activity of these enzymes [59]. Therefore, the function of PPIases seem not to be

limited to isomerisation of peptidyl-prolyl bonds, but also depends on the ability to participate in multiprotein complexes. Although PPIases take part in processes ranging from signal transduction to gene regulation, in some cases the deletion of PPIases does not have any physiological effects or the effects are restricted to specific conditions. For instance, the deletion of all 12 PPIases in *S. cerevisiae* did not show any prominent effect or the deletion of the periplasmic PPIases in *Yersinia pseudotuberculosis* did not demonstrate any measurable phenotype [60, 61]. So, the function of PPIases is very specific to the organism and niche which makes it difficult to predict their functions. In contrast, PPIases have distinct preferences in regard to the amino acid preceding the proline when it comes to their enzymatic activity. Accordingly, human FKBP12 reveals a preference towards leucine or phenylalanine whereas, its *Legionella pneumophila* homolog Mip isomerises the lysine-proline bonds more efficiently [62, 63]. Another remarkable feature of the function from PPIases is steric accessibility, for instance the human parvulin Pin1 that recognizes phosphotyrosine or phosphoserine residues, but if the targeting sites are inaccessible, PPIases cannot perform their functions [64].

The regulation of PPIases takes place via localization and catalytic or non-catalytic activities of different domains, the PPIases are also regulated through expression levels, on the one hand. For instance, some stress factors such as cold, heat or misfolded protein stress lead to the upregulation of PPIases [65, 66]. On the other hand, PPIases play also role in transcriptional regulation of other proteins [67]. To consolidate this kind of regulation with an example, it could be the human FKBP25 binds to the DNA and regulate transcription [21]. Whereas the FKBP12 homolog of *Botrytis cinerea*, a plant-pathogen fungus, is upregulated during infection and takes a part in infection of plant [68]. It is worth to mention that the PPIase mediated transcriptional regulation is thought to be in immunomodulatory processes, but it seems that these are also important in neuroprotection and neurodegeneration in humans, as well as in the development of different tissues in some plants [69-71].

Reviewing more than 30 years of research about PPIases reveals that this protein family is diverse, abundant and omnipresent so that they take part in almost every significant biological process. Protein folding and maturation, secretion, gene regulation, neurodegeneration, immunomodulation and infection are some of the

processes in which PPIases are involved. Different domains of PPIases and different substrate specifications make the PPIases difficult to study with, therefore some mechanisms especially in infection pathways remain poorly understood. Moreover, the unknown infection mechanisms promise novel therapeutics. Also, specific targeting of PPIases of pathogenic microorganisms will reduce the immunosuppressive side effects of inhibitors with broad PPIase spectrums. Accordingly, we studied about the cyclophilin PpiB in nosocomial pathogen *C. difficile*.

2.2.1. PPIases in *C. difficile*

. The PPIases of *C. difficile* have not been studied until now, except for the identification of a PrsA homolog in the cytoplasmic membrane in a proteome study where the lipoproteins were identified [72,14]. A bioinformatical search of the genome of the reference strain 630 yielded 6 putative PPIases, which are divided to 4 parvulins, 1 FKBP and 1 cyclophilin (Table 2.1). Two of the parvulins have a signal peptide required for translocation to the outside of the cell, whereas the others are most likely cytosolic proteins. But the parvulins, due to their homology to the PrsA of *Bacillus subtilis* and other species, seem to localize in the membrane, which is also shown by Charlton TM et al. [14]. However, the PrsA in *C. difficile* has additional amino acid stretches outside the PPIase domain comparing its *B. subtilis* homolog.

Table 2.1: Putative PPIases of *C. difficile*. C stands for cytosolic and Ext for extracellular localisation.

Name/Locus	Accession number	Class	Putative Localization
Trigger factor (TF)/ CD630_33060	Q180E9.1	FKBP	C
PrsA-like/ CD630_13570	YP_001087853	Parvulin	C
PrsA-like/ CD630_15570	YP_001088058	Parvulin	Ext

Table 2.1 Continuation.

PrsA-like/ CD630_22630	YP_001088777	Parvulin	C
PrsA-like /CD630_35000	YP_001090021	Parvulin	Ext
PpiB/CD630_03310	YP_001086800	Cyclophilin	C

Therefore, we aimed to study PPIases in this anaerobic pathogen, to find out interesting features of these enzymes and to predict some therapeutic agents. We started to pick up one of the putative PPIases in fact the cyclophilin PpiB. Reviewing cyclophilins leads us to find that they take part in various cellular processes. For instance, in cell division [23,24,73], transcriptional regulation [67], transport of proteins [74], signal transduction [75], stress tolerance [76], pre-mRNA splicing [77] and protein folding [78]. Although there are less examples about bacterial cyclophilin in the literature, an outstanding paper published about PpiB homolog is actually about *Staphylococcus aureus* [22], which is also a Gram-positive pathogen like *C. difficile*. In this study PpiB affects protein folding and the activity of secreted protein including virulence factors, has no effect on bacterial growth in rich medium but under starvation condition. Also, *Brucella abortus* needs its cyclophilins for the survival under stress conditions [79]. Lastly, PpiB has a role in cell division, biofilm formation and motility in *E. coli* [23,24].

Due to the promising functions of PPIases and the absence of knowledge about this protein family in the significant pathogen *C. difficile* we were led to study this topic. The explanation of the features of PPIases has been done but the importance of the model organism has not been reviewed yet.

3. MATERIALS AND METHODS

3.1. Materials

3.1.1. Instruments

Table 3.1: List of Instruments.

Name	Brand
Anaerobic Chamber	Coy Laboratories
Class II Type A2 Biological Safety Cabine	Hera
Agarose Gel Electrophoresis, Agagel	Biometra
SDS Gel Electrophoresis, Trans-Blot®, semi-dry transfer systems for blotting Electrophoresis power supply	Bio-Rad
Gel Documentation, GelDoc	Bio-Rad
Microplate Reader Varioskan Flash	Thermo Scientific
Incubator (CO ₂)	Thermo Scientific
Autoclave, LVSA 50/70	Zirbus
DNA Sequence HiSeq2500	Illumina
SP8 Confocal Microscope	Leica
LAS EZ imaging software	Leica
Spectrophotometer, NanoDrop™1000 by	PeQlab
Thermocycler, Tpersonal	Biometra
Thermomixer compact and comfort	Eppendorf
pH determination, C6840B	Schott
Photometer, C08000	Biochrom
High-Pressure Homogenizer	Glen Mills, French Press G-M
Homogenizer FastPrep®24 Instruments	MP Biomedicals
Centrifuges, 5804	Eppendorf
Biofuge primoR	Hereaus
Vortex Genie 2	Scientific Industries
Purification column systems	Sarstedt
Pipettes	Rainin
96- and 48-well plates	TPP
15 and 50 mL tubes	Sarstedt
10 mm cuvettes	Sarstedt
Shaker/Roller	Scientific Industries

3.1.2. Chemicals and Kits

- Enzymes:
 - T4 DNA Ligase
 - Q5 DNA Polymerase
 - Restriction enzymes and the corresponding buffers were purchased from New England Biolabs
- Kits:
 - QIAquick® Gel Extraction Kit
 - QIAquick® PCR purification Kit
 - QIAprep® Spin Miniprep Kit
 - By Qiagene®
- Molecular Weight Standards:
 - GeneRuler™ DNA LadderMix by Fermentas®
 - PageRuler prestained protein ladder 10 to 170 kDa by Thermo Fischer®
- Chemicals:
 - Cyclosporin A (Cay-12088) by Cayman Chemical®
 - Amoxicillin by Santa Cruz Biotechnology®
 - Other chemicals used in the experiments will be mentioned in the methods part and were all purchased from Sigma-Aldrich®

3.1.3. Strains, Plasmids and Oligonucleotides

Following strains were used in this study:

- *E. coli* CA434 *E. coli* HB101 carrying the Incβ conjugative plasmid R702
- *C. difficile* 630Δ*erm*, PCR ribotype 012 Erythromycin sensitive strain
- Cdi630Δ*erm*Δ*ppiB*:intron *ermB* (CD630_03310) 630Δ*erm* *ppiB* insertional mutant, site 290/291 antisense, *Erm*^r, created previously by Dr. Mareike Petra Berges.

Plasmids:

- pMTL82151 shuttle vector for transforming *C. difficile*, Chloramphenicol^r
- pDSW1728 shuttle vector for transforming *C. difficile*, Chloramphenicol^r

The plasmids were constructed differently and new versions will be demonstrated under the methods and results chapters.

Table 3.2: List of oligonucleotides

Name	Nucleotide sequence (5' → 3')	Annealing temperature	Features
EBS_univ_Rev	CGAAATTAGAACTTGCG TTCAGTAAAC	64°C	Primers for the determination of ClosTron gene knockout system [80]
ErmRAM_F	ACGCGTTATATTGATAAA AATAATAGTGGG	63°C	
PpiB_R50A_Rev	AGC ATG AAA TAT TAT TCC ATT GTA ATA TC	55 °C	Base exchange primers
PpiB_R50A_For	GTA ATA CCA GGA TTT ATG ATA C	54 °C	
CdPpiB_NStr ep_F	cgggtggctccaTTCCATAATTT ATTACCTTCCTTTTC	58 °C	Strep-Tag primers
CdPpiB_NStr ep_R	cagtttgaaaaaAATAAAAATCC TATAGTAACTATAGAAAT G	59 °C	
PpiB_For4	TCTgagctcATGAAAAGGAA GGTAATAAATTATG	56°C, SacI	Primers for knock in of <i>ppiB</i> to tetracycline-inducible system. Corresponding Restriction enzymes are given near temperatures.
PpiB_Rev4	ATAggatccTTAGTTTTTTTC TACATCTGAGTAA	56°C, BamHI	

3.2. Methods

3.2.1. Cultivation of Bacteria

Clostridium difficile were grown on ChromID Cdiff chromogenic agar (CDIF) bought commercially from bioMérieux® and BHIS medium (Brain-Heart-

Infusion supplemented with 0,5 % (w/v) yeast extract and 0,1 % (w/v) L-cysteine). L-cysteine was dissolved in 10 mL ddH₂O and freshly added to the medium after sterile filtration. For some experiments *C. difficile* were grown in Tryptone-Yeast Extract-Glucose medium (TYG; 20 g/l tryptone, 10 g/l yeast extract and 5 g/l glucose). *C. difficile* were grown under anaerobic conditions (95 % N₂/5 % H₂) at 37 °C. For liquid cultures 50 mL tubes were used, and cultures were not shaken. If necessary medium was supplemented with 15 µg/mL thiamphenicol (Sigma Aldrich; 15 mg/mL stock solution in methanol).

Escherichia coli CA 434 strains were grown in lysogenic broth (LB) containing 10 g/l peptone, 5 g/l NaCl, 5 g/l yeast extract and supplemented with 30 µg/l chloramphenicol (Roth®; 30 mg/mL stock solution in ethanol).

3.2.2. Minimal Inhibitory Concentration (MIC) Assay

Dilution series were used to determine the minimal inhibitory concentration (MIC) of active compounds to measure the necessary concentration to kill (bactericidal) or inhibit the growth (bacteriostatic) of microorganisms. MIC assays were performed in 96-well format. The bacteria were grown in anaerobic chamber overnight in 5 mL BHIS-medium. The next morning cultures were refreshed by inoculating 1:100 in 10 mL BHIS-medium. After this, the bacteria were adjusted to an OD_{600nm} of 0,1 in 15 mL BHIS-medium. As substances were generally dissolved in DMSO or ethanol and the final solvent concentration was 5 % (v/v), BHIS-medium containing 10% (v/v) DMSO was prepared for the following dilution series. The columns from 3-11 of a 96-well plate were filled with 100 µL BHIS-DMSO solution. In the 2nd column 190 µL of the same solution was pipetted. Into wells of the 2nd column 10µL of the substance of interest were given followed by serial 1:1 dilution down to the 10th column of the plate. After this, 100 µL of the bacteria suspension were given to transferred into each well. The 11th column was used for DMSO-control for each strain and medium background.

In order to determine the correlation between two compounds was done in 2D MIC assay by diluting the first compound vertically and the second compound horizontally.

3.2.3. *In vivo* cross-linking of interacting proteins

The cross-linking treatment was done in order to stabilize protein-protein interactions within complexes [81]. Formaldehyde was added as a cross-linking agent to the late stationary culture to a final concentration of 0,125% (v/v). The culture with formaldehyde

was incubated at 37 °C for 20 minutes. The termination of the cross-linking reaction was done by adding glycine at 130 mM final concentration and incubating at 37 °C for 5 minutes. Thereafter, the cells were harvested by centrifugation at 3000 *g*, for 20 minutes at 4 °C. The pellet was washed twice with PBS (4000 *g* for 20 minutes at 4 °C). Finally, the cells were resuspended in 25 mL PBS supplemented with 1 tablet of complete Protease Inhibitor Cocktail (Roche).

3.2.4. Protein Isolation via Strep-Tactin® Column

Cells were homogenized using a French Press or with glass beads and FastPrep®. Cell debris was removed by centrifugation (10000 *g*, 10 min, 4 °C), and filtering the supernatant through a 0,45 µm syringe filter. The supernatant was applied onto a 3 mL Strep-Tactin® column (iba Lifesciences) in a 20 mL syringe. After letting the protein solution flow through the column, the column was washed with 18 mL Buffer W, containing 100 mM Tris/HCl (pH 8.0), 150 mM NaCl and 1 mM EDTA. Then, 6 mL Buffer E was added for the elution of proteins in two 1,5 ml and two 2 mL fractions, containing 100 mM Tris/HCl(pH 8.0), 150 mM NaCl, 1 mM EDTA and 2.5 mM desthiobiotin. All fractions were collected for downstream analysis. Finally, the column was washed and regenerated. The wash of the column was done with Buffer W, whereas the regeneration of the column was done with Buffer R, containing 100 mM Tris/HCl (pH 8,0), 150 mM NaCl, 1 mM EDTA and 1 mM HABA (hydroxy-azophenyl-benzoic acid). After regeneration the columns was washed with ddH₂O and stored in 20 % (v/v) EtOH.

3.2.5. Determination of the Protein Concentration

Protein concentration of each fraction were determined with the Roti-Nanoquant (Roth K880.1) solution. Protein samples were diluted 1:5 with 1x Roti-Nanoquant-Solution and measured with a photometer, in this case with Varioskan, via the determination of OD_{590/420} ratio. If needed proteins were concentrated using ultrafiltration columns with 10 kDa molecular cut off (Merck-Millipore) according to the instructions of the manufacturer.

3.2.6 SDS Gels

This is an analytical method for the separation of proteins. The gels i.e. separating gel and stacking gel, were prepared according to Table 3.2.

Table 3.3: Preparation of 10% separating gel and 5% stacking gel. All values are given in mL.

10% separating gel	5 mL	10 mL	15 mL
ddH ₂ O	2,4	4,8	7,2
30% Acrylamid:Bisacrylamid (37,5:1)	1,25	3,5	3,75
1.5 M Tris pH8.8	1,25	3,5	3,75
10% SDS	0,05	0,1	0,15
10% APS	0,05	0,1	0,15
TEMED	0,002	0,004	0,006
5% stacking gel	2 mL	4 mL	6 mL
ddH ₂ O	1,46	2,92	4,38
30% Acrylamid:Bisacrylamid (37,5:1)	0,25	0,5	0,75
1 M Tris pH6.8	0,25	0,5	0,75
10% SDS	0,02	0,04	0,06
10% APS	0,02	0,04	0,06
TEMED	0,002	0,004	0,006

The gels are loaded with protein, which have about same concentration and mixed with Laemmli loading buffer (containing 5% (v/v) β -mercaptoethanol), and the electrophoresis was performed at with 45 mA per gel. Due to expecting protein size, the electrophoresis is run for 25 minutes.

3.2.7. Western Blot

Western Blot was used for the specific detection of PpiB. For this, the proteins previously separated on a SDS-gel were transferred with a Trans-blot system on a PVDF membrane. Transfer was done semi-dry for 8 minutes with 12V constant voltage. Membranes were blocked with 3% non-fat dry milk in 1X TBS-T (20 mM Tris-Cl pH 7,6, 150 mM NaCl and 0,02 % (v/v) Tween-20).1x TBS-T. The incubation with blocking solution was 1h at room temperature and then membranes were washed once with TBS-T. Thereafter, the membranes were incubated with TBS-T containing 1% non-fat dry milk 1h at room temperature and mouse anti-PpiB antibody diluted 1:2000. This antibody was generated against recombinant PpiB by the phage-display method and the subsequent cloning and production in mammalian cells in the Biozentrum of Technical University of Braunschweig. The membranes were washed three times with TBS-T, and anti-mouse antibody diluted 1:5000 in TBS-T containing 1% non-fat dry milk was added for 1h at room temperature. Then the membranes were washed three times with TBS-T and were incubated with alkaline phosphatase buffer (100 mM Tris-Cl pH 9.5, 5mM MgCl₂ and 100mM NaCl) for 10 minutes. Protein was visualized by adding NBT and BCIP diluted to 330 µg/mL and 165 µg/mL in alkaline phosphatase buffer, respectively.

3.2.8. Staining of SDS Gel

Coomassie® Blue Staining was done via staining the gel with SimplyBlue™ SafeStain over night after washing the gel several times with ddH₂O. The silver staining protocol is as follows:

- Fixation of the gel:
 - 50% EtOH
 - 12% acetic acid
 - 0.5 mL 37% formaldehyde
 - add ddH₂O to 1l
 - incubate for 1-3 hours
- Washing
 - 50 % EtOH 1l

- 2 times 20 minutes
- Sensitization
 - 0,2 g/l $\text{Na}_2\text{S}_2\text{O}_3 \times 5 \text{ H}_2\text{O}$
 - 1 minute
- Washing
 - dH₂O
 - 2 times 20 seconds immediately after sensitization
- Silver Staining
 - 2 g/l AgNO_3
 - 750 $\mu\text{L/l}$ 37% formaldehyde
 - 20 minutes
- Washing
 - dH₂O
 - 2 times 20 seconds
- Development
 - 30 g/l Na_2CO_3
 - 4 mg/l $\text{Na}_2\text{S}_2\text{O}_3 \times 5 \text{ H}_2\text{O}$
 - 0,5 mL 37% formaldehyde
 - 4-5 minutes
- Stopping
 - 1% glycine
 - 30 seconds
- Washing
 - dH₂O
 - 30 seconds
- Stopping
 - 1% glycine
 - 10 to 30 minutes
- Washing
 - dH₂O
 - 30 to 120 minutes

3.2.9. Mating of *C. difficile* and *E. coli*

The transfer of recombinant DNA into *C. difficile* is facilitated by conjugation with the *E. coli* strain CA 434 and shuttle plasmids of the pMTL-origin [82]. The recombined *E. coli* strains were grown in LB at 200 rpm and 37 °C overnight. In parallel *C. difficile* were grown anaerobically at 37 °C overnight. One mL of *E. coli* culture was harvested and washed two times with 500 μL PBS (8000 g, RT). The

pellet was transferred into the anaerobic chamber and resuspended in 200 μ L of *C. difficile* culture. This mixture was inoculated on a BHI-plate without any antibiotics and incubated at 37 °C. After 6 h, the mixture was scrapped off the plates with 1 mL BHIS or PBS and the bacteria were plated out on ChromIDplates supplemented with 15 μ g/mL thiamphenicol. Transconjugants were visible after 2-3 days at 37 °C.

3.2.10. Cytotoxic Effect Assay

The toxins produced by *C. difficile* affect the actin cytoskeleton via the glycosylation of GTPases [34]. Therefore, the effect of secreted toxins can be observed by visualizing the actin cytoskeleton in cell culture. To do that, 3T3 mouse fibroblast cells were cultured according to the instructions of NIH (National Institute of Health, product supplier) in DMEM (Dulbecco's Modified Eagle Medium) with 10% FBS at 37 °C and 5% CO₂. For the assay the 10⁵ cells/mL were seeded on glass cover slips in 24-well plates and cultured for two days. In parallel, *C. difficile* were grown in anaerobic chamber for 24 h, and the cell free supernatant containing secreted toxins was harvested by centrifugation (8000 *g*, 5 min, RT). This supernatant was diluted to 0,1 % (v/v) in cell culture medium. 3T3 cells were washed once with 1 mL PBS and 1 mL medium with bacterial culture supernatant was added. After incubation of confluent mouse fibroblasts with supernatant of bacteria for 2h, medium was removed and the cells were fixed for 15 min at RT with 2 % (w/v) PFA in 1x PBS. Cells were permeabilized with 0,1 % (v/v) Triton X-100 in 1x PBS with 1 % (w/v) BSA for 5 min. Excess Triton was removed by washing three times with 1 mL PBS. After this, the slides were placed on 20 μ L of staining solution containing 1 μ g/mL DAPI (Sigma Aldrich) for DNA and 1:1000 diluted Phalloidin-Alexa488 (Abcam) for actin staining. After 20 min in the dark, the slides were washed twice with PBS and placed on a microscope slide with fluorescent mounting medium (Dako). The cells were analysed with a confocal microscope (Leica® SP8). Objects were viewed with an oil immersion objective with 63x magnification.

3.2.11 Growth Curve

The bacteria were grown over night in a liquid medium, and the next day inoculated in 25 mL liquid medium at a final OD_{600nm} of 0,05. The growth was measured by monitoring the change in OD every 90 minutes.

4. RESULTS

4.1. The growth of *Clostridium difficile* 630 Δ *erm* strain compared to its *ppiB* deficient mutant on different media

To characterize the effect of PpiB on the growth of *C. difficile* under standard conditions, the wild type strain, its *ppiB* deficient mutant, and a *ppiB* variant encoding for a protein with an arginine to alanine substitution at position 50 (R50A) were cultured in BHIS medium and the OD_{600nm} was measured every 90 minutes (Figure 4.1). The base exchange was done by PCR mediated site directed mutagenesis and the sequence of the construct has been confirmed via sequencing (Appendix A). The construct has been polymerized with inverse PCR method via the primers (Materials). Due to the high homology and the conservation of the PpiB through the organisms, we targeted the amino acid arginine at the 50th position of peptide sequence from PpiB and substituted it by alanine.

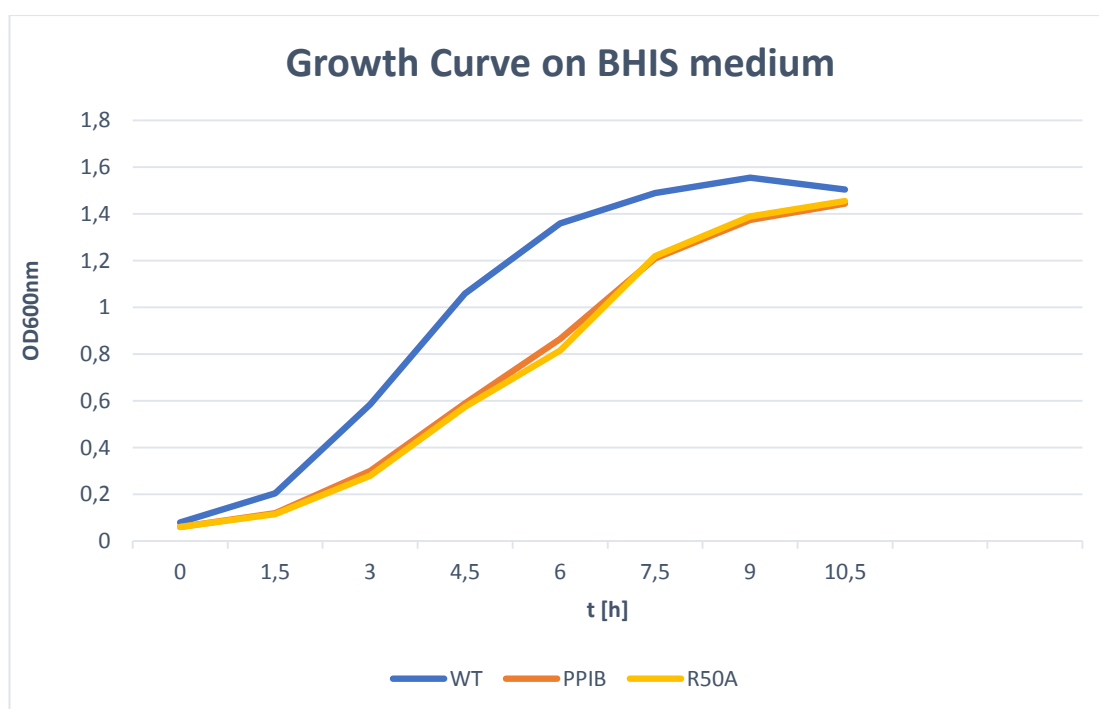


Figure 4.1: A representative growth curve of wild type strain, the *ppiB* deficient mutant, mutant complemented with R50A substituted PpiB on BHIS medium.

The PpiB in *C. difficile* involves in the growth of the bacteria, according to the Figure 4.1, the putative enzymatic active side looks like responsible for this growth. Also, the bacteria were grown in other media such as in TY (TYG without glucose) and compared the growth in that way (Figure 4.2).

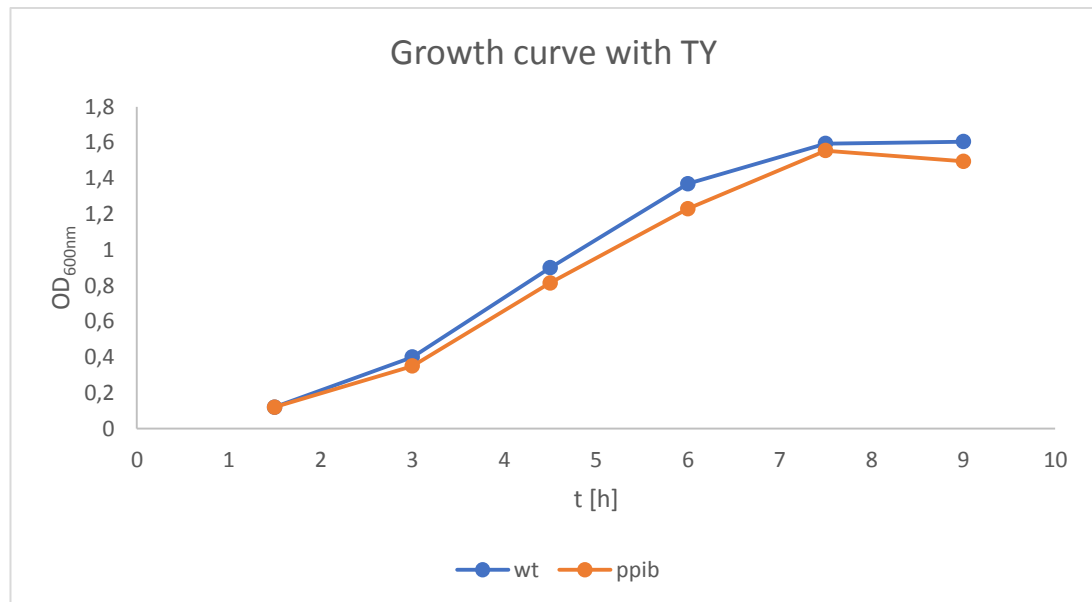


Figure 4.2: A representative growth curve of bacteria in TY (TYG medium without glucose). Wild type and $\Delta ppiB$ strains are compared, the graph illustrates absorbance at 600nm over the time.

The growth deficiency of the *ppiB* mutant was only observed in BHIS medium. In other media the growth effect, such as normal conditions, is not visible. Therefore, some compounds of the BHIS medium which are not included to the TY medium have been analysed.

4.2. PpiB confers cysteine-tolerance in *C. difficile*

In order to test the involvement of PpiB in cysteine-tolerance, we complemented the $\Delta ppiB$ -strain using a tetracycline inducible system. This system enables the tightly controlled expression of a gene with tetracycline or anhydrotetracycline. Due to the damage risk of tetracycline we used the harmless anhydrated form of the inducer. To construct this system, the gene encoding for mCherry in the plasmid pDSW1728 was replaced by wild type *ppiB* using the

restriction sites SacI and BamHI. (Figure 4.3). The mCherry is cut it out from the construct, the *ppiB* is polymerised with these sites encoding primers (Materials).

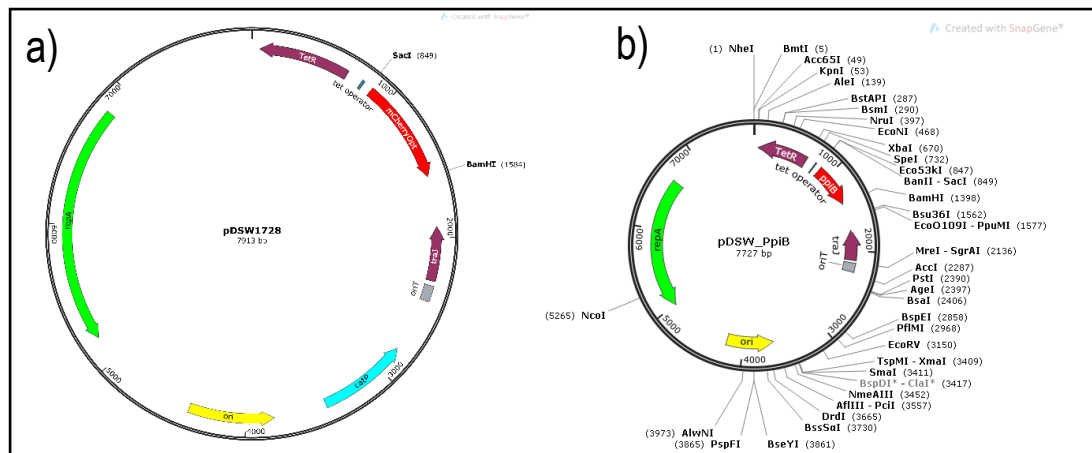


Figure 4.3: Tetracycline inducible system is constructed via changing the *mCherry* gene to *ppiB*. a) The construct with *mCherry* version and b) the altered and used tetracycline inducible *ppiB* are illustrated.

The suggestion was that PpiB has a role in cysteine metabolism. To test this, we performed two-dimensional MIC assay and measured the impact of L-cysteine concentration on the growth of the completed strain in respect the expression degree of *ppiB* in the tetracycline inducible system (Figure 4.4).

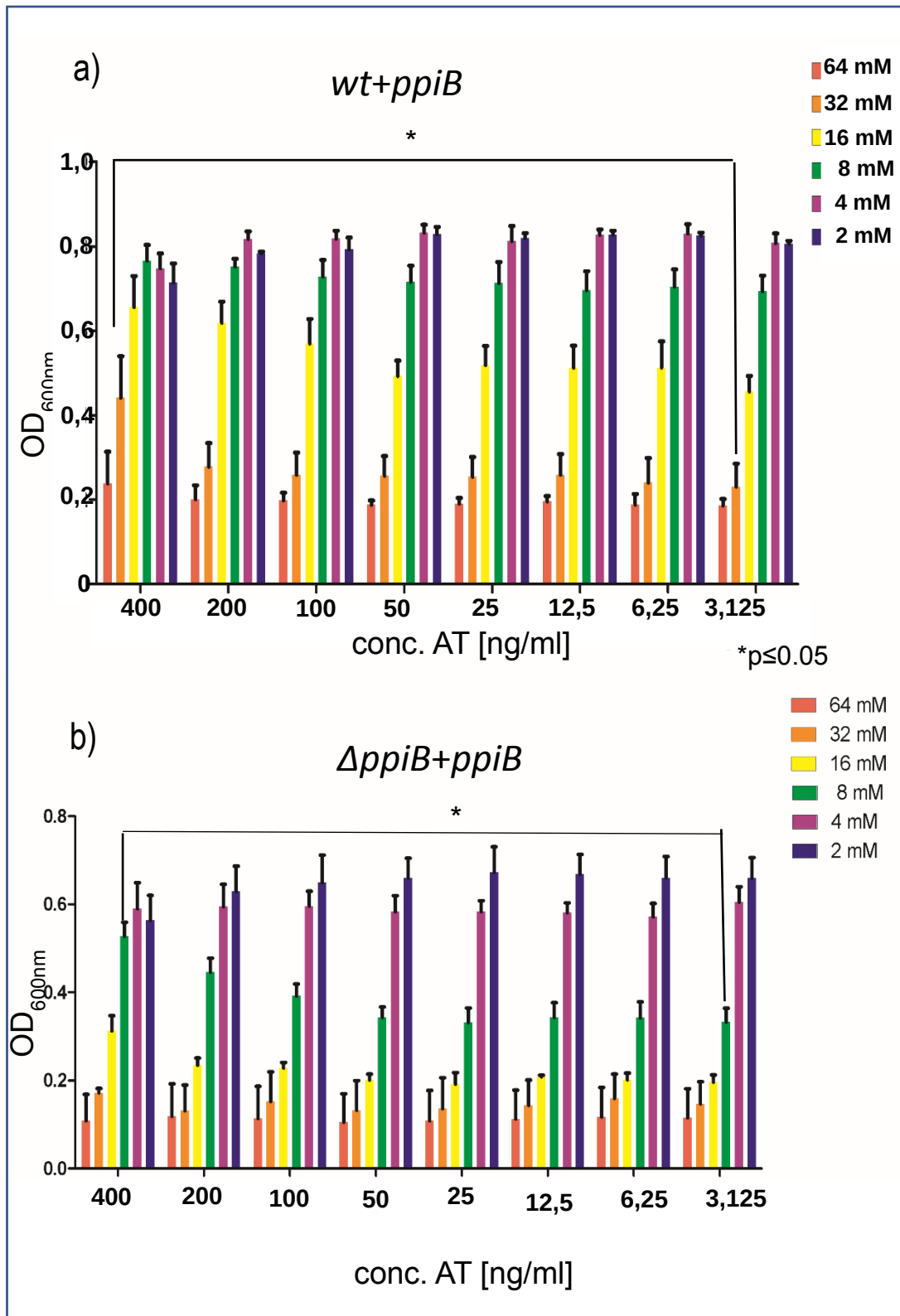


Figure 4.4: a) Growth of wild type strain complemented with *ppiB* under the control of the tetracycline-inducible promoter in the presence of decreasing amounts anhydrotetracycline and L-cysteine. b) Growth of *ΔppiB* strain complemented with wild type *ppiB* under the control of the tetracycline-inducible promoter in the presence of decreasing amounts anhydrotetracycline and L-cysteine.

Complemented $\Delta ppiB$ and wild type strains were grown in the presence of decreasing concentrations of L-cysteine (64-2 mM) and anhydrotetracycline (400-3,125 ng/mL). This resulted in a higher L-cysteine tolerance of the wild type strain as well as the of the tolerance level of the $ppiB$ -deficient mutant (Figure 4.4).

The experiments are performed with 6 replicates in a 96-well plate and this is repeated three times, the average of the results is taken and demonstrated in Figure 4.4. Standard BHIS medium used to cultivate *C. difficile* contains 8 mM L-cysteine. Therefore, focused on the 8 mM L-cysteine in $ppiB$ mutants i.e. complemented strains (Figure 4.4.B). The over expression of $ppiB$ (Figure 4.4.A) lead the bacteria to resist even 32 mM L-cysteine.

4.3. Identification of putative interaction partners of PpiB in *C. difficile*

In order to identify whether PpiB in *C. difficile* interacts with other proteins, *in vivo* cross-linking studies have been performed. Before that, a $ppiB$ complementation construct with a N-terminal Strep-Tag had to be generated in the vector pMT82151. PpiB is tagged via the primers (Materials) and the construct is confirmed with sequencing (Appendix A). This allowed the isolation and purification of PpiB and its interaction partners by affinity chromatography (Figure 4.5).

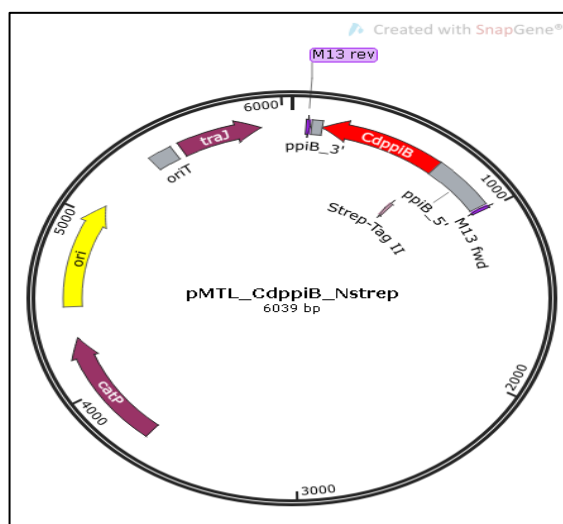


Figure 4.5: The $ppiB$ -Strep-Tag construct. Strep-Tag is recombined to the N-terminus of the $ppiB$.

The construct was used for the transformation of the *ΔppiB* strain. After this, bacteria were grown overnight and the proteins were cross-linked *in vivo*. Proteins were purified via Strep-Tactin® columns and let run on the SDS-Gel. The bands were visualized via silver staining and western blot methods (Figure 4.6). However, the samples were boiled just for 5 minutes at 95 °C before loading to the SDS-Gel.

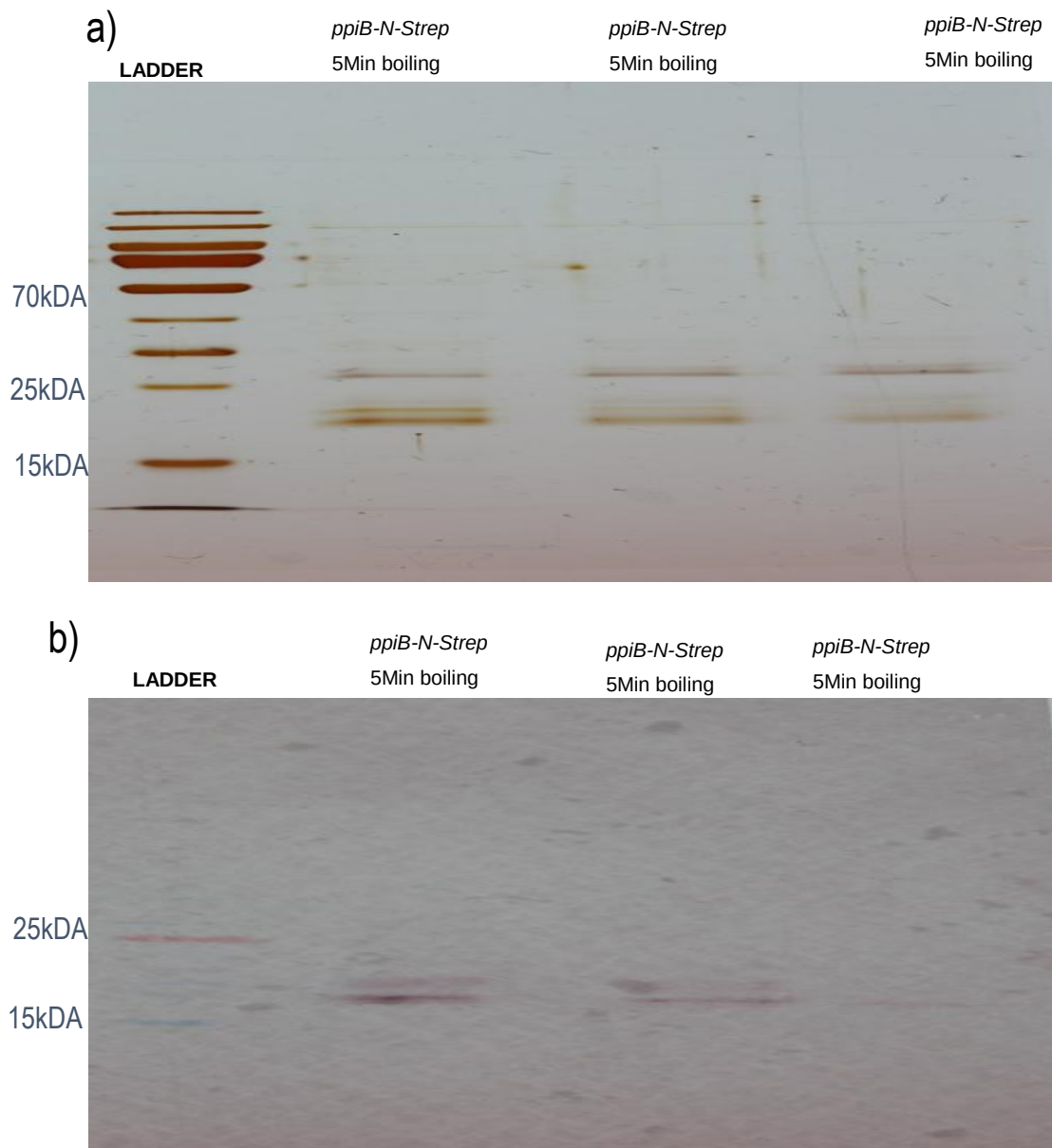


Figure 4.6: Proteins are cross-linked *in vivo* and purified via Strep-Tactin® column. a) is the silver staining of Strep-tag construct transformed to *ΔppiB* strains and b) demonstrates the western blot of the same gel. c) Coomassie staining and d) silver staining of the proteins pulled down from streptactin column and compared the denaturation of proteins. The last line from c and d is the comparison between boiled, nonboiled, Strep-Tag strains and *ΔppiB* strains.

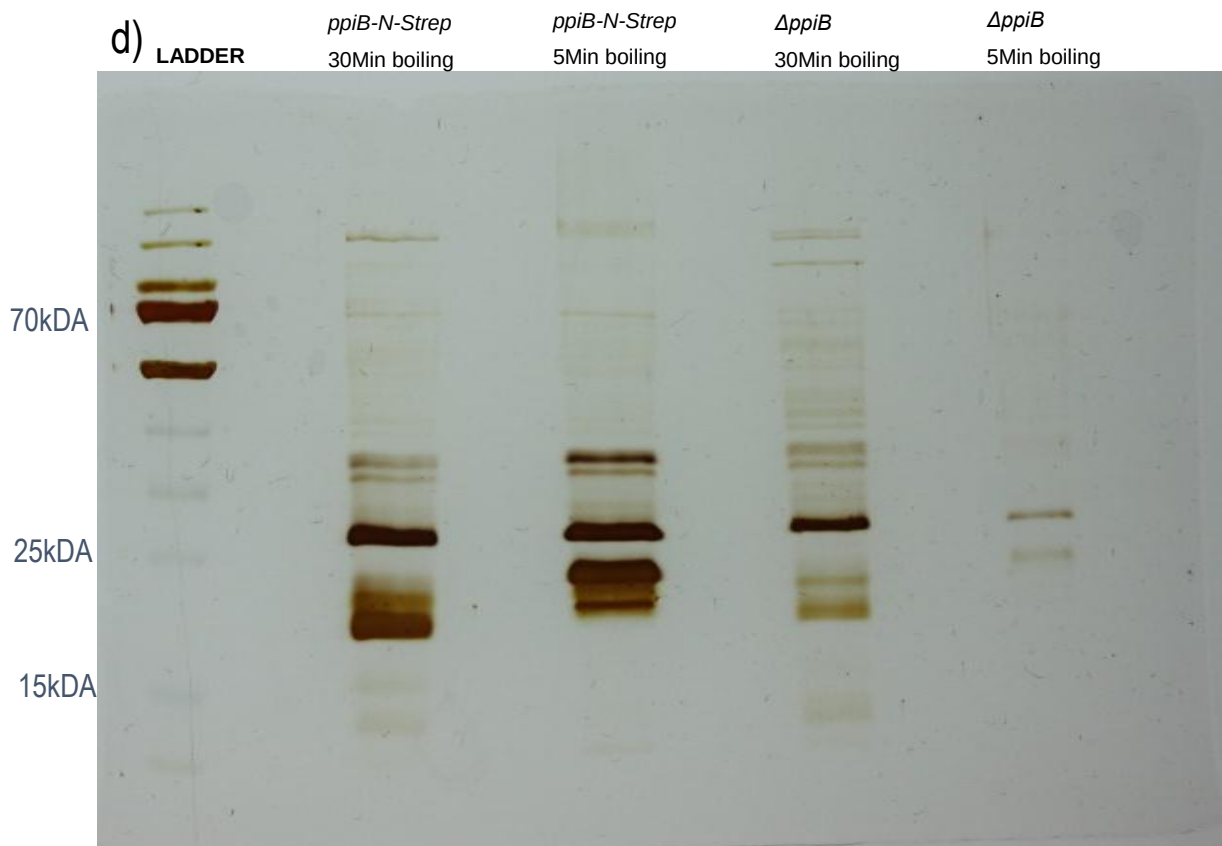
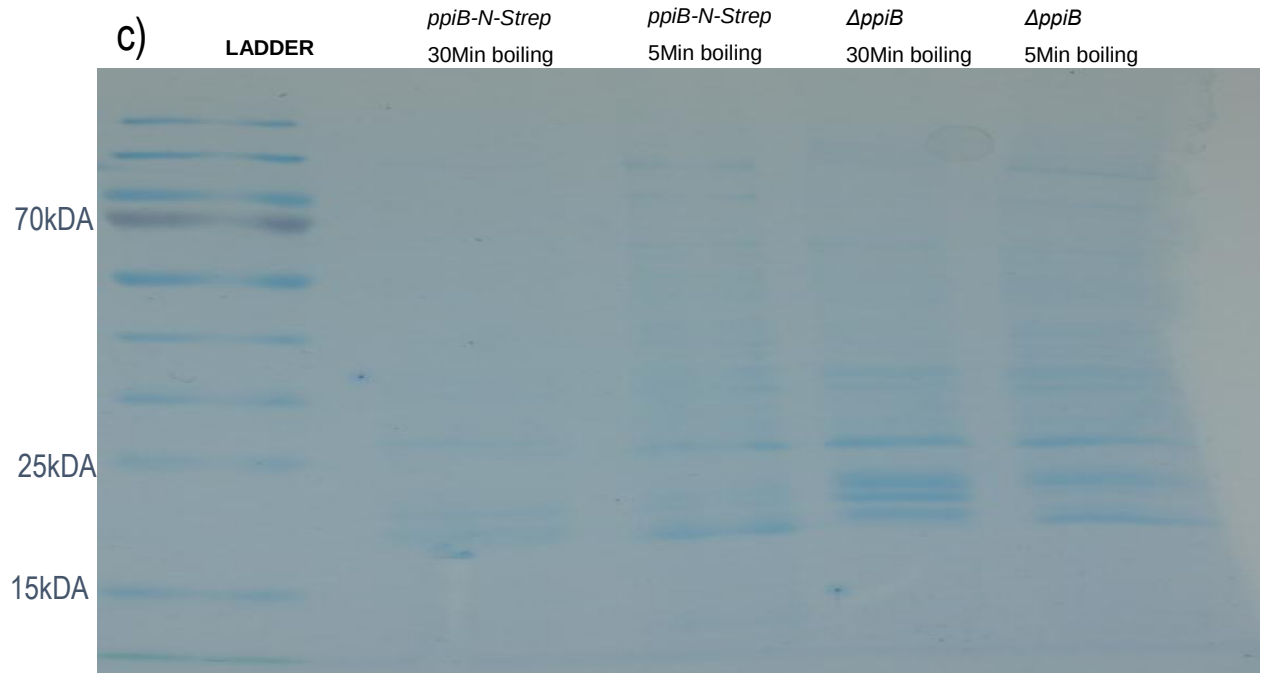


Figure 4.6 Continuation.

In order to overcome the problem of protein denaturation via boiling for 30 minutes, we compared samples with and without boiling. The samples were boiled 5 minutes and 30 minutes at 95 °C and let run on the SDS-Gel. Thereafter, the gel was stained with Coomassie® staining and silver staining (Figure 4.6.c) and Figure

4.6.d). Due to the low concentration of proteins, the samples are concentrated with and let them stain with Coomassie. However, the Figure 4.6.C shows us that the demanding amount of protein concentration for a Coomassie staining is not achieved. Nevertheless, the comparison between boiled and nonboiled samples on the silver staining shows us that PpiB forms a complex with other protein under *in vivo* cross-linking conditions.

4.4. PpiB modulates the toxic effect of *C. difficile* on eukaryotic cells

The biochemical activity of the two exotoxins, TcdA and TcdB, lead to disruption of colonic epithelial cells via actin depolymerization [34]. So, the maintenance of cytoskeleton and the contacts of the cells with other cells and/or extracellular matrix is disrupted. Thus, we tested the secretion of these toxins in wild type and the *ppiB* deficient mutant by monitoring the actin cytoskeleton integrity. The bacteria were grown for 24 h, the culture supernatant was diluted to 0,1% (v/v) in cell culture medium and applied to 3T3 mouse fibroblast cells. After a 2 h incubation the actin cytoskeleton was stained with Alexa488 coupled phalloidin (Figure 4.7). The objects were viewed with an oil immersion objective 63x magnification and to have the idea about the laser magnification the images are depicted with 10 μ m bars. In parallel culture supernatants of the mutant that was complemented with wild type *ppiB* under the control of the tetracycline-inducible promoter were prepared and used similarly.

After 2 h in untreated cells stress fibres typical for attached cells could be visualized with phalloidin-Alexa488 (Figure 4.7.a). However, when 3T3 cells treated with supernatant of the wild type strain were compared with the *ppiB* deficient mutant, a visibly higher degree of disorganization of the cytoskeleton was observed in case of the mutant (Figure 4.7.b) and Figure 4.7.c). In case of the supernatant of the complemented strain grown in the presence of 400 ng/mL anhydrotetracycline the disorganization of the cytoskeleton was comparable to the wild type and less than in the mutant (Figure 4.7.d) and Figure 4.7.e). Hence, PpiB influences the degree of toxicity of the *C. difficile* supernatants in 3T3 fibroblast cells.

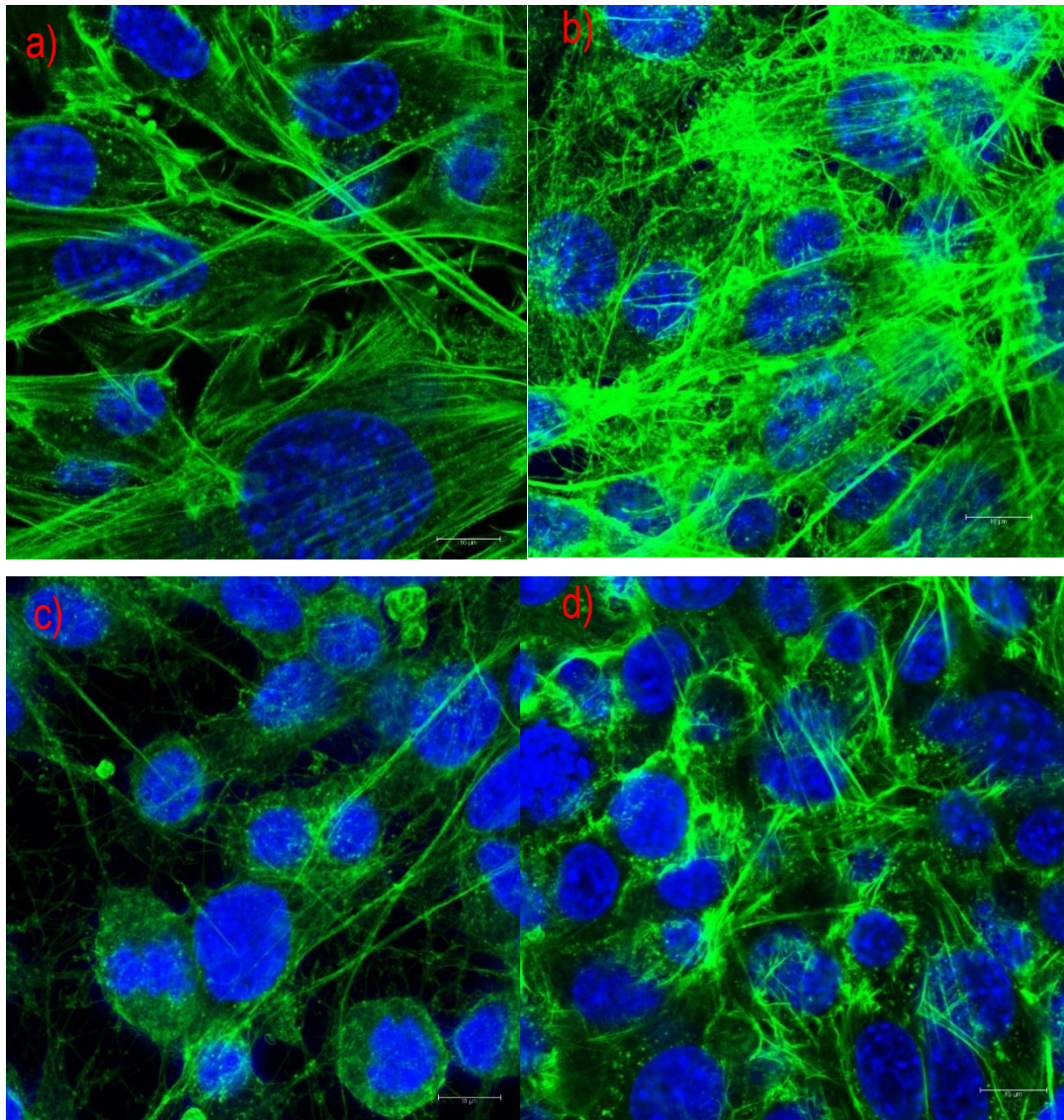


Figure 4.7: a) 3T3 mouse fibroblast cells without any treatment, b) wt *C. difficile* culture supernatants are given to 3T3 cells, c) $\Delta ppib$ *C. difficile* culture supernatants are given to 3T3 cells, d) wt *C. difficile* grown with 400 ng/mL anhydrotetracycline, culture supernatants are given to 3T3 cells and e) $\Delta ppib$ *C. difficile* grown with 400 ng/mL anhydrotetracycline, culture supernatants are given to 3T3 cells. The cells are stained with DAPI and Phalloidin-Alexa488. The bar corresponds to 10 μ m.

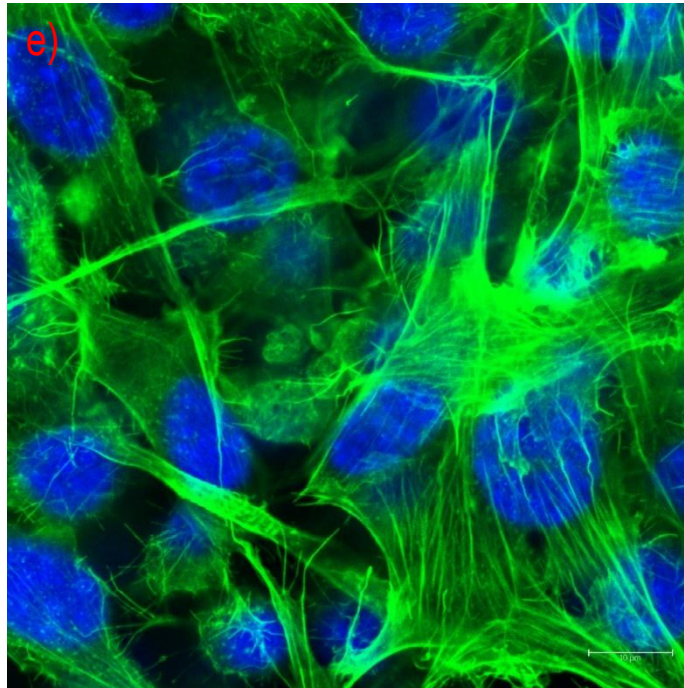


Figure 4.7 Continuation.

5. DISCUSSION

In this study, we addressed the characterization of the only cyclophilin of *C. difficile*, PpiB, in the context of fitness and cytotoxicity. Moreover, the protein-protein interactions are analysed which promises further study. *C. difficile* leads to CDI and the further analysis of PpiB, especially focusing on the protein-protein interactions and so the physiological roles of this protein, can lead us to find new diagnostics and treatment approaches for the diseases.

This cyclophilin could play a role about the growth of the bacteria. The putative active site of PpiB in *C. difficile* seems that it is responsible for the growth deficiency (Figure 4.1). This highly conserved active site (Appendix B), seems to play a role in this growth deficiency. On the other hand, the growth deficiency depends on the culture i.e. growing conditions (Figure 4.2), strictly speaking it depends on the L-cysteine (Figure 4.4). Thus, the tolerance against L-cysteine concentration depends on the PpiB. However, the cysteine tolerance tests such as in Figure 4.7, has not been performed with this active site. Therefore, to identify the role of this site in L-cysteine tolerance should be analysed in more detail. Besides, the other putative residues in the active site of PpiB (Appendix B), F110, should be analysed like R50 in the future. One can also find other putative residues in the active sites bioinformatically in the PpiB of *C. difficile*. So other active sites and R50 should be analysed according to the growth, L-cysteine tolerance, toxic effect and protein-protein interactions in this organism. But depending on the description of these effects in detail, these functions can be independent of the active sites in PpiB, but for now to conclude such a point is too speculative.

The inhibition of cyclophilin in *C. difficile* with cyclosporin A did not result in a growth deficiency, under the same growing conditions, compared to the *ppiB* mutant bacteria (data not shown). This inhibitor binds to the active site of the enzyme. On the one hand, this binding inhibits the PPIase activity of cyclophilins, and on the other hand, may facilitate the formation or ternary complexes with other proteins leading to cellular disorders [83]. Also, in *B. subtilis* this inhibitor prevents the binding of the protein to other proteins this organism rather than binding highly conserved amino acid residues [84]. Besides, it is also possible that the efficient amount of concentration from cyclosporin A is not achieved in our experiments.

As reviewed from the literature PPIases have a multitude of functions in biological processes (Figure 1.1). However, the pronounced effect of PpiB-deficiency in *C. difficile* lead us to think about the role of PpiB in gene/transcriptional regulation. Also, there are PPIases that are involved in transcription processes in different organisms [21]. Transcriptional analyses under different stress responses were performed in *C. difficile*, in which an upregulation of *ppiB* under amoxicillin stress was observed [85]. In our study, we found out that *ppiB* is indeed important in amoxicillin stress (data not shown). In this study, besides *ppiB* in total 264 genes were found to be deregulated during amoxicillin stress. Thus, it could be speculated that PpiB acts together with some of those gene products in order to cope with amoxicillin. This could be addressed in further studies. Among the deregulated genes, cysteine metabolism related genes, such as *iscS2*, *metK* etc. are also in accordance with our findings regarding the growth delay of the *ppiB* deficient mutant in the presence of L-cysteine (Figure 4.4). Interestingly, in the same study several genes that act as rod shape determining factors, such as *mreB*, were upregulated. It can be a clue of, that the lack of PpiB can be associated with the shape of bacteria which we could support by microscopical observations. The bacteria are stained with BacLight Red and observed under a confocal microscopy (Appendix C). One can easily determine the size of *ppiB* mutant strains are shorter than wild-type strains. However, it should be measured and calculated statistically to be sure about this point. Near that the growing phase of the bacteria should be also taken into consideration by the measurement of average size of mutant strains and wild-type strains. Another thought-provoking group of gene groups that are upregulated in the transcriptome analyses like *ppiB* is the cell cycle regulators of the bacteria such as *minE*, *ftsH2*(cell division protein) etc. It has been demonstrated that the PpiB in *E. coli* has a role in bacterial cell division [24]. Considering high conservation of PpiB among organisms, and the upregulated genes, PpiB can also intervene in cell cycle processes in *C. difficile*. Further analyses are needed in order to conclude any functional role of PpiB in the cell cycle of this anaerobic pathogen.

We could show that PpiB is involved in cysteine tolerance in *C. difficile* (Figure 4.4). Cysteine has a role in repression of toxin production in this organism [86]. On the other hand, in this study shows that the PpiB has also role in the repression toxic effect of *C. difficile* (Figure 4.7). So, according to the studies the

role of the PpiB in toxic effect of *C. difficile* is in line with previous findings from other laboratories. However, the cysteine is thought to be affecting toxic effect via the accumulation of cysteine by products in the bacteria [86]. Thus, the role of PpiB in toxic effect of *C. difficile* can be also in an indirect way via just regulating the cysteine metabolism in the organism. Therefore, the cysteine metabolism and PpiB should be studied in more detail to address the cysteine-PpiB-toxicity relationship.

The anaerobic pathogen produces two exotoxins which disrupts the actin of the host. So, the effects of the bacteria are measured via giving to mice the spores orally *in vivo* and some other toxicity assays *in vitro*. Our experiments were about the visualization of the toxic effect from *C. difficile* in host cells (Figure 4.7). The toxicity effect is also visible under light microscopy, but to illustrate the disruption of F-actin the phalloidin staining and confocal microscopy is more preferable. Figure 4.7.A shows us the normal mouse fibroblast cells comparison of the supernatant from wild-type *C. difficile* (Figure 4.7.B), some toxic effect is visible but this has not an enormous difference. This is also consistent with the literature due to the strain of *C. difficile*. The 630 Δ *erm* strain was used as wild-type, is less virulent than ribotype 27 strains [41]. Interestingly, the same amount of supernatant from *ppiB* mutants grown for 24h disrupts clearly more than the wild-type (Figure 4.7.C). So, the PpiB is responsible directly or indirectly in the toxic effect of *C. difficile*. In order to confirm this point, the mutants were complemented with tetracycline-inducible system and after complementation of the gene the rescue of the toxic effect was clearly visible in Figure 4.7.E. Moreover, the overexpression of *ppiB* (Figure 4.7.D) seemed to effect more than the wild-type, which let us assume that the expression level of the gene is regulated. Nevertheless, it would be better to test the toxicity in other tests such as lactate dehydrogenase (LDH) test in order to have the toxicity results quantitatively.

The cross-linked PpiB (Figure 4.6 A and B) shows multiple bands in the western blot that are also visible in the silver staining. This led us assume that PpiB can have matured and non-matured forms. However, the lack of signal sequence, for transport of the protein, in the primary form of the protein and due to the general features of prokaryotes, which modify proteins less compared to eukaryotes, this assumption is not in our scope. Accordingly, most probably cross-linked complexes were not fully dissociated. Because in the literature [81], you encounter to boil the samples for at least 30 minutes at 95 °C before loading to the SDS-gel. The PpiB has

a molecular weight of about 20 kDA. The boiling of the proteins is performed shorter than described in the literature [65], therefore it is more likely that the two bands, above the expected size of the PpiB, are PpiB complexes with other proteins. The suggestion about less boiling rather than maturation causes the formation of extra bands is approved by the Figure 4.6.D. Also, in the *ppiB* mutant strains lack PpiB as expected (Figure 4.6 C and D). In Figure 4.6.D. is clearly observable that the boiling and non-boiling protein mixture differs from the size. It would be better that the same samples are also loaded to western blots for the comparison the number of bands, but via comparing the silver staining in boiling and non-boiling protein mixtures the forming complexes can be determined. Moreover, the protein samples can be compared between *in vivo* cross-linked samples and without using this treatment, to defend the protein interaction thesis of PpiB in another way. Due to the time limit, we did not perform such an experiment. Nevertheless, the Coomassie staining with sufficient protein concentrations can be sent to MS (mass spectrometry) analysis and analyse the possible interacting protein partners. In this case, the transcriptome analysis [24] will be also helpful to investigate the protein partners in the PpiB complexes. Thus, PpiB clearly interacts with other proteins and the interacting partners should be analysed in more detail to conclude about the functions of this PPIase in *C. difficile*.

In conclusion, the cyclophilin PpiB was analysed in the anaerobic pathogen *C. difficile* at the molecular level. It could be shown that PpiB affects L-cysteine tolerance, has a role in the suppression of toxic effects on eukaryotic cells. Furthermore, interactions with other proteins in *C. difficile* could be demonstrated. However, in which way the functions are fulfilled in the organism should be analysed more in detail.

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BIOGRAPHY

Mustafa Safa Karagöz was born in 27th October 1992 in Detmold/Germany. He was graduated from Molecular Biology and Genetics of Gebze Technical University in 2015. In 2016, he started his MSc Education in Gebze Technical University, Graduate School of Natural and Applied Sciences, Molecular Biology and Genetics Department. He is a research assistant in Turkish German University and performed his experiments in Technical University of Braunschweig, a partner university of Turkish German University.

APPENDICES

Appendix A: ClosTron Gene Knockout System

Dr. Mareike Petra Belges constructed a knock-out to the *C. difficile* 630 Δ erm. The knocked-out gene is polymerized with PCR and the sequence is taken commercially with the polymerized primers (see materials).

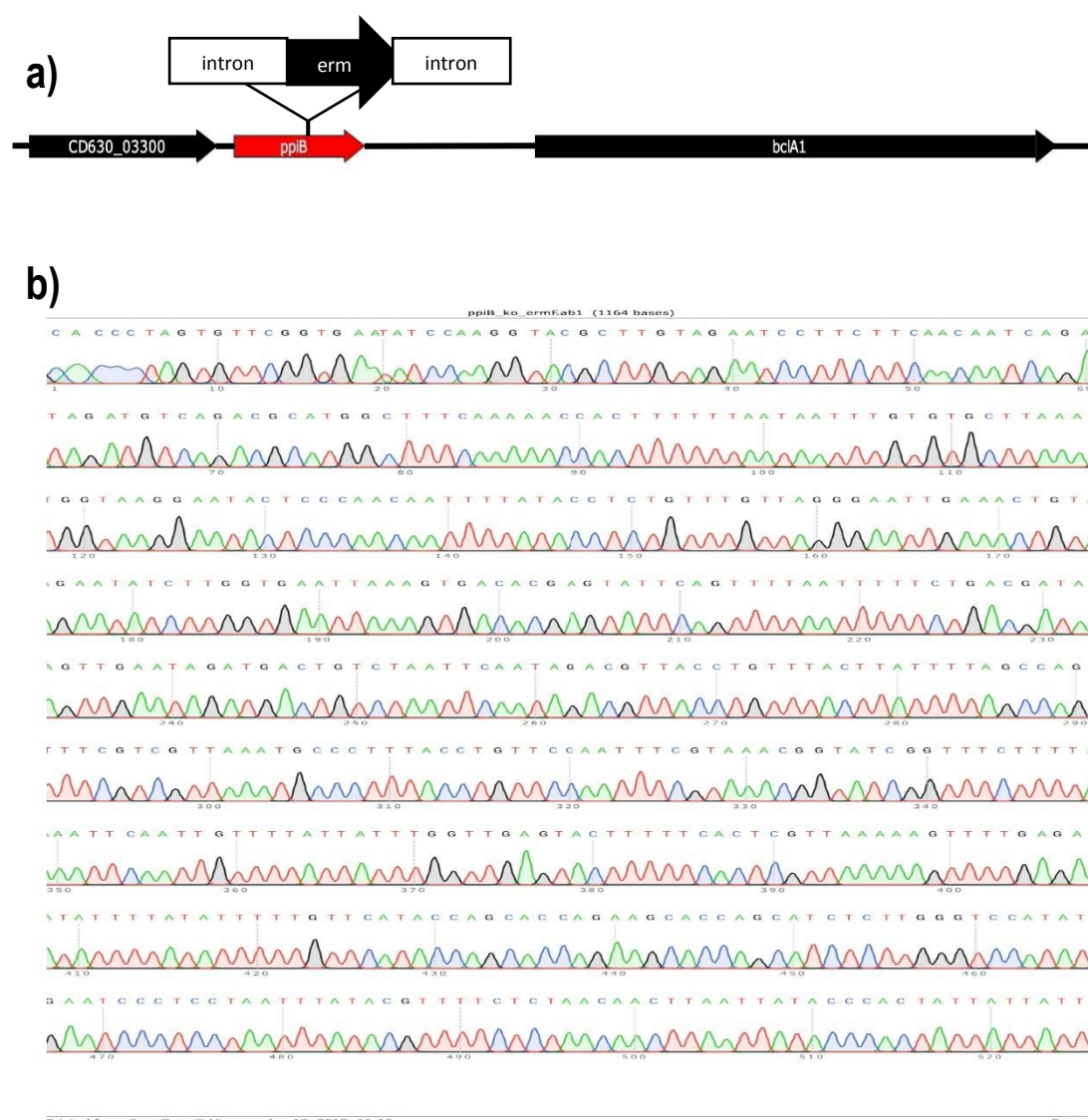


Figure Appendix A: The *ppiB* knocked-out region a), the DNA-sequence of the region b), the DNA-sequence of R50A point mutation construct region c) the red rectangle demonstrates the changed bases, the DNA-sequence of N-Strep tagged *ppiB* d) the red rectangle demonstrated the Strep-Tactin bases and the yellow rectangle demonstrates the start of *ppiB* gene.

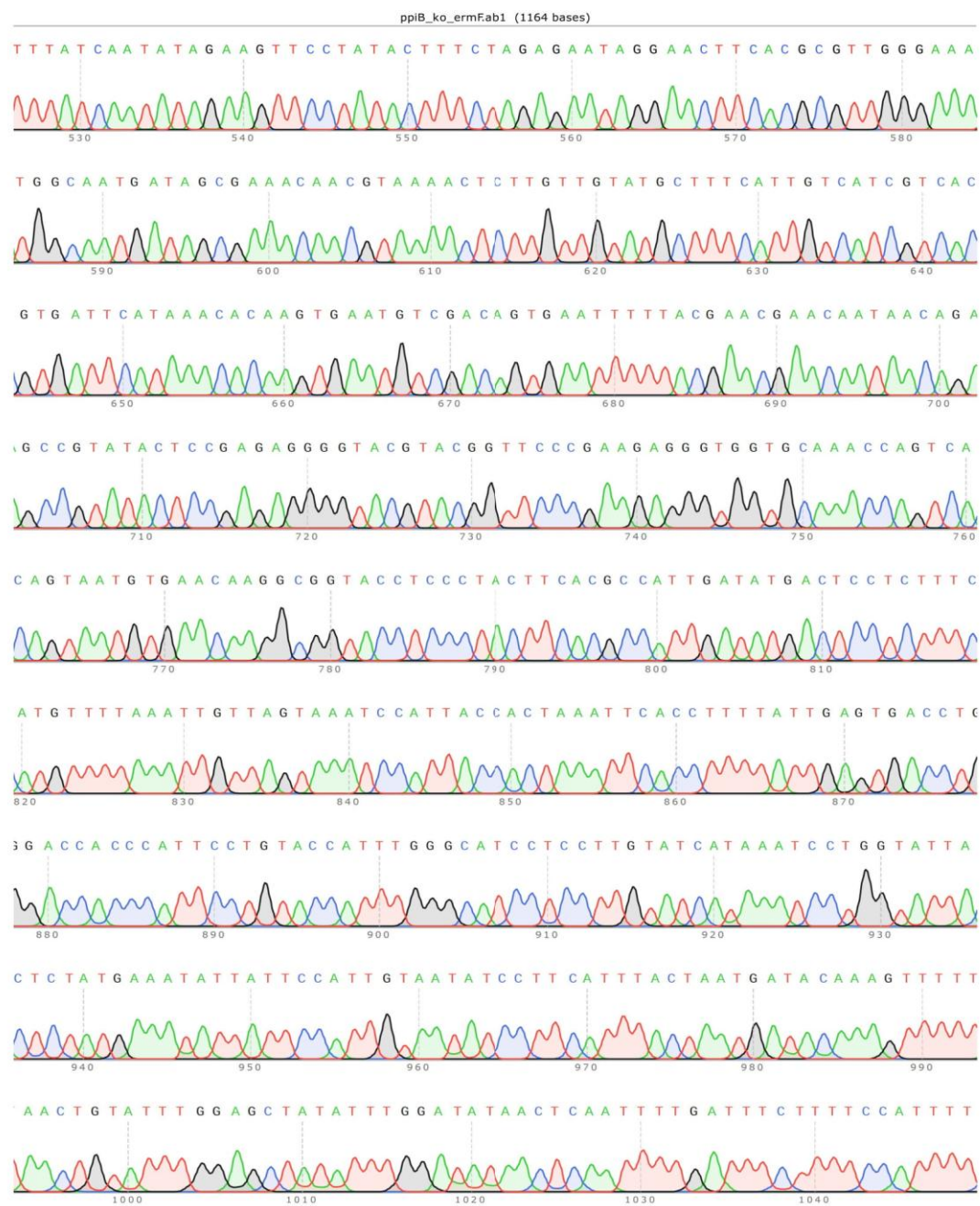


Figure Appendix A Continuation

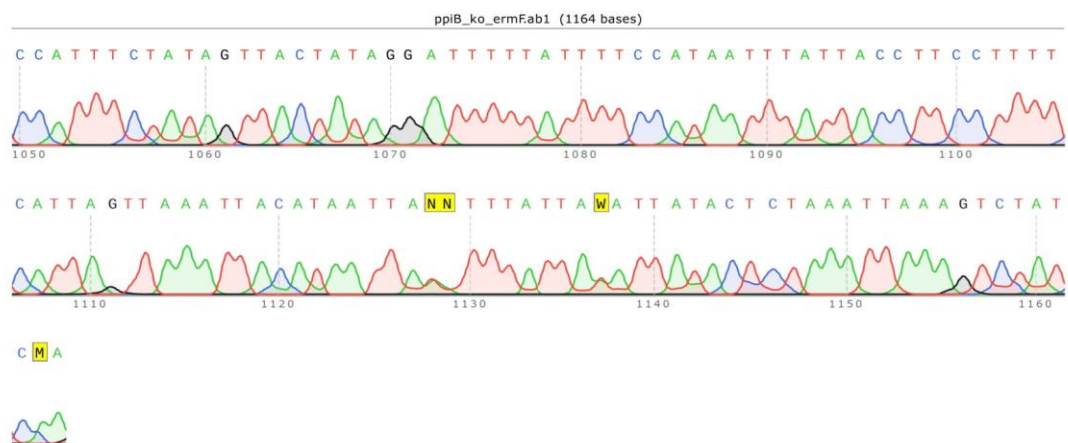


Figure Appendix A Continuation

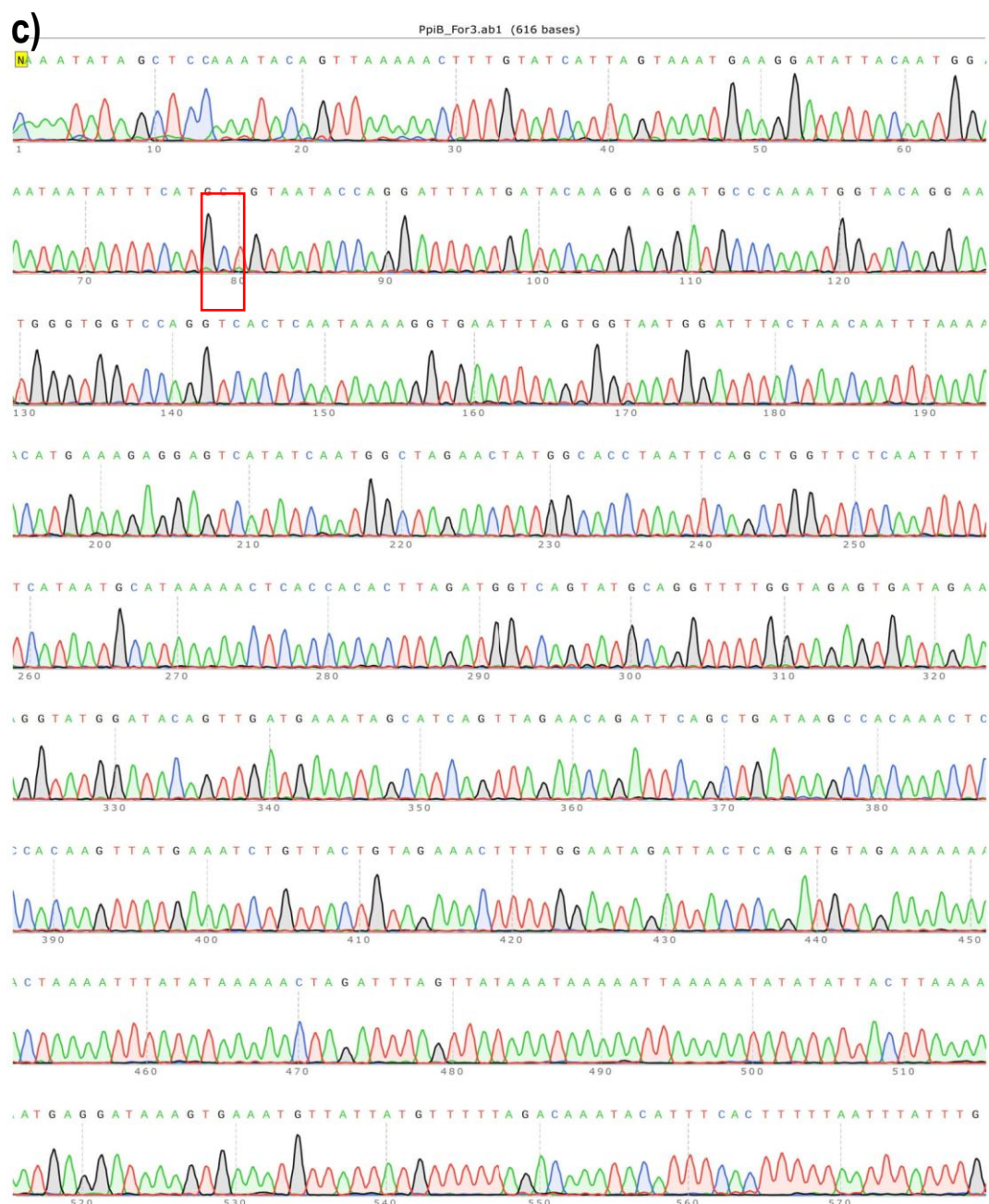


Figure Appendix A Continuation

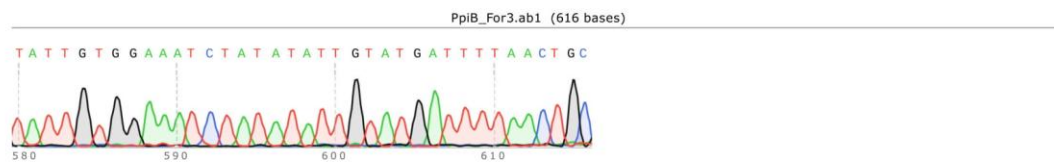


Figure Appendix A Continuation

d)

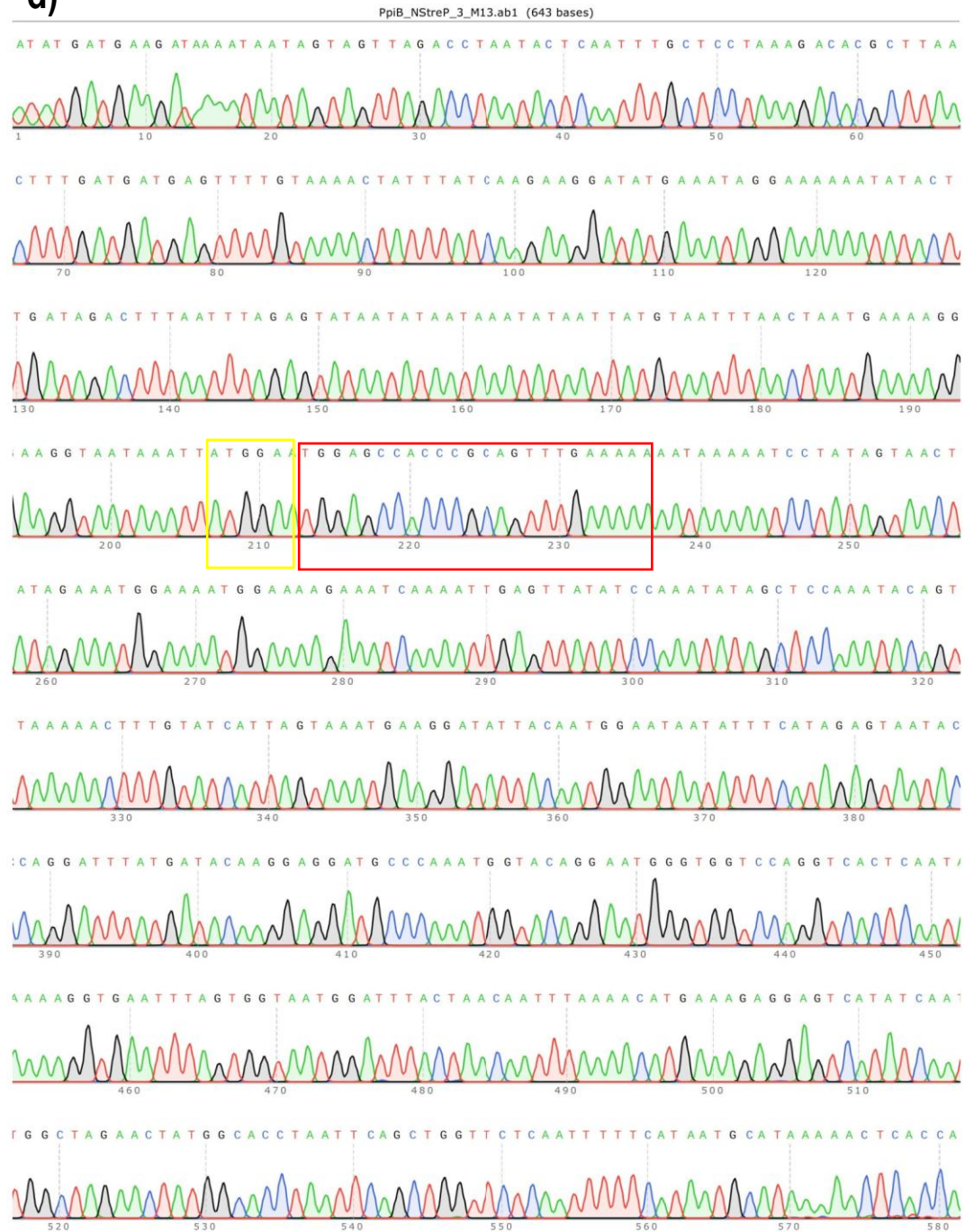


Figure Appendix A Continuation

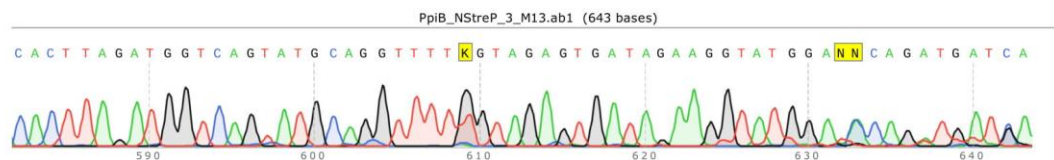


Figure Appendix A Continuation

Appendix B: Putative Active Site of PpiB in *C. Difficile*

The highly conserved amino acids are found bioinformatically. In *C. difficile* we see clearly the sites Arginine 50 and Phenylalanine 110 of PpiB are enzymatically active sites.

```

Ecoli      -----MVTF-HTNHGDIVIKTFDDKAPETVKNFLDYCREGFYNNNTIFHRVINGFMIQGG
Bsub_168   ----MKTGYFLLLEDGNKIEFELYPEAAPGTVANFEKLANEGFYDGLTFHRVIPGFVSQGG
Cdif_630   MENKNPIVTIEMENGKEIKIELYPNIAPNTVKNFVSLVNEGYYNGIIFHRVIPGFMIQGG
           :   :   . * : : : : * * * * .   . * : : .   * * * * * : * : * *

Ecoli      GFEP-GM---KQKATKEPIKNEANGLKNTRGTLAMARTQAPHSATAQFINVVDNDFLN
Bsub_168   CPHGTGTGGPGYTIKCETEGNPH---THEAGALSMAHA-GKDTGGSQFIVHEPQPHLN
Cdif_630   CPNGTGMGGPGHSIKGEFSGNGFTNNLKHERGVISMARTMAPNSAGSQFIMHKNSPHLD
           .   *       .   .   *   *       . :   * : : * : : .   . :   : * * * *   .   . * :

Ecoli      FSGESLQGWGYCVFAEVVDGMDVVDKIKGVATGRSGMHQDVPKEDVIESVTVSE-----
Bsub_168   -----GVHTVFGKVTSGLEFAKNMSNG-----DVMKEVRVEG-----
Cdif_630   -----GQYAGFGRVIEGMDTVDEIASVRTDSAD----KPQTPQVMKSVTVETFGIDY
           :   * . * . * : :   . : :   .           : : : . *   * .

Ecoli      -----
Bsub_168   -----
Cdif_630   SDVEKN

```

Figure Appendix B: PpiB peptide sequences alignment between *E. coli*, *B. subtilis* and *C. difficile*

Appendix C: The Confocal Images of *C. difficile*

C. difficile 630 Δerm (wild-type) and *ppiB* mutant strains are stained with BacLight Red and observed under a confocal microscopy.

a)

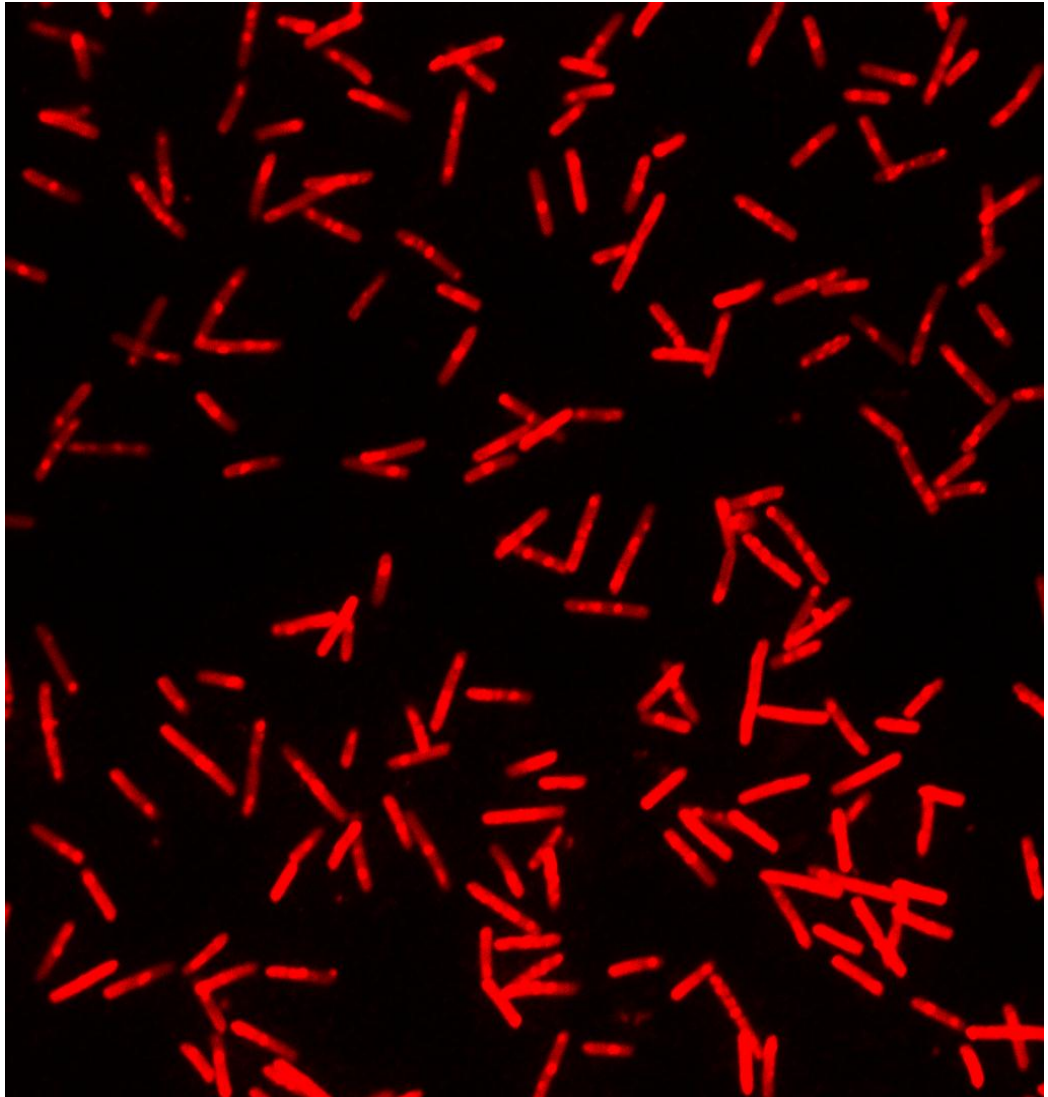


Figure Appendix C: The bacteria are stained with BacLight Red. a) demonstrates the wild-type bacteria and b) demonstrates the *ppiB* mutant bacteria. Bacteria were grown overnight and stained thereafter.

b)

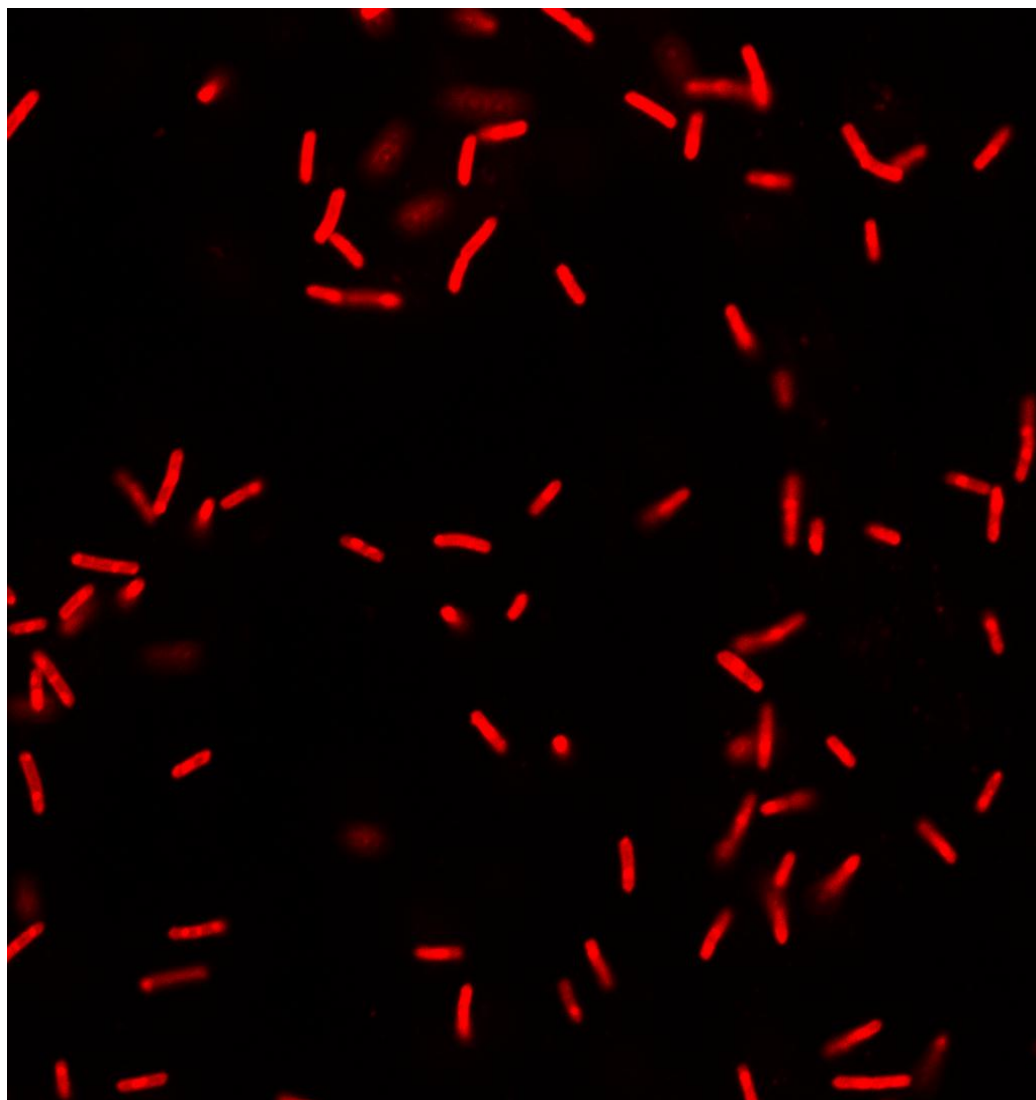


Figure Appendix C Continuation

Appendix D: Copyright Permissions

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