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New experimental approaches for human risk assessment of microbial pest control agents – exemplified by the bacterium *Bacillus thuringiensis*

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The reports are, however, published because the Danish EPA finds that the studies represent a valuable contribution to the debate on environmental policy in Denmark.

Content

Forord

Denne rapport beskriver resultaterne opnået i projektet 'Development of new in vivo and in vitro models for risk assessment of microbial pest control agents". som er blevet gennemført i 2008 til 2011. Projektet blev delvist finansieret af Miljøstyrelsens program for bekæmpelsesmiddelforskning. Projektets overordnede formål er at tilvejebringe ny viden som kan anvendes i dansk og international risikovurdering af mikrobiologiske plantebeskyttelsesmidler.

Resultaterne er fremkommet ved et samarbejde mellem, Aarhus Universitet, Institut for Miljøvidenskab (Bjarne Munk Hansen, Niels Bohse Hendriksen), Københavns Universitet, Institut for Fødevarevidenskab (Line Thorsen), Danmarks Tekniske Universitet, Fødevareinstituttet (Andrea Wilcks) og INRA, Genetique microbienne et Environnement, Frankrig (Christina Nielsen-LeRoux).

Til projektet har der været tilknyttet en følgegruppe, som takkes for gode råd og interesse gennem projektforløbet. Følgegruppen var sammensat af følgende personer: Jørn Kirkegaard, Christina Bøje (Miljøstyrelsen), Jesper Lund-Larsen (Fagligt Fælles Forbund 3F), Jesper Bælum (Odense Universitetshospital), Kirsten Friis (Bayer A/S), Michael Nielsen (Videncentret for Landbrug), Carsten Suhr Jacobsen (GEUS) og Jørgen Eilenberg (Københavns Universitet).

Sammenfatning

Det har været projektets formål, at undersøge tre forskellige typer af patogenecitets modellers evne til at vurdere virulens hos mikroorganismer, som indgår i plantebeskyttelsesmidler. De tre forskellige model typer er rundormen ***Caenorhabditis elegans***, larver af sommerfuglen ***Galleria mellonella*** (det store voksmøl) og funktionelle cellemodeller, hvor der anvendtes celler fra pattedyr.

Bakterien ***Bacillus thuringiensis*** (Bt) blev brugt som en model for mikroorganismer, som indgår i mikrobiologiske plantebeskyttelsesmidler. Den blev valgt fordi, den indgår i de mikrobiologiske plantebeskyttelsesmidler, som har den mest udbredte anvendelse og fordi den har et potentiale for at være patogen. Fire forskellige stammer fra produkterne Agree, Vectobac, Dipel og Novodor blev valgt, som de primære studie-objekter. Disse stammer er fra fire forskellige under-arter af ***B. thuringiensis***, og kan anvendes til bekæmpelse af henholdsvis sommerfuglelarver, myggelarver og billelarrver. Det er hovedsagligt Dipel og Vectobac, der finder anvendelse i Danmark.

For at have et sammenligningsgrundlag i forhold til virulens er der blevet udvalgt fire beslægtede stammer, som vi mener, har vist sig at være virulente (positive kontroller), og to stammer, som vi mener, må kunne betragtes som at være avirulente (negative kontroller). Valget af de positive kontroller er foregået ved, at med udgangspunkt i vores egne stammesamlinger, at finde ***Bacillus cereus*** stammer, som dels stammer fra sygdomstilfælde, og dels er identiske med eller meget nært beslægtede med stammer, som har været sat i forbindelse med sygdom. Slægtskabsanalysen er blevet gennemført ved hjælp af en metode kendt som ”Multiple locus sequence typing” (MLST). Desuden blev stammer som kan vokse ved 43°C valgt. ***B. cereus*** er så nært beslægtet med ***B. thuringiensis***, at mange forskere mener, at de bør anses for at være en og samme art; da fylogenetiske undersøgelser har vist, at nogle stammer af ***B. thuringiensis*** er nærmere beslægtede med stammer fra ***B. cereus*** end med andre ***B. thuringiensis*** stammer. Som negative kontroller har vi anvendt en ***B. thuringiensis*** stamme, som ved en mutation, har fået ødelagt evnen til at udtrykke de mest almindelige virulens faktorer, og en ***B. cereus*** stamme fra et probiotika.

Ni af de udvalgte stammer blev testet under forskellige ***in vitro*** betingelser for at undersøge deres evne til at overleve i mave-tarmkanalen hos mennesker – den første faktor nødvendig for at en ***Bacillus thuringiensis*** stamme kan give sygdom. Udover overlevelse under mave-tarm forhold, dvs. overlevelse under mave- og tyndtarmsforhold, blev stammerne også studeret for vækst ved lave og høje temperaturer. Tidligere studier har vist at sygdomsfremkaldende stammer gror bedre ved lave og høje temperaturer sammenlignet med miljø-isolater. Dette betyder at temperaturprofilen for en stamme kan give en første indikation af, hvorvidt stammen er i stand til at give sygdom hos mennesker eller ej.

I de ni undersøgte stammer er der en tendens til, at stammer isoleret fra enten fødevarer involveret i udbrud eller fra patienter er bedre til at gro ved høje

temperaturer, og har en bedre overlevelse i mave og tyndtarm end stammer isoleret fra kommercielle plantebeskyttelsesmidler. Men for at lave en endelig konklusion er det nødvendigt at undersøge flere stammer.

Caenorhabditis elegans er en rundorm, som lever af bakterier. Den er i de seneste år blevet anvendt som "forsøgsdyr" i mange forskellige eksperimentelle sammenhænge. Den har den store fordel som "forsøgsdyr", at den er nem at holde i kultur, reproducerer sig når den er 3-4 døgn gammel og har en levetid på 2-3 uger, desuden er dens fulde genom kendt, og den kan fås i en lang række forskellige typer (mutanter). F.eks. er der i forsøgene i dette projekt anvendt en mutant, som ikke kan reproducere sig ved 25°C. ***C. elegans*** har i de seneste år i stigende grad fundet anvendelse til at studere interaktioner mellem mikroorganismer og eukaryote organismer, og specielt til at studere potentielle humane patogener og deres virulens faktorer. Den har så vidt vi ved ikke tidligere været anvendt i forbindelse med undersøgelser af ***B. thuringiensis/B. cereus*** virulens overfor mennesker. I projektet er der blevet udvikler en metode, som på en relativ simpel, så lidt arbejdsbelastende, og reproducer måde kan anvendes til at undersøge ***B. thuringiensis/B. cereus*** stammers effekt på ***C. elegans***. Metoden vil sandsynligvis med fordel også kunne anvendes til undersøgelser med andre bakterier.

Resultaterne opnået med metoden, viser, at bakterierne med hensyn til virurlens over for ***C. elegans*** kan opdeles i fire kategorier, og at alle ***B. thuringiensis/ B. cereus*** stammerne har en højere virulens end en ***E. coli*** stamme, som betragtes som helt avirulent. Den mindst virulente stamme er Bt 50, som stammer fra produktet Agree; den efterfølges af stammerne Bc11, Bc14, Bt48, Bt52 og Bt53, en blanding af den negative kontrol stamme, produktions-stammer og positive kontrolstammer; den næste gruppe består af stammerne Bc25 og Bc38, to positive kontrol stammer, mens den fjerde og mest virulente gruppe består af en stamme Bt55, som er fra produktet Novodor.

C. elegans modellen kan således adskille ***B. thuringiensis/B. cereus*** stammer med hensyn til deres virulens. Modellen er relativt simpel at anvende, også med et større antal af bakterielle stammer, og data er relativt simple at analysere. Den kan bruges til at undersøge stammers potentielle virulens, men vi har ikke fundet nogen enkelt sammenhæng mellem patogenecitet overfor mennesker og virulensen overfor rundormen.

Galleria mellonella larvemodellen er et insekt, som ofte er blevet anvendt til virulensanalyser af både humane og insekt patogener. Fordelen ved denne model er, at den har en del af de fysiske barrierer og det immunforsvar som findes hos dyr og mennesker. Det drejer sig om tarmceller med et lag af mukus, immunceller der kan fortære mikroorganismer (makrofager) og forskellige faktorer der kan inhibere vækst af bakterier. Desuden er den let at dyrke og tåler vores kropstemperatur (37°C). ***Galleria*** er allerede anvendt til virulensanalyser af ***B. cereus*** gruppe bakterier (Salamitou ***et al.*** 2000, Fedhila ***et al.,*** 2010, Cadot ***et al.,*** 2010). Infektionen foregår både oralt og via injektion direkte ind i hemocoelen (larvens blod/lymfe system). Forsøgene blev udført med både sporer (et stadium hvor bakterien er sovende, hvilket er den form, der findes i de kommercielle Bt baserede produkter) og vegetative bakterier. Sporer som inokulum er relevant for visse situationer som for eksempel i forbindelse med forurening af fødevarer, hvor Bt har været anvendt til behandling af afgrøderne; men bakterierne kan også findes i deres vegetative form i forarbejdede fødevarer, samt når de er i kontakt med værtens

tarmceller. Derfor er det også nødvendigt at analysere virulens med det vegetative stadium. Foruden virulenstest blev der analyseret for sporernes spiringsevne og spiringshastighed i larvetarmen. Resultaterne viste en vis variation mellem stammerne især ved 3 timer post infektion, ved 24 timer post injektion var de fleste sporer spiret. Alle stammer blev analyseret oralt med en eller flere doser afhængig af stammerne. Direkte injektion blev gjort med flere doser. Der var generelt ikke signifikant forskel mellem stammerne ved oral infektion, dog var Bt53 mere virulent end de andre stammer i sporetest og alle stammer med undtagelse af den negative reference Bt48 (PlcR mutant) viste samme virulens med infektion med vegetative bakterier. Til gengæld var der en mere mærkbar stammeforskel i virulens niveau via injektion ind i hemocoelen. Det var således muligt at gruppere stammerne efter virulensniveau. Det kan derfor konkluderes at ***Galleria*** tilsyneladende kan bruges til adskillelse af virulens niveau via hemocoel injektion, og er bedst med vegetative bakterier. Det er interessant at notere at Bt sprøjtemiddel stammerne findes både i lavt og høj virulente Bc stamme-grupper. Selvom vi kun har analyseret få stammer var det muligt at udpege kandidatstammer for høj og lav virulens. For at validere modellen vil det være gavnligt at lave tilsvarende analyser med et større antal stammer.

In vitro/in vivo genekspressionsanalyser: En af de store udfordringer ved dette projekt var at kunne analysere genudtryk direkte ***in vivo,*** når bakterierne er i kontakt med værtscellerne. Dette aspekt blev analyseret i insekt modellen (***Galleria***) og i Caco2 tarmcellerne. Efter flere justeringer af forsøgsprotokollerne er det lykkedes at opnå kvantitative ekspressionsanalyser på enkelte stammer og gener, især for ***Galleria*** larve modellen, men også i Caco2. Alt i alt er det et meget positivt resultat, som vil have indflydelse på vores kendskab til Bt virulensgener, der er udtrykt ***in vivo***. Vi fandt blandt andet at enterotoksinerne (Hbl, nhE og CytK), i de 5 analyserede stammer var udtrykt i larve tarmen, men med forskellig styrke. Disse resultater er især af interesse for andre ***in vivo*** studier både med hensyn til analyse af bakterie faktorer samt insekt immunrespons.

Funktionells mammalle celle modeller

Tarmen er opbygget af epitelceller, som er bundet tæt sammen og danner en barriere. Har man diarré eller lider af en tarmsygdom, kan denne barriere være svækket. Ved hjælp af en cellemodel af tarmen er det muligt at måle den elektriske modstand over cellelaget (trans epithelial electrical resistance, TEER), som giver et udtryk for hvor tæt epitelcellerne er bundet. Det er tidligere vist at probiotiske bakterier kan øge TEER, mens sygdomsfremkaldende bakterier kan nedbringe TEER i model systemet. Yderligere, som et mål for bakteriernes virulens, er det muligt at undersøge adhesion til og invasion af epitelcellelaget samt at observere morphologiske ændringer ved mikroskopi, samt måle effekten på immuncellers viabilitet. Alle disse aspekter er blevet analyseret med de fleste af de udvalgte stammer. I indeværende arbejde er der anvendt to typer af epitelcellelinjer Caco-2, og HT29-MTX som begge er af human oprindelse. Caco-2 er en kræft cellelinje, som differentierer sig under vækst, så at den fysisk ligner epitelceller i tyndtarmen, og HT29-MTX er en "goblet" celle type der producerer et beskyttende lag mucus. Der er desuden anvendt en makrofag cellelinje PoM2, som er af svineoprindelse, til at måle bakteriernes evne til at omgå immunsystemet (overleve/undgå phagocytering).

Resultaterne af dette studie viser, at alle de undersøgte stammer (produkt stammer, en probiotisk og de sygdomsfremkaldende stammer) nedbringer den

elektriske modstand (TEER af Caco-2 og HT29-MTX celler). Alle stammerne kunne endvidere forårsage morfologiske ændringer/skader på epitelcellerne observeret ved mikroskopi. Resultaterne tyder på, at samtlige undersøgte bakterier inklusiv den probiotiske kontrol bc49 har sygdomsfremkaldende potentiale. Ved at undersøge effekt af dosis af bakterier blev det imidlertid klart at bakterierne differentierede sig med hensyn til den hastighed hvormed de lavede skade på de mammale celler, dette især ved lav infektionsdosis. Ved i indeværende arbejde at udføre infektioner med lave bakterietal, sås det, at bakterierne differentierede sig med hensyn til hvor hurtigt TEER blev nedbrudt, og hvor hurtigt epitelcellerne blev ødelagt (observeret ved mikroskopi). Ud fra forsøg udført med de funktionelle mammale cellemodeller, placerede to af produktstammerne (bt50 og bt52) sig primært i gruppen af bakterier med lav potentiale for virulens (var "langsommere" end den probiotiske bakterie). Til sammenligning var der én produktstamme (bt53) der uafhængig af type af model placerede sig som mere "hurtig" end de sygdomsfremkaldende bakterier (dog ikke ved meget lav infektions dosis). Én produktstamme (bt55) placerede sig i nogle modeller på linje med de sygdomsfremkaldende bakterier (bc25 og bc38), og i andre modeller blandt dem med lav potentiale for virulens (bc49). Hastigheden hvormed bakterierne udøver "skade" kan måske anvendes som et mål for bakteriernes potentiale for effektivt at kunne "klare" sig/lave skader under passagen i tarmen, hvor evne til sammenspil og konkurrence med mange andre bakterier vil være af stor betydning. At vurdere ud fra "hastighed" er tidligere gjort for produktstammen ***Bacillus subtilis*** (natto), der anvendes til fermentering af Natto (en japansk spise, der anses for at være gavnlig for helbredet). Således var hastigheden hvormed denne bakterie nedbringer TEER langsommere, sammenlignet med hastigheden af diverse sygdomsfremkaldende bakterier (Hosoi et al. 2003). Konkluderende synes TEER målinger at være mere differentierende og mere objektiv end adhesion/invasions modellen, der inkluderer subjektive observervationer ved mikroskopi. Modellen der undersøger effekt af dosis på tidspunktet for skade på cellelaget bidrager også med god information om forskelle i bakteriernes sygdomsfremkaldende potentiale. Det kan ikke udelukkes at den atmosfære (5% CO_2, 95% atmosfærisk luft), som er anvendt til udførsel af forsøgene har påvirket vækshastighed og gen ekspression, og måske dermed virulensen på en anden måde end hvis forsøgene havde været udført under anaerobe forhold. Det kunne derfor være interessant i fremtiden at arbejde med modeller med reduceret ilt, der minder mere om forholdende i tarmen. Resultaterne for gen-ekspression under infektion i cellemodellerne viser, at gener associeret til diarre (***cytK, nheA***) blev udtrykt i alle grupperinger af bakterier.
Resultaterne opnået for de funktionelle mammale cellemodeller i dette studie tyder på at hvis man skal vurdere andre typer af biologiske bekæmpelses midler vil der være behov for at udvikle modeller til netop disse grupper. Resultaterne for placering af produktstammer i forhold til sygdomsfremkaldende potentiale, tenderer de resultater, der er opnået med ***G. melonella*** og ***C. elegans***.

De opnåede resultater og erfaringer med de forskellige modeller bliver diskuteret i forhold til deres anvendelighed til risikovurdering af mikrobiologiske plantebeskyttelsesmidler.

Summary

The aim of the project has been to investigate three different types of pathogenecity models for their ability to assess virulence in microorganisms used as pest control agents. De three models were the nematode ***Caenorhabditis elegans***, ***Galleria mellonella*** larvae and functional mammalian cell-models.

The bacterium ***Bacillus thuringiensis*** has been used as a model for microorganisms used in pest control agents. This bacterium was chosen because it is an active ingredient in several plant protection products, which are the most successful worldwide. Further it is known that some strains are potential pathogens. Four different strains were chosen as primary objects for the investigations; these strains originate from the products Agree, Vectobac, Dipel and Novodor. The strains are from four different subspecies of ***B. thuringiensis,*** and can be used for control of lepidopteran, dipteran and coleopteran larvae. Mainly Dipel and Vectobac is in usen in Denmark.

To be able to compare these four product strains in relation to virulence, four related strains, which are supposed to be pathogenic were selected (positive control strains). Further were two strains supposed to be avirulent selected. The choice of the selected positive control strains were based on our of own selections of ***B. thuringiensis/ B.cereus*** strains were selected on the basis of their isolation in relation to cases of illness and their relationship to other strains isolated from "cases". The relationship to other strains was investigated by the use of a method known as "multiple locus sequence typing" (MLST). In addition were strains able to grow at 43°C selected. ***B. cereus*** is very closely related to ***B. thuringiensis***, and several researchers is of the meaning that these two species should be regarded as one species; some strains of ***B. thuringiensis*** is in a phylogenetic context more related to some ***B. cereus*** strains than to other ***B. thuringiensis*** strains. As negative controls were a ***B. thuringiensis*** strain mutated in the regulator of the most common virulence factors and a ***B. cereus*** strain from a probiotic chosen.

The nine selected strains were tested under different ***in vitro*** conditions to study their potential to survive in the gastrointestinal tract of humans – the first prerequisite of a ***Bacillus thuringiensis*** strain to cause disease. Besides the survival under gastrointestinal conditions, i.e. survival in gastric and small intestinal environments, the strains were also studied at low and high temperatures. Previous studies have shown that pathogenic strains grow better at low and high temperatures compared to environmental strains. This means that studying the temperature profile of a strain can give an indication on the predisposition of the strain to cause disease in humans.

There is a tendency in the nine strains investigated, that strains isolated from either food involved in outbreaks or from patients have better growth capacity at high temperatures and better survival at gastrointestinal conditions than strains used commercially in plant protection products. However, to make a firm conclusion, it is necessary to study more strains.

Caenorhabditis elegans model:

Caenorhabditis elegans is a nematode, which consumes bacteria. This nematode has during the last ten years been used as a model-organism in a number of different connections. Its significance as a model-organism depends on its easy culturability, that it starts reproduction within 3-4 days and have a lifespan on 2-3 weeks, further has its full genom been sequenced and it exists as a high number of types (defined mutants); e.g. is the experiments in this project carried out with a mutant unable to reproduce at 25°C. C. elegans has increasingly been used to study interactions between microorganisms and eukaryotes, and especially to investigate human pathogens and their virulence factors. According to our knowledge has it not previously been used for studies on the virulence of ***B. thuringiensis/B. cereus*** for humans. In the project a method, which in a relatively easy and reproducable way can be used for investigations on the effects of ***B. thuringiensis/B. cereus*** strains on ***C. elegans***. The developed methodology is most likely also applicable with other bacteria.

The results obtained with the develop methodology show, that the bacteria with regard to virulence toward the nematode, can be divided into four categories, and that they all got a higher virulence than an ***E. coli*** strain, which are considered to be avirulent. The strain with the lowest virulence is Bt50 from Agree, followed by the strains Bc11, Bc14, Bt48, Bt52 and Bt 53, a mixture of the negative control strain, strains from products and positive control strains; the next group consist of the strains Bc25 and Bc38, two positive control strains, while the fourth group onlt contains one strain Bt55, from the product Novodor.

The C. elegans model is, thus, able to distinguish between different ***B. thuringiensis/B. cereus*** strains with regard to virulence. The model is relatively easy to handle, and to use with more strains, the data is relatively easy to analyze. It can be used for investigatins on the potential virulence of strains, however a simple relationship between patogenicity towards humans and the virulence towards nematodes has not been found.

Galleria model:

Galleria mellonella insect larvae are often used to identify virulence and infection of both human pathogens and insect pathogens. An advantage of such a simple model is that it contains some of the physical barriers as well as innate immunity similar to those of man and higher animals. For instance the intestinal cells posses a protective gel-like layer, immune cells can ingest micro-organism and particular compounds can attack the surface of pathogens. And beside to this, larvae supports well human body temperatures and are easy to rear. ***Galleria*** is already used for virulence analysis of ***B. cereus*** group bacteria. (Salamitou ***et al.*** 2000, Fedhila ***et al.,*** 2010, Cadot ***et al.,*** 2010).

The analysis is conducted with two routes of infection: oral and by injection into the hemocoel (equivalent to blood/lymphe). Our tests were run with both spores (the dormant stage of the bacteria, the one which is present in the commercial ***B. thuringiensis*** based products) and vegetative bacteria (the form which can multiply). Using spores for infection is relevant to situations where the Bt based products may contaminate food, and vegetative bacteria can both be part of consumed food and be in this growth stage when in contact with the host cells (intestinal or immune cells). In order to get an idea about the speed of the strains to develop in the host, the spore germination capacity was

first analysed. The results showed a variation between strains at 3 hours post ingestion, while at 24 hours all strains had largely germinated. Virulence following oral infection was measured for all ten strains with one or several doses. Generally we did not find any strong difference among strains except for strain Bt53 (kurstaki) that was most virulent while using spores. All strains were equivalent virulent with vegetative bacteria except the negative control Bt48 (PlcR mutant). Meanwhile, we found a more pronounced strain difference when the strains were used for infection by injection into the hemocoel. Indeed, strains were found more or less virulent especially at lower dose.

From our results we may conclude that ***Galleria*** can probably be used as a model to estimate virulence potential and that such estimation is more easily obtained with vegetative bacteria. It is interesting to note that the Bt strains are both in low and high virulent groups. Meaning that some strains are potentially more virulent than others, if they are tested a particular situation. Thus although we only have analysed a few strains it was possible to identify a positive and a negative control strain, which might be used in the development of a new risk model protocol.

However, in order to validate the model it is important to make similar studies on a larger number of strains.

In vitro/in vivo gene expression analysis: One of the big challenges of this project was to analyse ***in vivo*** gene expression when the bacteria are in contact with the host using Q-RT-PCR. This aspect was analysed in the insect ***Galleria*** and in the intestinal cell culture Caco2. Upon optimisation we were finally able to measure expression of several genes in a few strains, while the bacteria were located inside the larval gut and in contact with the Caco2 cells. In summary it is very interesting results which highlights our knowledge to which genes are expressed ***in vivo.*** We found for instance that the entero-toxins (Hbl, NhE, CytK) were expressed at different levels in the analysed strains. These results are especially of interest for further ***in vivo*** studies both to analyse other bacterial factors but also to analyse host responses. But we do not recommend that for routine analysis, because of the complexity to standardize Q-RT-PCR.

Functional mammalian cell models

The intestine is composed of epithelial cells which are bound closely together, forming a barrier. If you have diarrhea or suffer from an intestinal illness, this barrier has been compromised. By using a cell model of the gut is it possible to measure the electrical resistance across a cell layer (trans-epithelial electrical resistance, TEER), which provides a measure of how close the epithelial cells are bound. It has previously been shown that probiotic bacteria may increase the TEER, while pathogenic bacteria can reduce the TEER in the model system (Klingberg et al. 2005). Further as a measure of bacterial virulence, it is possible to study adhesion to and invasion of the epithelial layer as well as to observe morphological changes by microscopy, and measure the effect on the viability of immune cells. All these aspects have been analyzed with most of the selected strains in the current work. The current work has used two types of epithelial cell lines, Caco-2 and HT29-MTX that are both of human origin. The Caco-2 is a cancer cell line that differentiates itself during growth, so that it physically and biochemically resembles the adsorptive epithelial cells of the small intestine. In comparison HT29-MTX is a "goblet" cell type that produces a protective layer of mucus during prolonged culturing. A

macrophage cell line PoM2 of porcine origin, has also been used to measure the bacteria's ability to circumvent the immune system (machrophage engulfment).

Results of this study show that all the investigated strains (***B. thuringiensis*** product strains, a probiotic and pathogenic strains) reduces the transepithelial electrical resistance (TEER) of Caco-2 and HT29-MTX cell monolayers. All the strains caused morphological changes / damage to the epithelial as observed by microscopy. The results indicate that all investigated bacteria including the probiotic control bc49 have pathogenic potential. By examining the effect of the dose of bacteria, however, it was clear that bacteria could be differentiated based on the speed with which they damaged the mammalian cells, particularly at low infectious dose. Thus in this work by performing infections with low bacterial numbers it was observed that bacteria are differentiated in terms of how quickly TEER was impaired, and how quickly epithelial cells were damaged (observed by microscopy). By use of the functional mammalian cell models, two product strains (bt50 and bt52) could be placed primarily in the group of bacteria with low potential for virulence (were "slower" than the probiotic bacterium). By comparison, there was one product strain (bt53) that independent of type of model positioned itself as more "rapid" than the pathogenic bacteria (but not by very low infectious dose). One product strain (bt55) positioned itself in some models in line with the pathogenic bacteria (bc25 and bc38) and in other models among those with low potential for virulence. We suggest that the rate at which the different bacteria performed "damage" may be used as a target for determining the potential to effectively cause damage during the passage of the intestine, interaction and competition with many other bacteria will be of great importance. "Speed" was previously used to differentiate the product strain ***Bacillus subtilis*** (natto) used for fermentation of Natto (a Japanese dining that is considered to be beneficial to health) from various pathogenic bacteria (Hosoi et al. 2003).

In conclusion TEER measurements seem to be more objective than the adhesion / invasion model that includes subjective observervations by microscopy. The model which examines the effect of the dose on the time for injury also contributes to good information about individual differences in the bacterial pathogenic potential. It is possible that the results we have achieved in the models were influenced by the choice of atmosphere (5 % CO2, 95% atmospheric air) affecting the growth rate and gene expression, and perhaps hence the virulence as compared to if conducted under anaerobic conditions. It could therefore be interesting in the future to work with models with reduced oxygen, which is more comparable to the conditions in the gut. The results for gene expression during infection in the cell models showed that genes associated with diarrhea (***cytK, nheA***) were expressed in bacteria that were categorized as having high and low potential for virulence.

The results obtained for the functional mammalian cell models in this study suggest that if we want to assess other types of biological control agents it will be necessary to develop new models for these types. The results for the product strains in relation to pathogenic potential are similar to the results obtained with ***G. melonella*** and ***C. elegans*** models.

The results and the experience witrh the three different approaches are discussed in relation to their applicabilty in raltion to risk assesment of microbial pest control agents.

1 Introduction

1.1 Introduction to Health risk assesment of microbial

Microbial plant protection agents based on fungi, bacteria or virus are used in Denmark in e.g. greenhouses for vegetables and flowering plants, and on vegetables in the field. Currently the microbial plant protection products that are allowed for use in Denmark are strains of the following microorganisms (active ingredients):

Fungi:	***Beauveria bassiani***
	Coniothyrium minitans
	Lecanicillium muscarium
	Phlebiopsis gigantean
	Streptomyces griseovirides
	Trichoderma harzianum
	T. polysporium
Bacteria:	***Bacillus thuringiensis*** subsp***. kurstaki***
	B. thuringiensis subsp***. israelensis***
	Pseudomonas chloroaphis
Vira:	***Cydia pomonella***

Before a microbial plant protection agent can be used in Denmark, it has to be approved in the EU, and put on the Annex 1 list of EU directive 91/414/EC, that list all active ingredients approved. In 1993, the European Commission launched a Community-wide review process for all active ingredients used in plant protection products within the EU. From the end of 2003, EFSA (European Food Safety Authority) deals with risk assessment issues and the European Commission is responsible for the risk management decisions.

The EU Directive 91/414/EC lays out a comprehensive risk assessment and authorisation procedure for active ingredients and products containing these substances. Each active ingredient has to be proven safe in terms of human health, including residues in the food chain, animal health and the environment, in order to be allowed to be marketed. It is the responsibility of industry to provide the data showing that a substance can be used safely with respect to human health and the environment.
However, the existing guidelines for risk assessment of human health of microbial plant protection agents are based on guidelines for chemical pesticides. Microbials are living organisms and therefore more complex than chemicals, and therefore the methods used in the existing guidelines are not always the best to reveal potential pathogenic properties in microbes. However, the guidelines have been developed to include infectivity and persistence as one of the factors that have to be studied in the animal studies.

The human health risk assessment of microbial plant protection agents is based on a tiered approach. The Tier 1 studies aims at determining whether high concentration of the active ingredients cause any harm in a series of test, including acute oral, acute pulmonary, acute intraperitoneal, acute dermal, skin and eye irritation, and dermal sensitivity. If the Tier 1 testing results in any uncertainty, Tier 2 studies must be performed. These are primarily the

same as the Tier 1 studies, but require multiple consecutive exposures, especially if the Tier 1 studies have shown infectivity or toxicity.

One of the weaknesses of the existing methods, are that no positive controls (pathogenic isolates) are used, giving the uncertainty whether a pathogenic isolate would give a response in the animal models used. In a former research project for the Environmental Protection Agency, we showed that using a known human pathogenic ***Bacillus cereus*** strain in an oral rat study, did not give rise to illness or diarrhea in the animals (Wilcks ***et al.***, 2006). So there is a strong need to develop new models and methods to assess the potential human pathogenicity of microbial organisms used in plant protection products.

In this project we are primarily focused on establishing better methods and models to assess pathogenicity in humans after oral exposure of microbes used as plant protection agents.

1.2 Bacillus Thuringiensis as an example of a micrbial pest control agent

In this work ***Bacillus thuringiensis*** has been selected as an example of a pest control organism. This selection is made first of all because it is the most thoroughly investigated organisms on the market today. Second, as it is important to include negative and positive controls in the model development, we can benefit from the close relationship with ***B. cereus,*** which more or less is genetically identical with ***B. thuringiensis*** (Hansen and Hendriksen 2002)***,*** and which is represented by both pathogenic strains, and strains used as probiotics (Hansen and Salamitou, 2000). ***B. cereus*** is generally known to be an opportunistic human pathogen producing a number of virulence products, which also are known to be produced by ***B. thuringiensis*** (Damgaard 1995). Although this group of bacteria has been studied for years, it has still not been possible to link specific genotypes with highly virulent phenotypes, and consequently, risk assessments rely primarily on ***in vivo*** testing. Recently, however some studies have divided a collection of ***B. cereus*** group species strains into 7 phylogenetic groups, with different probabilities for virulence. The groupings are based on temperature growth range, toxin gene occurrence, cytotoxicity assays and grouping with outbreak strains. The groupings comply with groupings by key genes such as the ***PanC*** gene (Guinebretiere et al. 2008; Guinebretiere et al. 2010). In the present study we use multi locus sequence typing in combination with growth at 43°C, (See also sections 3.1.3 and 3.1.4).

1.3 Effects of physical and chemical factors on the survival of mibrobial pest control agents in the gut

In order for an ingested microbial pest control agent to infect human beings, the strain must be able to survive the harsh conditions through the upper gastrointestinal tract, and be able to grow and express its potential pathogenic traits at the temperature present in the gut (app. 37° C). Many of the fungi used as microbial pest control agents are unable to grow at 37° C and are therefore unable to cause infection in humans. However, e.g. ***Bacillus***

thuringiensis that is closely related to ***Bacillus cereus*** is able to grow at body temperatures, and therefore it is relevant to study its potential to cause disease in humans after ingestion.

The first barrier, the agent meets on its way to the intestine, is the stomach. The stomach is characterized by containing hydrogenchloride (HCl) that has a very low pH (approx. 2) and the presence of the enzyme pepsin. Most microorganisms are killed by the low pH. However, studies have shown that some food stuff can enhance the survival of microorganisms as ***B. cereus*** through the stomach (Clavel ***et al***., 2004; Clavel ***et al***., 2007).

After the stomach, the food and surviving microorganisms enters the small intestine. Bile acids excreted by the pancreas kills many microorganisms, but again the microorganisms can be protected by being imbedded in food. Furthermore, the sensitivity against bile acid differs among different strains of microorganisms.

At present, oral studies with rodents are used in assessing the potential of a microbial pest control agent to survive and give rise to infections in humans. However, many of the end-points in these studies can be investigated by ***in vitro*** experiments, and that is what we have done in this project. Firstly, it is important to study whether the microorganism has at all, the potential to survive the passage through the stomach. If that is not the case, the potential of the microbial plant protection agents to cause any toxicity or pathogenicity in humans can be regarded as insignificant. Furthermore, if the agent is very sensitive to bile acid, it has also a very low potential to cause any harm in humans by ingestion.

1.4 *Caenorhabditis Elegans, Galleria Mellonella* and mammalian cell models as tools in risk assesment

1.4.1 C*aenorhabditis elegans* as a model to study host – microbial interactions

The nematode ***C. elegans*** is a small eukaryotic organism, which during the last years has been used increasingly for research purposes. The genomic DNA sequence is known, and numerous mutants are available. Further, ***C. elegans*** is easy to maintain in laboratory cultures, can reproduce in an age of approximately three days, and has a lifespan of about 2-3 weeks. Besides being used for genetic investigation, the nematode has also been used to study microbial-eukaryotic interactions, and especially have been used increasingly to study bacteria pathogenic to humans.

1.5 Use of *Galleria mellonella* as a model to study host – microbial interactions

Using insects to identify pathogens and to determine important virulence factors and to understand infection processes of pathogens and parasites as different as, virus, fungi, and nematodes have been done with the insect larvae ***Galleria mellonella***. It has mainly been performed with respect to insect pathogens but also human pathogens like the Gram negative bacteria ***Pseudomonas aeruginosa*** (Finlay, 1999), the fungus ***Candida albicans***, (Kavanagh & Reves, 2004) and gram positive bacteria as ***Streptoccoccus, Enterococcus*** (Gaspar et al. 2009) and ***Listeria monocytognes*** (Susan et al.

2010) as well as ***Bacillus cereus*** (Fedhila et al. 2010). Most of published infections have been conducted by injection of the bacteria into the larval hemocoel, (which can be considered similar to blood/lymphe of higher animals) but for ***B. cereus*** it has been possible to use the ***Galleria mellonella*** larvae as a model for oral infections using the so called synergy between the bacteria (as spores or vegetaive) associated with a Cry1C toxin (Salamitou et al., 2000, Fedhila et al., 2002). Indeed, ***G. mellonella*** larva is only slightly susceptible to this toxin or spores alone, but strong mortality will occur when the toxin is mixed with spores or vegetative bacteria (Salamitou et al., 2000). Using this approach several virulence factors have been identified and particularly the role of the transcriptional regulator PlcR, which is found in all ***B. cereus*** and ***B. thuringiens*** sequenced strains, has been investigated. Since this regulator is controlling the expression of several of the enterotoxins and a metalloprotease which are involved in virulence of ***B. cereus*** and ***B. thuringiensis*** (for review Stenfors Arnesen et al. 2008; Johnston et al. 2010, and Fedhila et al., 2002) it is of interest to use ***G. mellonella*** as a model for comparing virulence of ***B. cereus*** and ***B. thuringiensis*** strains particularly when questions are related to interaction with the intestinal barriers. ***Galleria*** is also attractive as a model since it tolerates human body temperatures (eg. 37°C to 45°C). In addition this insect is easy to maintain in rearing and the larva has physiological and immunity features and barriers, which are similar to vertebrates. For instance some parts of the intestine is composed of cells with microvilla and a mucus like structure, called peritrophic matrix which is protecting the intestinal cells from direct contact with food and ingested pathogens (Terra et al., 2006). ***Galleria*** also has cellular immunity like phagocytosis by hemocytes (similar to human macrophages) and antimicrobial peptides are produced in hemocoel (Kavanagh & Reves, 2004). Thus, although this insect larvae is not a vertebrate model it is expected that some of the factors which permits the bacteria to overcome the host resistance immunity might be similar in an insect and in a higher animal model or man (Gely –Vallet, et al., 2008).

1.5.1 Use of mammalian cells as in vitro models to study host – bacterial interactions (in the gastro intestinal tract)

The gastrointestinal tract (luminal surface) is mostly lined with a single layer of highly polarized epithelial cells. The epithelial cells in the intestinal region consist of enterocytes or absorptive cells (main cell type), goblet cells that secrete mucin, endocrine cells, paneth cells, M cells, tuft, and cup cells (Balimane et al. 2000). The enterocytes are polarized cells with a distinct apical and basolateral membrane. Tight junctions between the enterocytes separate the apical (facing the lumen) and basolateral domains (facing the underlying basement membrane) and enable the epithelial monolayer to maintain polarization and impermeability to digestive enzymes and foreign substances (e.g. bacteria) (Balimane et al. 2000; McCormick 2003). During invasion by pathogens, food intolerance reactions, and inflammatory bowel diseases, an increased permeability of the epithelial barrier may occur, resulting in disease.

Animal models (e.g. rodents and mice) have often been used to study host pathogen interactions in the (human) gastro intestinal tract. However rodents and mice do not always react to pathogens in the same way as humans do, e.g. rodents did not develop diarrhoea when exposed to a diarrhoeal strain of ***B. cereus*** (Cencic and Langerholc 2010; Wilcks et al. 2006). Functional mammalian cell models use mammalian cell lines (human or animal) that can

easily be cultured in a laboratory, are of relatively low cost, and have no ethical issues. They are as such an interesting alternative to the animal models (which are expensive and have ethical issues, and demand specially trained personnel). Many mammalian cell lines are available and they have their pros and cons, and the choice of cell line is dependent on the purpose of the studies (Cencic and Langerholc 2010). The mammalian cells lines (Caco-2, HT29-MTX and PoM2 cells) and models used in the present work are presented in the sections below.

One of the most widely used cell lines as an ***in vitro*** model of the intestinal barrier is the Caco-2 cell line. The Caco-2 cell line is a human colon adenocarcinoma cell line obtained from a 72 year old patient (Sambuy et al. 2005). Caco-2 cell show features that are very important for i***n vitro*** model studies of the intestine. Thus the Caco-2 cells grow into a polarized monolayer (as in the intestine), and differentiates spontaneously in long term cultures expressing morphological and biochemical characteristics of small intestine columnar absorptive enterocytes (Pinto et al. 1983). Caco-2 cells are often grown on plastic surface for the purpose of studying adhesion and invasion. Adhesion to and invasion of the epithelial layer, can be used as a measure of bacterial virulence (Andersson et al. 1998; Minnaard et al. 2004). However, if Caco-2 cells are grown on filter supports permits (instead of on plastic surfaces) uptake of nutrients will (apart from the apical) occur via basolateral feeding, mimicking the ***in vivo*** setting where epithelial cells take up nutrients from underlying blood vessels (McCormick 2003). Further the free access of ions and nutrients to the two sides of the cell monolayer grown on permeable filter supports improves morphological and functional differentiation (McCormick 2003). The level of Caco-2 polarization when grown on filter supports can be determined by trans epithelial electrical resistance (TEER) measurements (McCormick 2003). Development of a high TEER is normally linked to the generation of tight junction complexes, and increases during prolonged culturing (Bravo et al. 2004). TEER measurements of Caco-2 cells grown on permeable filter supports have been widely used to demonstrate host pathogen/probiotic interactions (Commane et al. 2005; McCormick 2003). For example, it has been shown that probiotic bacteria were able to strengthen the intestinal barrier (increase the TEER), while pathogenic bacteria such as the Gram positive bacterium ***Listeria monocytogenes*** permeabilized the barrier (decreased the TEER) in the Caco-2 model system (Klingberg et al. 2005). A weakening of the epithelial barrier (a decrease in TEER) has further been observed after infecting with Gram negative pathogenic bacteria including ***Salmonella*** enterica serovar ***typhimurium*** (Tafazoli et al. 2003), ***Escherichia coli*** (Philpott et al. 1996; Resta-Lenert and Barrett 2003), ***Pseudomonas aeuriginosa*** (Wu et al. 2004) and the pathogenic yeast species ***Candida albicans*** (Frank and Hostetter 2007).

As mentioned earlier, the intestinal epithelial layer also consists of mucus secreting goblet cells. The mucus has many functions; one of them is to cover the microvillus surface which contains glycoprotein components/carbohydrates that may act as receptors of toxins and pathogenic bacteria (Walker 1985). There are 12 known mucins in the intestinal tract. The secreted mucin MUC2 is the main secreted mucin in the healthy human intestine, and the surface associated mucins MUC1, MUC3, MUC4, MUC12, MUC13 and MUC17 are also identified (Corfield et al. 2001). The production of mucin is affected by environmental stimuli, and some mucins are strongly produced in cancer cells. MUC1 deficiency has been shown to

result in higher rates of systemic infection in ***Campylobacter jejunii*** infections in a murine model. Further increased MUC3 production has been positively correlated with a decreased binding of enterohaemorrhagic ***Escherichia coli***. Harmful microorganisms and enterotoxins however, are able to attach to and penetrate the mucosal barrier, gaining access to the enterocytes or interstitial space resulting in clinical disease (Walker 1985). As mentioned earlier the Caco-2 cell line is suitable for studying host-pathogen/probiotic interactions due to its ability to e.g form a polarized monolayer. A drawback of the Caco-2 cell line however is that it is not able to secrete a suitable mucus layer, though it does expresses various surface associated mucins (MUC1, MUC3, MUC4, MUC5A/C, MUC12, MUC13 and Leb) (Linden et al. 2007; VanKlinken et al. 1996). To investigate the importance of the mucus layer in bacterial infection or probiotic interactions other cell lines are recommended. One of them is a cell line of human carcinogenic intestinal origin, named HT29-MTX. This cell line is a strong producer of mucus (produces a dense mucus gel) (Kerneis et al. 1994) of mainly gastric immuno-reactivity (Lesuffleur et al. 1990). More specifically HT29-MTX produces MUC1, and expresses the genes encoding MUC2 (increases with time), MUC3 (expression is stable from day 14) and MUC5C (increases from day 7 in culture)(Lesuffleur et al. 1993). Although the mucus produced by the HT29-MTX cell line does not exactly match the mucus of the small intestine, the cell line has been widely used as a model to study host pathogen interactions in the intestinal tract, including adhesion and invasion studies, and studies involving TEER measurements although it does not form a highly polarized monolayer compared to Caco-2 (Kerneis et al. 1994; Laparra and Sanz 2009; Stanley et al. 2007; Tamang et al. 2009).

If the epithelial intestinal layer is permeabilised due to e.g. pathogenic bacteria, then macrophages will be one of the first barriers against infection. As part of the immune response different cytokines will be produced that mobilize the innate immune defense, that signals to T-cells activating a response against the pathogenic bacteria. Apart from the production of cytokines, and anti microbial substances (e.g. nitrogen oxide and hydrogen peroxide), the macrophages can phagocytise and kill pathogenic bacteria. If the macrophages however are killed by bacteria, then it indicates that these bacteria are highly virulent (Ivec et al. 2007). Among available macrophage cell lines, are the PoM2 cells, monocytes/macrophage cell line established from the peripheral blood of a pig. The PoM2 cells are positive for markers of myeloid cells, and functional characteristics (positive for MHC class II, membrane glycosylation patterns, presence of non-specific esterases, acid-phosphatase, amido-black staining, activation ability (phagocytosis, NO and ROS production)(Cencic and Langerholc 2010).

To summarize, functional mammalian cell models have previously been used to study the pathogenic or probiotic properties of both gram positive and gram negative bacteria (Andersen et al. 2007; Andersson et al. 1998; Botic et al. 2007; Klingberg et al. 2005; Laparra and Sanz 2009; Minnaard et al. 2004; Nissen et al. 2009; Riedel et al. 2006; Wijnands et al. 2007; Zheng et al. 2006), pathogenic properties of fungi (yeast) (Frank and Hostetter 2007) and virus (Botic et al. 2007; Ivec et al. 2007; Maragkoudakis et al.). Functional cell models are therefore expected to be useful for evaluating microorganisms including bacteria, fungi and even virus used as biopesticides.

2 Materials and methods

2.1 Collection, screening and selection of strains

2.1.1 Initial strain collection

The initial strain collection contains pathogenic and non pathogenic strains from the ***B. cereus*** group besides a number of ***B. thuringiensis*** product strains. The primary purpose for establishment of an initial strain collection is to have a number of potentially pathogenic and non-pathogenic strains among which we could select positive and negative control bacteria. As it is rather expensive to buy strains and as distribution of pathogenic bacteria is restricted, we decided to choose among pathogenic bacteria already present in our own strain collections. The non pathogenic strains were probiotic strains and a strain inactivated in the regulation of virulence functions. Although most of the product strains are available from strain collections, product strains in this study were all isolated from commercial products, to ensure the highest possible similarity with the actual bacterial content of products. The initial strain collection contains 46 potential pathogenic strain, three non-pathogenic strains and six product strains, which sums up to 55 strains in total. The bacteria are listed in appendix 1. Strains from the initial strain list which are selected and included in the selected strain list have further been characterised by the ability to grow at 43°C, and by the genetic relationship, determined by Multi Locus Sequence Typing (MLST).

2.1.2 Growth at 43ºC

2.1.2.1 Growth in liquid substrate

All 55 strains from were tested for their ability to grow at 30 and 43ºC under both aerobe (oxygen rich) and micro aerobic (oxygen poor) conditions. One bacterial colony from a plate was transferred to 5 ml BHI (Brain Heart Infusion, Oxoid) media. From here 200 µl were transferred to microtiter plates and incubated at 30 and 43ºC under both aerobic and micro aerobic conditions. Growth was tested after 24 hrs and scored in a scale up to three, where 1 was poor growth and 3 was good growth. The experiment was performed twice.

2.1.2.2 Growth on solid substrate

The sizes of the colonies of the 55 strains inoculated on solid T3 substrate (Travers et al., 1987) were determined after three days incubation at 30°C and 43°C. The day before inoculation of the solid substrates, the petri-dishes were placed at 30°C or 43°C to ensure the right temperature during incubation at 30°C and 43°C. From petri-dishes inoculated and cultivated with the 55 bacteria for three days at 30°C, small amounts of bacteria were transferred with a toothpick first to the “43°C” petri-dish, and then to the “30°C” petri-dish (without “re-inoculation” of the toothpick). As all 55 bacteria are known to be able to grow at 30°C, this procedures has a build-in internal control for successful transfer of bacteria to the solid substrate in the petri-dishes, as the toothpick is first dipped in 43°C agar and then in the 30°C agar. Also, to avoid temperature fluctuations, only 10 petri-dishes at a time were taken out of the 30°C and 43°C incubators for inoculation and

measurement of colony size. Further, in case no growth was observed at 43°C after three days incubation, the petri-dishes were transferred to the 30°C incubator for 24 hours as a control of successful inoculation.

2.1.3 Multi Locus Sequence Typing (MLST)

MLST is a nucleotide sequence based approach for the characterisation of bacterial isolates. The procedure characterises isolates of bacterial species using the DNA sequences (400-600 bp) of internal fragments of seven household genes. The advantages of MLST are that the data does not depend on techniques or differences in protocols. The DNA sequence of a gene is unique and can be stored and compared in databases available via the internet. The MLST procedures and available data for the ***B. cereus*** group are found at http://pubmlst.org/bcereus/. The primers for amplification and sequencing of the seven household genes are listed in Tabel 2.1. PCR is performed using recombinant ***Taq*** polymerase from Fermentas (#EP0404), the conditions recommended by Fermentas and the annealing temperatures are given in Table 2.1.3.1. The PCR reaction use an initial denaturation for 2 min at 94°C, 35 cycles (30 sec at 94°C, 30 sec at annealing temperature, 45 sec at 72°C) and a terminal 5 min at 72°C, after which the temperature was lowered to 4°C. A subsample of the PCR product was analysed on a 1.5% agarose gels, and the remaining PCR product was send for sequencing to SDGenomics A/S (Aarhus-Beijing). The fragments were sequenced in both directions. The data returned from SDGenomics were automatically generated sequences and the corresponding chromatograms. The suggested sequences were controlled and verified/modified using the corresponding chromatograms, and a final sequence was generated combining the sequences from the bi-directional sequencing. Using the ***B. cereus*** group MLST homepage (http://pubmlst.org/bcereus/) analytical tools, the individual sequences were assigned an allelic number if the sequence was present in the database. If it was an unknown sequence the closest related allelic number was returned from the database. By submitting the allelic numbers to the ***B. cereus*** group MLST homepage a specific sequence type (ST) number was returned if the sequence type was known in the database, otherwise the closest related ST number was returned. Having the ST number, the history and characteristics of isolates with the same and/or closely related ST number can be downloaded from the ***B. cereus*** group MLST homepage. 770 sequences were analysed during the MLST analysis of the 55 isolates in the initial strain collection.

Tabel 2.1 Primers used for MLST

Primers[A]	Primer sequence	Annealing Temperature	Fragment size[B]
glpF-F *glpF*-R	GCGTTTGTGCTGGTGTAAGT CTGCAATCGGAAGGAAGAAG	59	549
gmk-F *gmk*-R	ATTTAAGTGAGGAAGGGTAGG GCAATGTTCACCAACCACAA	56	600
*gmk*_F3-F *gmk*-R	GAGAAGTAGAAGAGGATTGCTCATC GCAATGTTCACCAACCACAA	55	565[C]
ilvD-F *ilvD*-R	CGGGGCAAACATTAAGAGAA GGTTCTGGTCGTTTCCATTC	58	556
*ilvD*_2-F *ilvD*_2-R	AGATCGTATTACTGCTACGG GTTACCATTTGTGCATAACGC	58	618[D]
ilv4F-F *ilvD*_2-R	GCAGAGATTAAAGATAAGGA GTTACCATTTGTGCATAACGC	50	569[E]
pta-F *pta*-R	GCAGAGCGTTTAGCAAAAGAA TGCAATGCGAGTTGCTTCTA	56	576
pur-F *pur*-R	CTGCTGCGAAAAATCACAAA CTCACGATTCGCTGCAATAA	56	536
pycA-F *pycA*-R	GCGTTAGGTGGAAACGAAAG CGCGTCCAAGTTTATGGAAT	57	550
tpi-F *tpi*-R	GCCCAGTAGCACTTAGCGAC CCGAAACCGTCAAGAATGAT	58	553

A): "-F" and "-R" means forward and reverse primer, respectively. The amplified household genes are: *glpF* (glycerol uptake facilitator protein), *gmk* (guanylate kinase, putative), *ilvD* (dihydroxy-acid dehydratase), *pta* (phosphate acetyltransferase), *pur* (phosphoribosylaminoimidazolecarboxamide), *pycA* (pyruvate carboxylase) and *tpi* (triosephosphate isomerase)
B). Fragment size is calculated based on results returned after Nucleotide Blast search with the primers on http://blast.ncbi.nlm.nih.gov/Blast.cgi.
C) This primer combination is used if (*gmk*-F + *gmk*-R) are unable to give a product
D) This primer combination is used if *(ilvD*-F + *ilvD*-R) are unable to give a product
E) This primer combination is used if neither(*ilvD*-F + *ilvD*-R) nor (*IlvD*_2-F + *IlvD*_2-R) are able to give a product

2.1.4 Selected strain collection

A realistic number of strains to analyse in the models were decided to be 10, of which four strains were intended as positive controls, two strains as negative controls, and finally four product strains. The four positive controls were selected on the basis of their history/origin, growth characteristics at 43°C and the presence of related pathogenic isolates, based on the MLST analysis. The two negative controls are selected based on similar criteria. The four product strains will represent the four serotypes most frequently used for plant protection.

2.2 Quantitative reverse transcriptase PCR (Q-RT-PCR) detection of virulence expression

2.2.1 Primers for RT-PCR

Primers used for RT-PCR reactions were designed using the primer Express software (Applied Biosystems) or the CLC main workbench software version 5 (CLC Bio, Aarhus, Denmark), and are shown in Table 2.2. For detailed information on the development and test of the primers on genomc DNA and cDNA see Appendice 4.

2.2.2 Sampling for RT-PCR from liquid cultures

A volume of 0.5 ml was sampled from cultures grown in 10 ml LB broth pH 7.5, 37°C, 120 rpm when the OD650 reached 1 (time 0h). Thereafter 0.5 ml was sampled at 1, 2 and 4 h. The samples were centrifuged immediately after sampling at 10,000xg for 4 min, 37°C. The pellet was dissolved in 1 ml RNAlater (Ambion). The samples were stored according to the manufacturer overnight at 4°C, before being transferred to -20 or -80°C.

2.2.3 RNA purification from RNAlater samples and reverse transcription

Samples in RNAlater (Ambion) were thawed and centrifuged for 8 min at 10,000 g, 4°C. Bacterial pellets were resuspended in 1 ml Tri Reagent (Ambion) at KU-LIFE and at INRA samples were thawed from –70°C and the following steps were more or less identical and as follows: The suspension was ransferred to 2mL RNAse-free microtubes containing approximately 300 mg acid washed glas sbeads (<106 µm) (Sigma). Cells were disrupted in a FastPrep machine (FP120, BIO101; Kem- En-Tec A=S, Taastrup, DK) for 45 sec at speed 6. Tubes were immediately placed on ice for 2 min, before being centrifuged for 2 min at 13,000 g. Supernatants were processed according to the protocol provided for extraction with Tri Reagent (Ambion). RNA samples were treated with the Turbo DNAse kit (Ambion) according to the manufacturer's instructions to remove residual DNA. At KU-LIFE the quantity and quality of the total RNA was analysed by Nanodrop equipment. At INRA the quality and concentration of the total RNA were analysed by Nanodrop equipment and Nano agilent biochip. Before reverse transcription, RT-PCR with SYBRG was used to verify the absence of DNA in the RNA samples, using the tpi or purH primer set (see section 2.2.1, Table 2.2). At Ku-Life reverse transcription was performed on 1 µg of RNA using The High Capacity cDNA RTPCR kit according to the guidelines given by the supplier (Applied Biosystems). At INRA the Stratagen Affinity Script QPCR cDNA synthesis Kit (La Jolla, CA, USA) was used for production of cDNA.

Table 2.2 RT-PCR Virulence and housekeeping gene Primers. Genes in regular letters are virulence genes, and genes in Bold are housekeeping genes

Gene product	Gene	Primer navn	Primer sekvens	Designed by
Cytotoxin K	*Cytk*	Cytk1_F Cytk1_R	5'-AGAAACGGGCGCTGTTATCTT-3' 5'-CCTGGCGCTAGTGCAACAT-3'	INRA CNL
Hemolytic toxin L2 component	*hblC*	hblC_F hblC_R	5'-TTTTGATACCAATGTGGCAACTG-3' 5'-CTGGTATTAATGCAATTTGCTGTAAATC-3'	INRA CNL
Hemolysin	*HlyII*	hlyII_F hlyII_R	5'-GGTATACACTTGGTGGAAGTGTTAAAGT-3' 5'-TCCAAGCAAAGCTACCTGTGATACT-3'	INRA CNL
Zinc metalloprotease	*InhA2*	inhA2_F inhA2_R	5'-AACTATGCGGGATCAGATAATGG-3' 5'-GATGCACCCCAACCCAGTTA-3'	INRA CNL
Iron-regulated leucine rich surface protein	*ilsA*	ilsA_F ilsA_R	5'-TCCAGCTATTGGATATGATCACAAA-3' 5'-CCTGAATTAGAGTTATCATCTGGTTTCTC-3'	INRA CNL
Non hemolytic enterotoxin	*nheB*	nheB_F nheB_R	5'-AAGACTTTAATTACAGGGTTATTGGTTACA-3' 5'-TCTGTTTGCCCCTCCTTAGC-3'	INRA CNL
Transcription regulator/activator	*plcR*	plcR_F plcR_R	5'-GAAACAGTTATCCGATAATATATGTCATCA-3' 5'-TTGCTGCGATACCTTGCAAT-3'	INRA CNL
RNA polymerase ß-subunit	***rpoB***	rpoB_SYBRG_BCF rpoB_SYBRG_BCR	5'-TCAGTGGTTTCTTGATGAGG-3' 5'-CTTTTACACGAAGTGGTGCT-3'	Cadot et al.,2010
	16SrRNA	16SA1_RT PCR 16SA2_RT PCR	5'-GGAGGAAGGTGGGGATGACG-3' 5'-ATGGTGTGACGGGCGGTGTG-3'	(Martineau et al., 1996)
triosephosphate isomerase	***tpi(1)***	tpi_SYBRG_BCF1 tpi_SYBRG_BCR1	5'-ACGGCGGTAGCGTAAAAC-3' 5'-CCCCAGAAGACCTAAGAACGA-3'	KU-LIFE/BMH DMU
triosephosphate isomerase	***tpi(2)***	tpi_SYBRG_BCF2 tpi_SYBRG_BCR2	5'-GCGGTAGCGTAAAACCAGAAA-3' 5'-AAGAACGAAGCAGGCTCTAAGC-3'	KU-LIFE/BMH DMU
phosphoribosylaminoimid azolecarboxamide	***pur***	pur_SYBRG_BCF pur_SYBRG_BCR	5'-CGTAAACTAGCAGCGAAAGTATTCC-3' 5'-GTCACAGTTAATGTTTCTGGACTTTCTT-3'	KU-LIFE/BMH DMU
phosphate acetyltransferase	***pta***	pta_SYBRG_BCF pta_SYBRG_BCR	5'-CATGGTACGTGAAGAAGAGAA-3' 5'-CGCAGAACCTTTTGTAGAGA-3'	KU-LIFE/BMH DMU
glycerol uptake facilitator protein	***glpF***	glpF_SYBRG_BCF glpF_SYBRG_BCR	5'-CGGGGCAGTTATCGTATATTT-3' 5'-ATGTGTTCGGAATTGCTGG-3'	KU-LIFE/BMH DMU

2.2.4 Setup of RT-PCR endpoint and Q-RT-PCR for analysis of gene expression

At KU-LIFE RT-PCR was set up in MicroAmp Fast Optical 96-Well Reaction Plates with Barcode, 0.1mL (Applied Biosystems) using the Power SYBRgreen Master Mix (AppliedBiosystems). The reactions were prepared in triplicates in a total volume of 20 µL; the final concentrations of primers were 500nM. Plates were covered with MicroAmp Optical AdhesiveFilm (Applied Biosystems). Amplification of PCR products and recording of fluorescence were done with the 7500 Fast Real-Time PCR system (Applied Biosystems) using the following program: 1 cycle at 95°C for 15 min, 40 cycles at 95°C for 15 sec followed by 75°C for 1min, and finally a dissociation stage consisting of 1 cycle at 95°C for 15 s, 57°C for 1 min, 95°C for 15 s. At INRA: Q-RT-PCR analysis are also realized by SYBR green system Kit BrilliantII SYBR Green QPCR master Mix using the equipment from Applied Biosystems 7900HT, on the experimental Platform at INRA, Jouy en Josas. The experimental setup is identical to the one used at KU-LIFE, except that we only make duplicates for each sample on a Q-PCR plate (but 2 technical replicates for each 2 or 3 biological replicates) and the final primer concentration is 300nM and test were run with 1 ng cDNA per well. PCR started with initial denaturation at 50°C 2 min, 95°C 10 min, followed by 40 cycles of denaturation, hybridisation and elongation (95°C, 15 sec, 60°C 1 min). Finishing by elongation at 60°C for 15 sec and dissociation at 95°C for 15 sec. Quantitative expression was performed by relative expression as based on Ct delta Ct. The results are analysed by the software SDS2.3 (Applied Biosystems).

2.3 Temperature growth characteristics and survival under gut/stomach conditions

2.3.1 Temperature growth characteristics

2.3.1.1 Growth at low temperatures

One colony from a fresh LB (Luria-Bertani, Oxoid) plate was streaked onto a new LB plate, and incubated aerobic and micro aerobic at 10ºC. Growth was observed once a week for up to three weeks. The experiment was performed twice.

2.3.1.2 Growth at high temperatures

One colony from a fresh LB plate was streaked onto a new LB plate, which was incubated aerobic and micro aerobic at 37, 43, 45 and 50ºC for a period up to 5 days. The experiment was performed up to three times.

2.3.2 Survival under gut/stomach conditions

2.3.2.1 Survival under simulated gastric conditions

One ml of an overnight culture was added to 100 ml SGF (Simulated gastric fluid; 2 g NaCl and 3.2 g pepsin (Sigma, P7000) in 7 ml 12 M HCl. Sterile MilliQ was added up to 1 l (Anonymous, 2003). pH was adjusted with HCl and NaOH to the desired values) in 250 ml sterile bottles. The bottles were incubated up to 4 hours at 37ºC. Samples (200 µl) were taken regularly and diluted in MRD (Maximum Recovery Diluent, Difco) and spotted (20 µl) on LB agar plates. The plates were incubated overnight at 30ºC. SGF with three different pH values: 2.0; 3.4 and 5.0 was tested twice.

2.3.2.2 Survival under simulated small intestinal conditions

One ml of an overnight culture (grown at 37ºC) was added to 40 ml pre-warmed (37ºC) SGF, pH 5.0 and incubated for 30 min at 37ºC. To the culture 50 ml double strength LB (37ºC) and 10 ml sterile filtrated bile acid (B8631, Sigma) solutions (1.5 or 3.0 g/l) was added. The culture was incubated under micro-aerophilic conditions at 37ºC for up to 4 hours. Samples (1 ml) were drawn at 0, 1, 2 and 4 hours, diluted in MRD and spotted on LB agar plates.

2.4 Detection of enterotoxin HBL using a commercial kit

2.4.1 Detection of HBL expression

Overnight cultures were established in BHI broth supplemented with 1% glucose (BHIG) at 270 rpm and 32°C. For production of enterotoxic substances, 20 µl overnight bacterial culture was added per ml BHIG, and the cultures were cultivated at 270 rpm and 32°C for six hours. The culture was centrifuged at 15000 g for 3 min at 4ºC, the supernatant was sterile filtrated and for detection of the L2 component of HBL, the BCET-RPLA toxin detection kit from Oxoid (TD0950) was used as recommended by the manufacturer.

2.5 Development of a Aenorhabitis Elegans model

2.5.1 Maintenance and synchronisation of *C. elegans* populations

The nematode ***Caenorhabditis elegans*** used in the development of a model was the temperature-sensitive ***C. elegans*** pha-1 (e2123ts), which can multiply at 15°C but not at 25°C (Schnabel and Schnabel, 1990). ***C. elegans*** pha-1 (e2123ts) was kindly supplied by Line E. Thomsen, Faculty of Life Sciences, University of Copenhagen. The nematodes were cultivated at 15°C on solid Nematod Growth Medium (NGM) in 9 cm Petri-dishes and were fed ***E. coli*** OP50 (Stiernagle 2006). For maintenance, the nematodes (and OP50) were simply washed off the Petri-dishes with sterile water and a few drops of nematode suspension were transferred to fresh NGM Petri-dishes. Alternatively, slices of agar were cut and transferred from established nematode cultures to fresh NGM Petri-dishes. For synchronisation, Petri-dishes with established cultures having high number of nematode eggs were washed gently 5 times with 2 ml sterile water, whereby all nematodes were removed leaving most of the eggs. The Petri-dishes with eggs were incubated at 3-4 days, where after the nematodes where used to test microbial pathogenicity

2.5.2 Microbial pathogenicity test with the *C. elegans* model

Synchronised nematode populations were washed from the NGM substrate with sterile water. 10 µl nematode suspensions were transferred to a number of wells in a 96 well microtiter plate, and the number of nematodes in the suspension was determined by microscopy (40 times magnification). The density of nematodes in the suspension was adjusted to 15 – 20 nematodes per 10 µl. 10 µl nematode suspensions were transferred to each well in 96 well microtiter plates, typically on a Friday, and the plates were incubated at 15°C

in a sealed box at 100 % relative humidity to avoid evaporation of water from the 10 µl nematode suspensions in the microtiter wells. The following Monday the numbers of living nematodes in the wells were counted. Also late Monday afternoon, the bacteria to be tested were inoculated in 2 ml liquid BHI substrate in 14 ml Falcon tubes, which were inoculated at room temperature and 275 rpm. Tuesday, 20 µl (BHI, NGM, T3 or water) +/- 50 µg/ml ampicillin +/- 2, 5% Laked Horse Blood (Oxoid SR0048C) were added to the wells. The ampicillin was added to inhibit growth of ***E. coli*** OP50, and only allowing the ***B. cereus*** group bacteria to grow, as an absolute majority of ***B. cereus*** group bacteria are resistant to penicillins. The horse blood was added to imitate a situation where bacteria had got access to blood in the human body. Also on Tuesday, the bacteria were diluted in water (125, 250 or 500 times) and 10 µl diluted suspensions were added to all wells. In control wells with the ***E. coli*** OP50 no ampicillin was added. The following days, the numbers of living nematodes in the wells were counted. In liquid, living nematodes had at least two bends, while dead nematodes had only one or no bend.
The survival data were analyzed by ANOVA on ranked data.

2.6 Evaluation of a Galleria Mellonella model

2.6.1 Preparation of spores and vegetative cells

The spore preparations used for infection were done with a dose (1-3 10^6 spores/larva) of spores obtained from growth in 100 ml of HCT sporulation medium in 1 liter flasks until spore liberation (48-72 hours, at 37°C 175 rpm). Following centrifugation the spores are washed twice in cold sterile water and suspended in 10 ml. This suspension is heat treated for 12 min at 78°C to eliminate vegetative bacteria.

The vegetative bacteria preparations are performed with suspensions of bacteria obtained from a liquid culture (LB medium) of exponential growing bacteria OD (600nm=1). The doses $5x10^6$ bacteria (spores and vegetative bacteria) per larva was chosen to give about 50 % mortality for the wildtype ***B. thuringiensis*** 407 (positive control) and a higher dose of $5x10^7$ vegetative bacteria are used in order to have a larger amount of bacteria present in the midgut before larval death, which is searched for the ***in vivo*** gene expression approach (RT-PCR). Thus, the assays are done for two purposes: estimation of virulence and for gene expression in the midgut. All doses are verified by plating of inocula dilutions on LB medium followed by colony counting.

2.6.2 G. mellonella infections

Infections assays were run both with spores and vegetative bacteria. 25 larvae per dose (weighting about 200 mg each) are infected orally by the use of a needle (round tip) and syringe, (using a micro-injector devise) with 10µl of either a suspension with spore or vegetative bacteria alone or mixed with Cry1C toxin at 3 microgram per larva (the toxin is used as a synergy factor to obtain high level of infection (Salamitou ***et al.*** 2000). Mortality is scored at 6, 24 ad 48 hours post infection following incubation at 37°C. All tests are repeated at least 3 times. Control larvae are fed with PBS (phosphate saline buffer) buffer alone or Cry1C in buffer alone. Although the project are mainly concerned with infection related to interaction with the intestinal barriers, the relative virulence among strains was also investigated following infection by

injection of spores and vegetative bacteria into the hemocoel of the insect larvae. 10 microliter of spore suspension containing various doses of spores (3.000, 10.000, 30.000) in PBS buffer using a cutting headed needle. Larvae are incubated at 37°C and mortality is scored over 3 days.

2.6.2.1 Determination of spore germination in larvae

Spore germination assays were performed with 2 x 2 larvae per strain (repeated three times) by homogenisation of whole larvae in 10 ml of PBS buffer 3 and 24 hours post infection. The suspensions are divided into two, and one is submitted to heat chock (78°C for 12 min). These analyses are run with and without addition of Cry1C toxin to the spores. Determination of level of spore germination is done by plating of various dilutions of the two suspensions. The CFU (colony forming units) were counted and the percentage of spores germinated (not heat resistant) in larvae was determined.

2.6.3 Sampling of bacteria from infected *Galleria mellonella* for RNA preparation

The infections are done with high doses of vegetative bacteria from LB culture. About 15 ml at OD650nm between 1.2 to 3 are centrifuged and suspended in Cry1C toxin (0.3 mg/ml). The obtained dose is (5 $x10^7$ to 2 $x10^8$). 20 larvae are infected and incubated at 37°C. 6 Hours post infection 5 larvae are dissected and the midgut (part of the whole intestine) is immediately and gently homogenised in 100 microliter buffer. 10 microliter of this is sampled for estimation of bacterial content, and the remaining is transferred to liquid nitrogen and stored at -70°C. The five midguts are pooled together before total RNA extraction and bacterial estimation is done on the 5 pooled 10 microliter samples. The remaining 5x 90 microliter are frozen in liquide nitrogen and stored at -70°C until extraction of total RNA following the same methology as indicated in section 2.2.3 using the Tri Reagent (Ambion) RNA extraction followed by DNAase treatment etc. Extraction was done if the amount of bacteria in the pool was at least $1x10^{8.}$

2.6.3.1 Expression of bacterial virulence genes in G. mellonella

The reverse transcription (RT), was also done by using the Stratagen Kit and random hexamers primers (like indicated in 2.2.4). Thus, the obtained cDNA was a mixture of fragments amplified from mRNA from both bacteria and ***G. mellonella***. The quality of RNA and cDNA, was verified by Nano and Pico Agilent chips. Extractions and analysis were done twice from 5 larvae infected with the same bacteria preparation and at least one time from a second infection for each strain. Before running SYBR-green Q-PCR, endpoint PCR was performed in order to get a first indication for gene expression. Q-PCR was only performed if a positive result was found by endpoint PCR.

2.7 Development of functional mammalian cell models

2.7.1 Mammalian cell lines and maintenance of cells

The human colon adenocarcinoma cell line Caco-2 was purchased from the Deutche Sammlung von Mikroorganism und Zellkulturen (DSMZ, Braunschweig, Germany). Routine maintenance and seeding of wells for infection experiments was performed as previously described (Klingberg et al. 2008).

Mucus producing HT29-MTX cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) with GlutaMAX^{TM-1}, pyruvate, 4.5 g glucose (Gibco®), 10% FBS, penicillin (100 U/ml), streptomycin (1 mg/ml). For infection experiments, cells were grown for 28-29 days to obtain sufficient mucin production. Further the monolayer was not washed with PBS prior to infection to protect the mucin. Mucin production was verified by Alcian blue/periodic acid staining using a modification of the method described by (Bancroft and Gamble, 2008). Briefly cells were fixed with 96% ethanol at 4^{o}C for three days. The cells were then rehydrated with PBS buffer for 5 minutes before incubating with 1ml 3% glacial acetic acid, 1% Alcian blue (Sigma-Aldrich, St. Louis, Maryland, USA) in PBS for 5 minutes. After washing with MilliQ water (MQ-water) the cells were incubated with 1ml 1% aqueous peracetic acid for 5 min. After peracetic acid treatment the cells were rinsed in MQ-water and then incubated with 1ml Sciffs reagent for 15 min. Finally the cells were washed with MQ-water before being photographed using a Real Time Viewing (RTV) cooled camera (Q-imaging, Surrey, BC, Canada) coupled to a reverted microscope (Zeiss Axiovert 25). Images were collected using the software programme Image ProPlus vs. 5.0 (Media Cybernetics, Inc., Bethesda, Maryland, USA).

Monocytes/macrophages from pig blood (PoM2) were kindly provided by the University of Maribor, Slovenia (Gradišnik et al., 2006). PoM2 cells were maintained as described above, except that the growth media was Advanced DMEM (Gibco®), 10% FBS, L-glutamine (2mmol/L), penicillin (100 U/ml), streptomycin (1 mg/ml). Cells used for infection experiments were seeded at 10^6cells/ml and grown in growth media without antibiotics until a monolayer was obtained (2-3 days).

2.7.2 Preparation of bacteria for infection experiments

For infection experiments, 10 ml of LB-broth (Bertani 1951) pH 7.5 in 100 ml conical flasks was inoculated with 10-25 µl overnight culture (37°C, 225 rpm) in LB-broth pH 7.5, and incubated at 37°C,120 rpm in water baths to an OD650 of 1-1.8. The cultures were then centrifuged at 10,000 g, 4 min at 37°C and re-suspended in either MEM, 10% FBS, 1% NEAA (Caco-2) or DMEM, 10% FBS (HT29-MTX) or Advanced Dulbecco´s medium, 10% FBS (Pom2). The bacterial cultures were inoculated onto HT29-MTX, Caco-2, or PoM2 cells at the appropriate cell density. Growth rates in LB-broth at 37°C was calculated by using the MicroFit software,

2.7.3 The effect of bacterial cell density on Caco-2 cell morphology

Caco-2 cells were seeded in 24 well plates (Nunc, Denmark) at a density of $1*10^5$ cells per well and incubated for 7-22 days. Growth media without antibiotics was changed every 2-3 days. Effect of bacterial cell density on morphology of Caco-2 cells was observed once every hour from 0 h of infection using bright field microscopy.

2.7.4 Adhesion and invasion

Adhesion and invasion experiments using Caco-2 (17-18 days) or HT29-MTX (28-29 days) were performed using 24 well culture plates (Nunc). Infection was with approximately 10^4 bacteria per well and for 3 hours at 37°C, 5% CO_2, 95% air. The number of adhering bacterial cells per well was determined by removing the growth media, washing the wells 3 times with

PBS, pH 7.0, lysing with 1 ml sterile MQ-water and by using the drop plate method. The number of invading cells per well was determined by removing the growth media and incubating at room temperature for 40-50 minutes together with 1 ml 100 or 200 µg gentamicin/ml PBS. Wells were washed with PBS, cells were lysed with MQ-water and CFU/well was determined as described above for adhesion. Experiments were repeated at least 2 times in duplicate.

2.7.5 Transepithelial electrical resistance measurements

Measurements of the TEER of Caco-2 or HT29-MTX monolayers seeded on Transwell filter inserts (0.4 µm pore size, 12-mm) in 12-well plates (diameter: 22.1 mm; Corning Incorporated, Sigma Denmark and infected with approximately 10^3 CFU of ***Bacillus***/ml on the apical side was conducted as previously described (Klingberg et al. 2008; Klingberg et al. 2005). Each assay was conducted at least twice (more than two different passages) with triplicate determinations. Average TEER values at time 0 h was 940 Ωcm^2 for Caco-2 and 270 Ωcm^2 for HT29-MTX.

The statistical significance of the effect of bacterial infection on TEER (HT29-MTX) was analyzed by two way ANOVA combined with a Bonferroni post test using the Graphpad Prism Software version 5 (GraphPad Software, Inc. La Jolla, California, USA). The level of significance was $p = 0.05$.

2.7.6 Mitochondrial activity of PoM2 exposed to *Bacillus* spp.

Mitochondrial function may serve as an index of living metabolically active cells. The effect of various ***Bacillus*** spp. on the mitochondrial function of PoM2 macrophage/Monocytes was examined using the Methylthiazolyldiphenyl-tetrazolium bromide (MTT) –assay (3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (Sigma) as described by Bergamini et al. (1992). Initially PoM2 cells were seeded in 96 well plates, $0.33cm^2$ well areas (Nunclon™ surface, Nunc, Denmark) at a density of $2*10^5$ cells per well and incubated for 3 days at 37°C, 5% CO_2, 95% air. The monolayer was washed 3 times with PBS pH 7.0, and fresh media was added before infection with bacteria. Bacteria were added at 10^2 or 10^4 cells per well (moiety of infection of 1:600 or 1:6) and incubation was for 22 hours at 37°C in a humidified atmosphere of 5% CO_2, 95% air. After infection for 22 h, the wells were carefully washed with PBS, and media with antibiotics was added (gentamicin 100 µg/ml, penicillin 100 U/m), and streptomycin 1mg/ml). Alternatively gentamicin was added after 1 h of infection at a concentration of 100 µg per ml in order to kill the bacteria. Then twenty µl MTT at 15 mg/ml was added to wells containing 180 µl growth media, and incubation was for 75 min at 37°C, 5% CO_2. Then 100 µl was removed and replaced by 100 µl of 10% Triton X-100, 0.4% concentrated HCl in isopropanol to dissolve the formazan crystals. The plate was read at 590 nm using a multiscan plate reader. The experiments were performed in triplicates on 2-5 separate occasions.

Percent survival was calculated: 100* OD590nm (exposed cells)/OD590nm (control cells).

2.7.7 Expression of bacterial virulence genes in the mammalian cell models

Sampling for RNA extraction from bacterial cells was performed for adhesion/invasion experiments with Caco-2 cells. Sampling was performed at 3 and 4 hours of adhesion/invasion by removing the supernatant from the wells (without washing). Thereafter 1 ml RNAlater (Ambion) was added, and pipeting was performed until the Caco-2 layer was disrupted. The samples including supernatant (spinned down at 4000 g, 2 min, 37°C) and resuspended in 1 ml RNAlater were then stored overnight at 4°C and then at -20°C or -80°C until further use. Sampling was performed on 3-4 separate occasions.

Samples were also collected from a TEER experiment, where RNAlater (Ambion) was added to the apical side of the monolayer 2 hours after infection with bacteria. The suspension was pipetted up and down, until the monolayer was disrupted.

3 Results and discussion

3.1 Collection, scereening and selection of strains

3.1.1 Introduction

For the purpose of developing models for human health evaluation of microbial pathogenic traits, it is important to have closely related negative and positive control strains. The ideal situation would be having microbial strains that without doubt were proven to be pathogenic to humans. This is however not possible as re-infection experiments with humans are unacceptable. The best alternative is to use microorganisms isolated from diseased patients, or food expected to contain the disease causing microorganism. However, such strains might simply been present in the patient by chance, or the patient might even have had a wrong diagnosis. Partly to overcome such uncertainty, we have chosen to select the presumed positive control strain on the basis of more criteria. First of all, the history and origin of the strains are important factors. Selected strains will have some connection to human health problems, either as they have been involved in gastro-intestinal disease, or they have been isolated from tissue of diseased humans. Second, bacteria able to infect humans must at least be able to grow at 37°C. Unpublished data have shown that ***B. cereus*** group bacteria having pathogenesis connection generally formed much larger colonies at 43°C than environmental isolates. Similarly it has been published that ***B. cereus*** group bacteria from mammals can grow at temperatures up to 50°C (Guinebretière et al., 2008). Further, a genetic method, Multilocus sequence typing (MLST), has been used for typing isolates. MLST is a DNA based technique for the typing of typically seven household genes. Approximately 400-600 bp internal fragments of each gene are used. Using the ***B. cereus*** group MLST database at http://pubmlst.org/bcereus/ is possible to investigate whether related/similar strains can be found in the database. As the origin and history of the isolates are given in the database, it is possible to see whether a specific isolate is similar to or group close to known pathogenic bacteria. Unfortunately it is not so simple that all pathogenic ***B. cereus*** group bacteria group closely together. The pathogenic trait has been allocated to a wide diversity of bacteria distributed among diverse MLST types. However, if similar/identical bacteria have been described in the MLST database, the history and origin of such isolates could be useful in judging whether a specific isolate could be expected to have human pathogenic traits.

3.1.2 Initial strain collection

The initial strain collection is primary based on isolates already present in our strain collections. The collection is presented in appendix 1. The list contains 46 isolates having an origin in food poisonings or somatic infections. At least some of these 46 isolates are expected to be true pathogens, among which four will be selected as positive controls. Negative control bacteria are much harder to find. It was only possible to identify three potential negative controls, two of which have been used as probiotics (see appendix 1). The third negative control candidate was an isolate in which a pleiotrophic virulence regulator, PlcR, had been inactivated. This inactivation is expected

to keep a low expression of genes expected to be involved in pathogenesis of ***B. cereus*** group bacteria. (Unfortunately, late in the project it was discovered that one of the Probiotic strains, ***B. cereus toyoi*** by accident had been contaminated by another ***B. cereus*** during production of the probiotic product. The product was stopped before it was used commercially as probiotic for animal production, but we only realized this late in the project.. The ***B. cereus toyoi*** has number bc49 in the consecutive number system, and has number Bt1254 in the "Internal DMU strain number" list. Later, the right ***B. cereus toyoi*** strain was included in some experiments. This was given the "Internal DMU strain number" 1306) For the product strain list, it was attempted only to use ***B. thuringiensis*** strains re-isolated from commercial products to be able to work with bacteria as identical to products strains as possible. We, however, only succeeded to get free product samples in two cases. Three other product strains were from our strain collection. The last strain was re-isolated from a freeze dried bacterial stock from 1972 with the original ***B. thuringiensis*** subsp ***kurstaki*** HD-1, which was the first isolate of significant commercial importance. The product strains are also listed in appendix 1.

3.1.3 Growth at 43ºC

3.1.3.1 Growth in liquid substrate

The results are shown in Appendix 2. Most of the strains grow well at both temperatures and under both oxygen conditions. In general, the strains used in plant protection products, grow less well at 43ºC and under micro aerobic conditions, which could indicate that the commercial strains are less well adapted to survive in the human body.

3.1.3.2 Growth on solid substrate

As described in M&M, care was taken to keep Petri-dish temperature as constant as possible. Each day the diameters of the bacterial colonies were measured. Of the 54 strains analysed, only four were not able to grow at 43°C. Except for strain bt53 (***B. thuringiensis*** subsp. ***kurstaki***, ABTS-351) all product strains were inhibited in growth at 43°C. Contrary, only six of the potential pathogenic strains were inhibited in growth at 43°C. The data from growth on solid substrate are also presented in appendix 2.

3.1.4 Multi Locus Sequence Typing (MLST)

Partial sequencings in both directions of the seven household genes were performed by a Chinese/Danish company only once due to limited financial resources. The consequence of this is that not all 7 sequences in all strains are perfect. However, a perfect set of sequences is not necessary to be able to assign specific isolates to a closely related sequence type (ST). The results from the MLST analysis are presented in Appendix 3, where the sequence numbers and sequence types for each of the analysed strains are given. When looking at the first 46 strains representing potentially pathogenic strains, 52% of the related ST types are represented by isolates originating from human sources, 29% are represented by isolates originating from food sources, 11% does not inform about origin, and 8% of related ST types are only represented by environmental isolates. Among the ST types related to the Product strains, only the ***kurstaki*** ST 8 contains one single isolate related to animals, as it was found in swan faeces. Strain no bc11 (Bt676, MADM 1291) was originally isolated from a birthday cake in Brazil. This strain is special in that none of its household gene sequences have ever been seen, and as such, strain bc11 must

represent a completely new type ***B. cereus***, at least in relation to MLST characterisation**.**

3.1.5 Selected strain collection

Based on the strain history, temperature characteristics and relations to other strains as indicated by the MLST analysis, four potentially pathogenic strains were selected to be used as positive control strains (Tabel 3.1). Other strains could have been chosen, but strain bc 11 (Bt 676, MADM 1291) was selected as it at least in the MLST analysis represented a completely new type, and it had been involved in a gastro-intestinal case. Strain bc14 (Bt 698, MADM 1561) had also been involved in gastro-intestinal disease, and closely related strains produce the emetic toxin cereulide. The third, strain bc25 (Bt 1202, B-05) has been isolated from blood, and closely related related to strains involved in somatic infections. The fourth, strain bc38 (Bt 642, B4-ac) has been involved in gastro-intestinal cases, has for years been considered a human pathogen and closely related bacteria have been involved in both somatic and gastro-intestinal disease. Of the potentially negative control bacteria, strain bt48 (Bt 959, ***B. thuringiensis*** 407 PlcR) was selected as it has been interrupted in the pleiotrophic regulator PlcR, and as such should be unable to produce most known ***B. cereus*** group virulence products. The two remaining potential negative controls were both categorised as probiotics. Of these two, unfortunately strain bc49 (Bt 1254, ***B. cereus toyoi***) was selected as it first of all was considered non-pathogenic as it has been approved as probiotic, second as it was able to grow at higher temperatures than the other probiotic candidate. Very late in the project, we however found that we by mistake from the producer had received a powder contaminated by another ***B. cereus***. Although it was attempted to re-do experiments with the right strain (Bt 1306), only few data are available with this strain. The selected negative reference strains are listed in Tabel 3.2. The four selected product strains are chosen as they represent the four most frequently used ***B. thuringiensis*** type strains. The product strains are listed in Tabel 3.3.
Recently a grouping of ***B. cereus*** group strains suggesting a relationship between the phylogenetic affiliation to food poisoning has been proposed (Guinebretiere et al,, 2008; 2010). The strains are divided in to seven different phylogenetic groups on the basis of AFLP and sequence analysis. We have tried to affiliate our strain collection to these groups. This was done by the use of of the Norwegian “Hypercat Superdatabase (http://mlstoslo.uio.no/), which summarizes all available AFLP and sequence data on ***B. cereus*** group bactera. By finding strain identical to our strains, they were affiliated to the follwing clusters: Bc14 to cluster III, Bc25, Bc38, Bt50 and Bt53 to cluster IV, Bt 52 and Bt 55 could not our current knowledge be affiliated to any group, however all other B. thiuringiensis israliensis and B. thuringiensis tenebrionis strains are affiliated to cluster IV; it is not possible to affiliate Bc11 to any cluster as it is unique, it might be a strain which do not belongs to any of the seven knwwn cluster (other strain of this type exists). On the homepage (https://www.tools.symprevius.org/Bcereus/english.php) on these clusters a brief description of each cluster is given:

Group III
Mesophilic group corresponding to ***B. thuringiensis III, B. cereus III*** (emetic strains included) or ***B. anthracis*** species, depending on whether strains contain parasporal crystal or not, or whether strains contain virulence factors associated to anthrax. Growth temperature range ***15-45°C***. There are not yet phenotypic features that could be exploited to easily and rapidly identify

theses strains, except presence of ces gene for emetic strains. Strains of this group are generally cytotoxic, many of them are highly cytotoxic.

Group IV :
Mesophilic group corresponding to ***B. thuringiensis IV*** or ***B. cereus IV*** species, depending on whether strains contain parasporal crystal or not.
Growth temperature range ***10-45°C***. There are not yet phenotypic features that could be exploited to easily and rapidly identify theses strains.
Strains of this group are generally cytotoxic, some can be highly cytotoxic.

Tabel 3.1The four pathogenic strains selected as positive control strains.

Consecutive strain number	Internal DMU strain number	Original Strain name	Origin	Reference or source
bc 11	Bt 676	MADM 1291	Birthday cake	Marilena de Muro, Brazil
bc14	Bt 698	MADM 1561	Cooked chicken	Marilena de Muro, Brazil
bc25	Bt 1202	B-05	Blood, Patient 8	Gaur et al., 2001
bc38	Bt 642	B4-ac	Gastro-intestinal	Agata et al., 1995

Tabel 3.2. The two strains selected as negative control strains.

Consecutive strain number	Internal DMU strain number	Original Strain name	Origin	Reference or source
bt48	Bt 959	Bt 407 PlcR	Inactivated PlcR regulator	Salamitou et al., 2000
bc49	Bt 1254	*B. cereus* var. *toyoi* (CNCM I-1012/NCIMB 40112)	Probiotic	A free sample from Rubinum, Spain

Tabel 3.3. Selected product strain list

Consecutive strain numbers	Internal DMU strain number	Product name	Serotype	Original Strain name	Reference or source
bt50	600	Agree 50WP	*aizawai*	GC-91	Certis
bt52	1253	Vectobac-12AS	*israelensis*	AM 65-52	A free sample from Borregaard, Bioplant, DK
bt53	1255	Dipel	*kurstaki*	ABTS-351	Valent BioSciences
bt55	1256	Novodor	*tenebrionis*	NB-176	Free sample from "Andermatt-Biocontrol AG", CH

3.1.6 Discussion

In general there is a good agreement between the results from the growth studies in liquid and solid media. In general, the strains used in plant protection products, grow less well at 43ºC and under micro aerobic conditions, which could indicate that the commercial strains are less well adapted to survive in the human body. The strains growing less well in liquid media also give smaller colonies on solid media. Some strains (e.g. 43, 47 and bt52) do not grow up at all on solid media, whereas in liquid media growth, although poor, is observed. This could be due to differences in media used, and in general bacteria grow better in liquid than on solid media. The results from the temperature screening, where together with the MLST data and the history of the strains, used to select the ten strains used in further experiments. Whether the selected positive control strains were the right can of course be questioned, as it of obvious reason is impossible to categorise an opportunistic human as a true pathogenic bacterium. However, if it turns out that the 10 selected strains will show variable pathogenicity in a model, it has at least been shown that the model can distinguish among the isolates, and as such the model must be considered to be useful. Whether it had been possible to select the right positive and negative control strains is another question. But if a distinguishing model has been identified, it merely a question of testing more strains to find the optimal positive and negative control strains.

3.2 Quantitative reverse transcriptase PCR (Q-RT-PCR) detection of virulence expression

3.2.1 Introduction

One of the aims of our study is to understand a possible difference in virulence among stains from the ***B. cereus*** group and particularly between ***B. thurigiensis*** trains used as biopesticides and ***B. cereus*** strains from clinical issues. Thus one of the first approaches to explain an eventual difference would be to study the presence, absence of "known" virulence factors such as enterotoxins complexes (Nhe, Hbl) and cytotoxin K (CytK) (for review, Stenfors–Arnesen, et al. 2008) as well as hemolysin II (hlyII) (recently suggested to be a virulence marker (Cadot et al. 2010, Tran et al., 2010) and metalloprotease inhA2 (shown to be important for insect virulence (Fedhila et al. 2002, Guillemet et al. 2009) and finally ilsA (surface protein shown to be important for iron acquisition during infection (Daou et al., 2009). Some of these factors are regulated by PlcR a transcriptional regulator, which was shown to be important for virulence in insect and in mice (Salamitou et al.2000). One of the studied strains is the Bt407cry- delta ***plcR*** (Bt48), which is deleted for ***plcr***, resulting in the reduction of production of some virulence factors. Using the PCR we are able to show whether a virulence gene is present in a particular strain, with RT (reverse transcriptase) reaction coupled to PCR , we are able identify if a gene is expressed (production of a virulence factor) and by coupling this method to Q-RT we will be able to (Q) quantify the level of expression and thus to estimate whether a gene is more or less expressed during infection, (or in other conditions), in a particular strain and among strains. Such findings might help in suggesting if a strain is more or less virulent. In this project we will thus try to correlate the presence of particular genes and their expression

with virulence on different levels in some of the models (Cell culture and ***Galleria mellonella*** larvae).

3.2.2 Detection of bacterial gene expression in LB-broth by RT-PCR

Results for virulence gene expression by a selected number of ***B. cereus*** and ***B. thuringiensis*** in LB-broth at 37°C are shown in Table 3.4. Except for ***B. thuringiensis*** BT407 that did not transcribe ***inhA2*** and bc49 (***B. cereus*** var. toyoi) that did not transcribe ***cytk,*** most of the ***Bacillus*** spp. strains transcribed all of the genes investigated at one or more of the time points investigated. Similar results were obtained for growth experiments in LB-broth on a different occasion using qPCR. The only difference obtained was, that the ***plcR*** gene was not expressed in BT407, while ***inhA2*** was (results not shown).

Table 3.4 Gene Transcription (average Ct values of 3 wells) during growth in 10 ml LB-broth 37°C, 120 rpm. T0 is at OD650 = 1, t1 = one hour post OD650 =1, t2 = 2 hours, and t4 = 4 hours post OD650 =1. Blue markings indicate that this gene was not present in the strain as detected by PCR on Genomic DNA (Appendice 4). The experiments are either single or double experiments (in triplicate wells). Gene expression was determined with SYBRG technology.

	Virulence							Housekeeping	
Strain /time	*plcR*	*nheB*	*hblC*	*cytK*	*ilsA*	*InhA2*	*hlyII*	*tpi*	*purF*
bc 11 t0	nd/-	nd/(35.1)	nd/-	nd/35.8	nd/37.6	nd/-	nd/(35.8)	nd/33.4	nd/34.9
bc 11 t1	nd/-	nd/35.5	nd/-	nd/32.3	nd/35.3	nd/-	nd/32.4	nd/29.9	nd/33.2
bc 11 t2	nd/-	nd/35.4	nd/-	nd/32.3	nd/35.3	nd/-	nd/-	nd/30.4	nd/32.0
bc 11 t4	-/-	32.8/33.2	-/-	32.5/30.0	(32.5)/32.7	-/35.9	-/32.0	33.2/27.5	31.1/28.1
bt48 t2	-	34.7	33.2	30.3	33.7	30.9	33.3	29.5	29-2
bt48 t4	-	34.0	34.2	(34.2)	-	-	(35.1)	32.8	-
BT407 t0	28.8	29.7	34.8	28.0	30.8	-	33.1	27.8	30.0
BT407 t1	29.8	23.4	25.3	23.5	24.1	-	(32.1)	27.3	27.3
BT407 t2	32.2	23.8	29.3	25.4	20.4	-	32.9	23.9	28.3
BT407 t4	33.5	27.3	35.5	35.2	30.9	-	-	33.6	33.0
bc49 t0	33.8/(34.9)	30.7/-	35.1/-	-/-	30.6/-	-/-	-/-	26.0/34.3	31.3/-
bc49 t1	nd/-	nd/33.0	nd/-	nd/-	nd/-	nd/-	nd/-	nd/(30.8)	nd/(33.7)
bc49 t2	nd/-	nd/33.4	nd/(34.3)	nd/-	nd/-	nd/33.1	nd/-	nd/30.2	nd/31.3
bc49 t4	33.9/(28.3)	32.2/24.6	30.7/30.0	-/-	30.2/-	-/26.9	-/-	28.4/22.6	32.2/(23.1)
bt52 t0	28.2/ 31.2	31.9/ 29.5	31.7/ 29.5	26.3/ 26.3	37.7/ 35.4	31.8/27.3	26.7/-	19.0/23.8	32.1/28.4
bt52 t1	26.8/13.01	18.2/16.7	30.0/16.7	17.2/13.1	37.0/-	18.8/15.8	23.1/-	17.3/16.4	25.7/16.6
bt52 t2	nd/ 31.6	nd/30.4	nd/30.4	nd/ 28.6	nd/ -	nd/22.0	nd/-	nd/23.1	nd/27.9
bt52 t4	31.2/(35.3)	24.0/ (36.5)	32.2/ (36.5)	30.7/34.6	32.4/-	23.9/28.1	27.1/-	27.7/25.8	25.5/(34.2)
bt53 t0	34.1	30.2	34.6	34.0	-	31.0	-	28.5	32.8
bt53 t1	(36.3)	34.1	-	(31.6)	(36.6)	34.1	-	31.1	(30.7)
bt53 t2	-/-	31.2/35.7	-/-	(32.7)/-	-/-	33.7/-	-/-	32.3/30.2	31.3/36.2
bt53 t4	35.5/(37.1)	-/-	-/-	30.2/(34.6)	34.4/35.3	31.1/-	-/-	26.1/30.2	29.0/32.4

3.2.3 Discussion of set up of RT-PCR and virulence gene expression in LB medium

This part of the project was very time consuming and much more difficult to manage than thought, mainly due to the fact that we are working with non sequenced strains and because we scheduled to analyse a large number of strains and genes. Meanwhile, although some of the results need more repetitions in terms of quantification we are confident with the results showing expression or no expression in the tested conditions and the chosen primers seems globally to fit to the different strains, except for the ***inhA2*** primer set. However we would have expected more stable expression of the housekeeping genes in the RNA extraction from bacteria cultures in liquid LB medium, as these are supposed to vary less during the various bacterial growth stages. Thus this needs to be optimized. The virulence gene primers were tested on both genomic DNA and cDNA and worked well for both. Thus, these initial tests should indicate that if a gene is expressed, at a detectable level, during infection, these primers should be able to function. Although our final aim is to detect expression when the bacteria are in contact with the host cells (cell lines or invertebrate models) we have here shown that most of the strains are able to express the searched genes, although the expression level varies from one strain to another and also among genes as observed by the indicative Ct values in (Table 3.4). To find the virulence genes in most strains are not really a surprise (Guinebretiere et al., 2010) it is more interesting that some genes are absent such as ***cytk*** from the probiotic strain (Bc49), the finding that some genes (***cytK and hlyII*** in Bt53) were not transcribed in the analysed conditions, while the plcR regulated factors were found as transcipts in the plcR mutant (Bt48) were surprising. Meanwhile the indications from the Quantitative analysis (the higher the Ct the less expression) show that the level of expression of the PlcR regulated factors in strain Bt48 is lower in the Bt407 wildtype strain indicating that low quantity of RNAm/cDNA was present in the mutant strain, which corresponds to PlcR independent expression. Thus this strain may be used as a negative control for expression of a number of the analysed virulence factors, at least in these ***in vitro*** conditions. Altogether the tools and methods here tested on cDNA from pure bacterial cultures are ready to be used under infection conditions in the insect model ***Galleria mellonella*** (section 3.7.6) and in cell culture models (section 3.8.6).

3.3 Temperature growth characteristics and survival under gut/ stomach conditions

3.3.1 Introduction

In order for at microbe to cause disease in humans, the microbe has to survive the passage through the stomach (low pH), be resistant towards bile acid present in the small intestine, and be able to grow and/or express toxins at the temperature present in the gut (app. 37°C). These conditions were tested in this part of the project with the purpose that those ***in vitro*** experiments can be a supplement, or in best case be a replacement for oral animal studies. Unpublished studies at AU-DMU have shown that disease-causing ***B. cereus*** isolates grow much better at higher temperatures than environmental isolates.

The ten selected strains were tested for growth at 37, 43, 45 and 50° C to study whether there is a difference at higher temperatures between the virulent and the commercial and non-virulent strains. The strains were also differentiated by looking at growth at low temperatures.

In an earlier Environmental Protection Agency project we showed that spores of ***B. thuringiensis*** survive even at pH 1.5, whereas the vegetative cells quickly die off at this value (Wilcks ***et al.***, 2008). A pH value of 1.5 is only present at the beginning of a meal, and thereafter rapidly increases. So, in this part of the project we studied the survival of vegetative cells in simulated gastric fluid at pH values from 2.0 to 5.0.

Finally, we investigated the ability of vegetative cells to grow and express enterotoxins in the small intestine. The vegetative cells were first incubated in simulated gastric fluid, since this could maybe induce higher resistance to bile acid, and reflects the real life situation more. Afterwards, the cells were incubated with different concentrations of bile extract from pigs having high similarity to human bile acids.

3.3.2 Temperature growth characteristics

3.3.2.1 Growth at low temperature

All nine strains grow well at 10°C under both aerobic and micro aerobic conditions, see

Table 3.5.

Table 3.5 Growth of the ten selected strains at low temperature

Strains	Growth observed at week	
	10°C aerobic	10°C micro aerobic
Bt48	1/1	1/1
Bt50	1/2	1/2
Bt52	1/1	1/1
Bt53	1/1	1/1
Bt55	1/1	1/1
Bc11	1/1	1/1
Bc14	1/1	1/1
Bc25	1/1	1/1
Bc38	1/1	1/1

* Disease causing isolates: red; Commercial strains: green; Probiotic strains: blue. - no growth. The experiment was performed twice, and the results from the two experiments are separated by /.

3.3.3 Growth at high temperatures

The four strains that have been involved in human illness (bc11, bc14, bc25 and bc38) all grow fine at Day 1 under all temperatures and oxygen conditions. The commercial strains and bt48 (Bt 407 ΔplcR) grow slower when the temperatures reaches 45°C and above. And for the bt52 (Vectobac) and bt55 (Novodor) strain an effect by the oxygen conditions can be observed at 45°C, where the micro aerobic conditions restrict growth, whereas growth is unaffected under aerobic conditions. See

Table 3.6 Growth of the selected strains at higher temperatures

Strain	Growth observed at day							
	37°C Aerobic	37°C micro	43°C aerobic	43°C micro	45°C aerobic	45°C micro	50°C aerobic	50°C micro
Bt48	1/1	1/1	1/1	1/1	1/1	1/1/	1/6/6	1/6/-
Bt50	1/1	1/1	1/1	1/1	1/1	1/1	5/-/-	1/-/-
Bt52	1/1	1/1	1/1	1/2	1/2	2/6/6	5/3/-	5/6/2
Bt53	1/1	1/1	1/1	1/2	1/1	1/2	1/6/-	1/-/6
Bt55	1/1	1/1	1/1	1/1	1/2	1/6/6	2/-/6	5/6/-
Bc11	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1
Bc14	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1
Bc25	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1
Bc38	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1

*Micro: micro aerobic conditions. Disease causing isolates: red; Commercial strains: green; Probiotic strains: blue. No growth: -. The experiment was performed up to three times, and the result of each experiment is separated by /

3.3.4 Survival under simulated stomach/gut conditions

3.3.4.1 Survival under simulated gastric conditions

Only results from pH2 are shown (Figures 3. 1-3.3), since at pH3.5 and 5.0 no pronounced difference could be observed between the three categories of strains (virulence attenuated (bt48), product and disease isolates). At pH5.0 all strains where growing, and at pH3.4 an initial drop of 0.5 – 3 log was observed for all strains, and afterwards the strains where stably maintained.

At pH2, all strains have an initial drop of 2-3 log values. Most of the strains stabilize after this drop; this includes all the disease isolates (except bc14), the positive strains and the strains from bt50 (Agree) and bt52 (Vectobac). The strain from Dipel (53) is most affected by the low pH, and is undetectable after four hours.

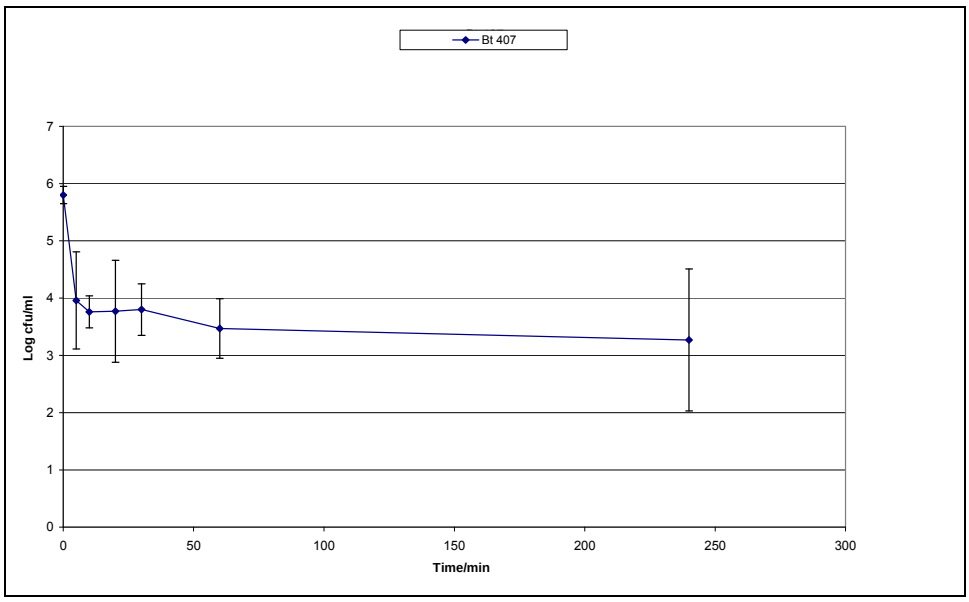


Figure 3.1. Persistence of the negative reference strain (PlcR mutant Bt48) in simulated gastric fluid at pH 2.0. Experiments were performed twice, and SEM's for each time point are shown.

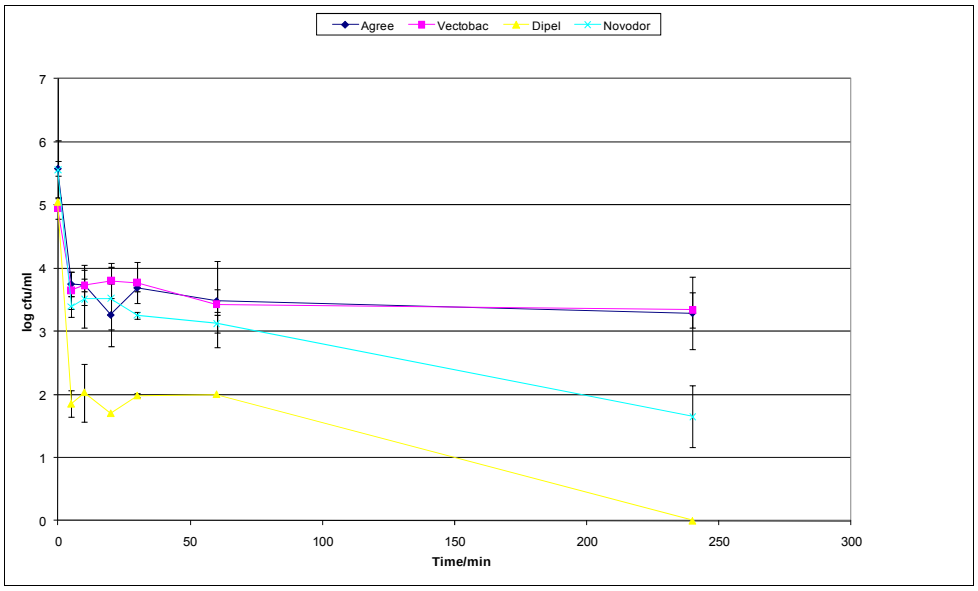


Figure 3.2 Persistence of the four product strains (bt50, bt52, bt53 and bt55) in simulated gastric fluid at pH 2.0. Experiments were performed twice, and SEM's for each time point are shown.

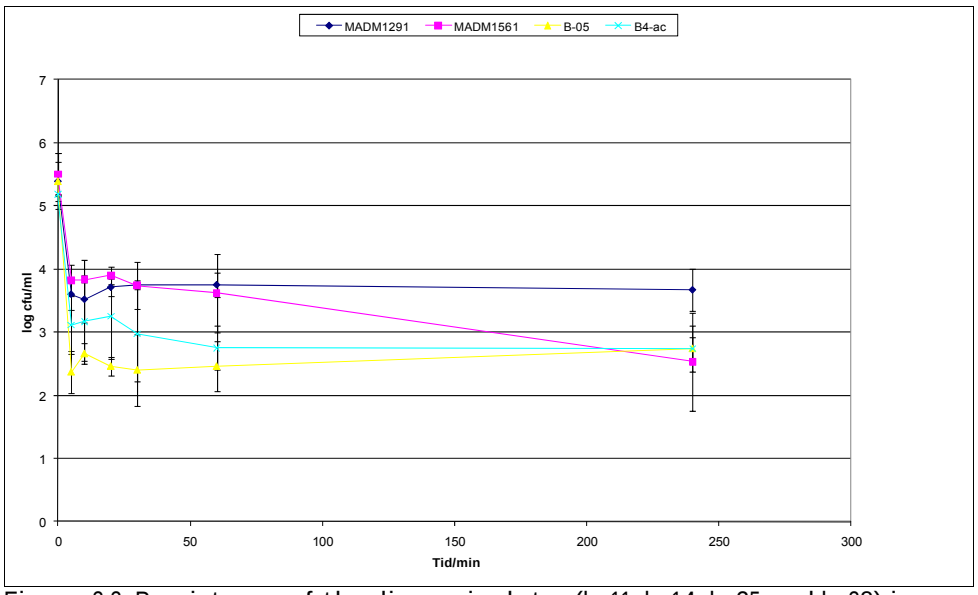


Figure 3.3. Persistence of the disease isolates (bc11, bc14, bc25 and bc38) in simulated gastric fluid at pH 2.0. Experiments were performed twice, and SEM's for each time point are shown.

3.4 Survival under simulated small intestinal conditions

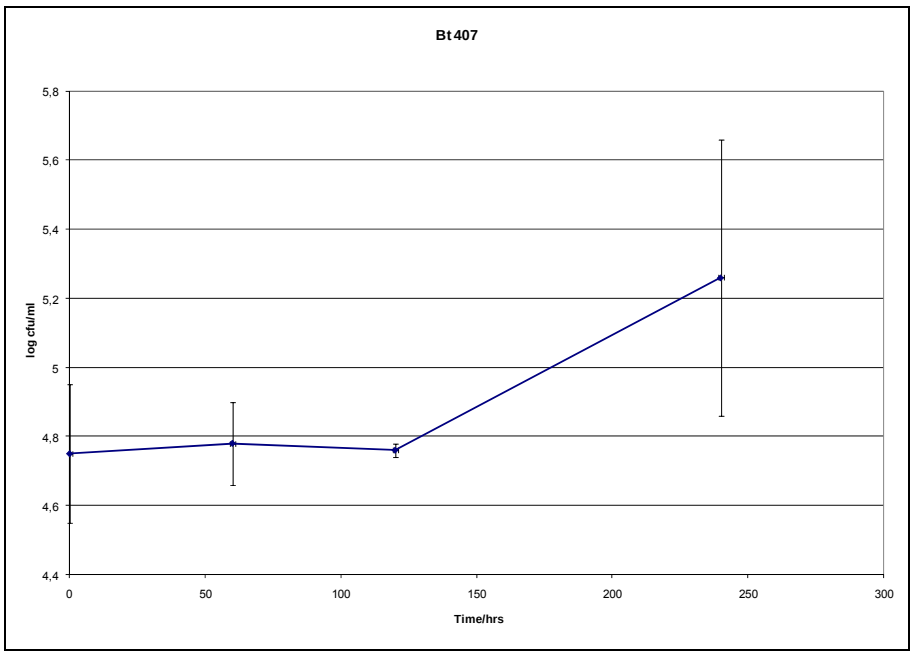


Figure 3.4 Persistence of the negative reference strain (PlcR mutant Bt48) in 0.3 g/l bile acids. Experiments were performed twice, and SEM's for each time point are shown.

Adding bile salt to a concentration of 0.15 g/l does not have an effect on the strains, all grow well (data not shown). At 0.3 g/l the growth of the strains is affected (Figures 3.4-3.6), however there are no pronounced differences between the strains, and maybe the experiment should have been performed with a higher concentration.

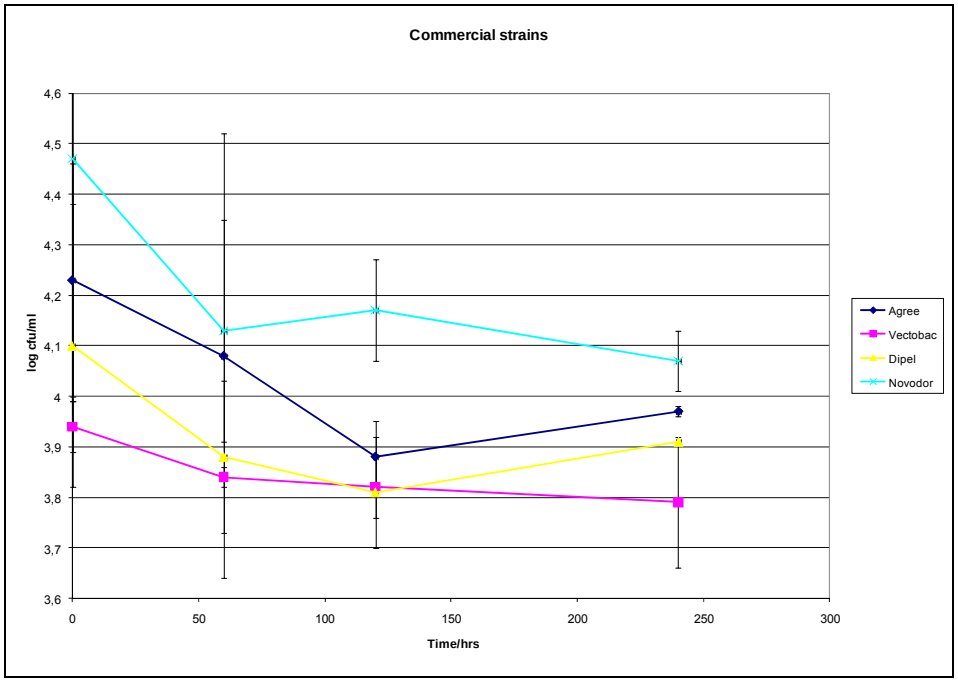


Figure 3.5 Persistence of the four product strains in 0.3 g/l bile acids. Experiments were performed twice, and SEM's for each time point are shown.

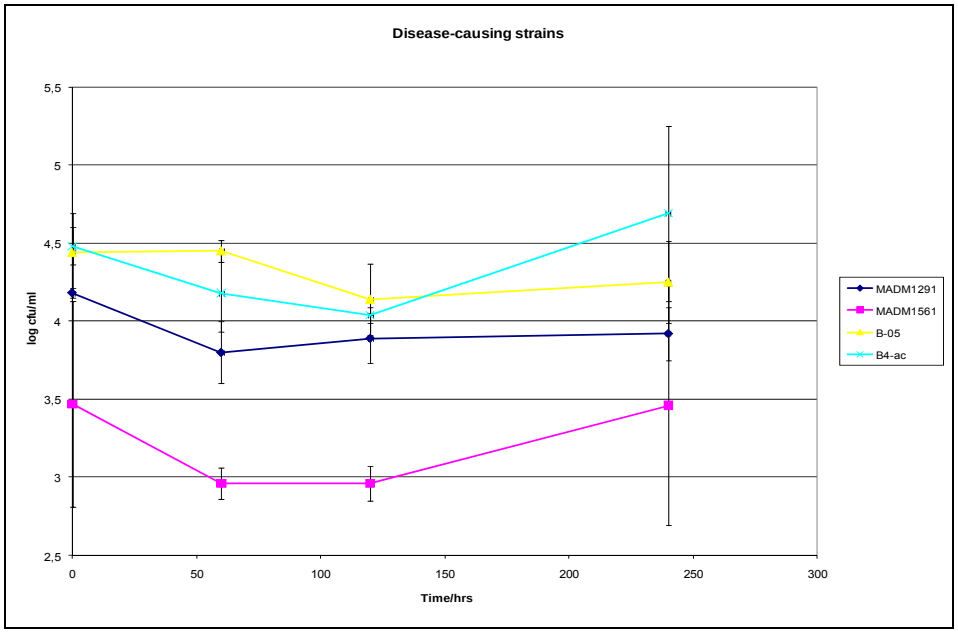


Figure 3.6 Persistence of disease isolates in 0.3 g/l bile acids. Experiments were performed twice, and SEM's for each time point are shown.

Discussion

Table 3.7 gives an overview of the results obtained for the nine strains tested under various temperature conditions, and under stomach and gut conditions. There is a tendency that in at least our few strains investigated, that strains isolated from either food involved in outbreaks or from patients have better

growth capacity at high temperatures and better survival at gastrointestinal conditions than strains used commercially in plant protection products. However, to make a firm conclusion, it is necessary to study more strains.

Guinebretiere ***et al***. have shown that ***B. cereus*** strains can be placed into seven major phylogenetic groups differing in their temperature preference (Guinebretiere ***et al***., 2008). Among 143 strains investigated, belonging to the group able to grow at the lowest temperatures none have been associated with food poisoning. This is in accordance with another study showing that mesophilic strains of ***B. cereus*** germinate and grow faster in simulated gastrointestinal conditions than psychrotrophic strains, indicating that mesophilic strains are more important in causing diarrhea than psychrotrophic strains (Wijnands ***et al***., 2006).

Table 3.7 Overview of results obtained in temperature and stomach/gut experiments. The amounts of + indicates how well the strain survived under the different conditions.

Strain	Low T	High T	SGF	SIF
Bc11	+++	+++	+++	++
Bc14	+++	+++	+++	++
Bc25	+++	+++	+++	+
Bc38	+++	+++	+++	+++
Bt48	+++	+	+++	+++
Bt50	+++	+	+++	++
Bt52	+++	+	+++	++
Bt53	+++	+	+	++
Bt55	+++	+	+	+

3.5 Detection of enterotoxin HBL using a commercial kit

3.5.1 Introduction

The way, many bacteria are pathogenic to humans, is by expressing toxins in the small intestine. This is also true for ***B. cereus*** where the hypothesis is that ingested spores germinate in the small intestine, followed by growth of vegetative cells and expression of enterotoxins. Therefore we were interested to investigate, whether the nine strains were able to produce enterotoxins. This was studied using optimal laboratory conditions, and using a commercial kit from Oxoid that is able to detect the L2 component of the Haemolytic BL enterotoxin.

3.5.2 Expression of enterotoxins

In Bc14, no toxin component was detected (Tabel3.8). The 407 with the deleted PlcR was, as expected, also very low in toxin expression. Bc11 expresses medium level of the toxin component, Bt58 very low, whereas the rest of the strains produce high amounts of enterotoxin component under the conditions tested. In a previous study financed by the Environmental Protection Agency, we showed the opposite, namely that subsp. ***israelensis*** (Vectobac) produced a lot of the component, whereas subsp. ***kurstaki*** (Dipel) and ***tenebrionis*** (Novodor) produced less (Wilcks ***et al***., 2006). The difference may be due to the fact that not exactly the same strains were used.

Tabel3.8 Detection of the L2 component of HBL in the ten selected strains under optimal growth conditions using the Oxoid BCET-RPLA kit.

Strain	Dilution factor*
Bt48	4/2
Bt50	64/64
Bt52	8/8
Bt53	128/128
Bt55	128/128
Bc11	16/32
Bc14	ND
Bc25	128/128
Bc38	64/128

* Reciprocal value of the highest dilution factor where the assay was still positive. ND: not detected. The strains were tested twice, and the results of the two experiments are separated by /.

3.5.3 Discussion

The study showed that most of the nine investigated strains were capable of producing enterotoxins, or at least the L2 component of enterotoxin HBL under optimal laboratory conditions. It could be interesting to study whether this component is also expressed under the small intestinal conditions tested in the previous chapter.

3.6 A Caenorhabditis Elegans model

3.6.1 Introduction

The nematode ***C. elegans*** has increasingly been developed and used as model to study host-pathogen interactions relevant for humans (Schulenburg et al., 2004; Thomsen et al., 2006). The nematode used for development of a ***B. cereus*** group pathogenicity model is a temperature sensitive mutant (pha-1 (e2123ts)), which can reproduce hermaphroditically at 15°C, but not at 25°C. This enables us to multiply the nematodes at 15°C, and expose the nematodes to ***B. cereus*** group bacteria at 25°C without interference from newly hatched nematodes, even though the nematode-bacteria exposures are observed for several days. As no reproduction occurs, the nematode populations will die from old age at 25°C. The disadvantage of many published ***C. elegans*** model procedures is that the nematodes are picked manually, one by one, and are transferred to agar substrate with the bacterium to be tested. As such, establishing a ***C. elegans*** procedure can be very laborious. To overcome these problems, it was decided to develop a model where nematodes were handled in suspension and not one by one.

3.6.2 Development of a *C. elegans* model

The methods selected/developed for maintaining and synchronisation of the ***C. elegans*** populations is described in section 2.5.1, and the procedure for testing the pathogenicity of microbials is described in section 2.5.2. One of the first challenges was to obtain a synchronised population of nematodes. A simple method for this was to treat a mixed suspension of nematodes and eggs with bleach (sodium hypochlorite) and sodium hydroxide, whereby living nematodes were killed, leaving only the eggs alive (Stiernagel 2006). Transferring the eggs to NGM agar inoculated with ***E. coli*** OP50 resulted in almost synchronised germination and growth of nematodes. A major disadvantage with this method was that dead nematode bodies heavily contaminated the newly hatched nematode population. As we had decided to avoid the ergonomically problematic manually picking of nematodes and only working with suspensions, we tried a number of unsuccessful methods to separate the eggs from the dead nematode bodies. The most convenient way to obtain an egg population free of living and dead nematodes was to remove nematodes with multiple gently washing of the agar substrates, leaving a majority of the eggs attached to the agar (Figure 3.7). Another challenge was to fit the procedure to a normal working week. As the nematodes only slowly were killed by the ***B. cereus*** group bacteria, the numbers of living nematodes were counted several times during a week. It was found to be most optimal to wash off the nematodes on a Tuesday, leaving the eggs to germinate during the week, dispense the nematodes in water to 96 wells microtiter plates on a Friday and incubating the nematodes at 15°C until Monday, where the numbers of living nematodes were counted. The advantage of counting the nematodes Monday is that nematodes with minor damages due to the physical handling will be dead, and only physically healthy nematodes will be alive. As the nematodes were suspended in water, the ***E. coli*** OP50 did not multiply, and the nematodes did not grow. Monday afternoon, the bacteria to be tested were inoculated in liquid cultures and grown overnight, diluted and added to the nematodes Tuesday and the number of surviving nematodes where counted each of the following three days (Figure 3.8). An advantage of working with the nematodes in liquid substrate was that in liquid, dead nematodes were straight or had only one bend, while living nematodes had 2 or more bends. See Figure 3.9. Another interesting experience during developing the nematode assay was that at some time, a (back?) mutation had occurred, enabling the nematodes to multiply also at 25°C. This experience taught us always to keep frozen nematode stocks (Stiernagel 2006). Another problem took rather long time to solve. At a time, the nematode survival pattern changed dramatically when exposed to ***B. cereus*** group bacteria. Finally we found that the nematode population had been infected by two different (based on morphology) bacteria, which however did not harm the nematodes on NGM agar, but when BHI substrate was added, these infection turned into effective nematode pathogens. Attempts to sterilize the nematode population with antibiotics and the bleach/NaOH treatment failed.

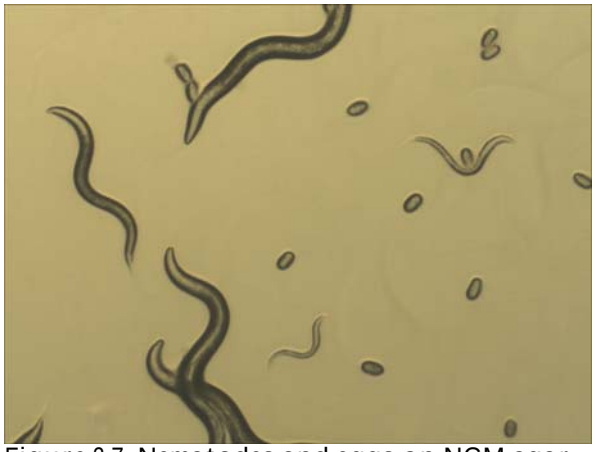


Figure 3.7. Nematodes and eggs on NGM agar

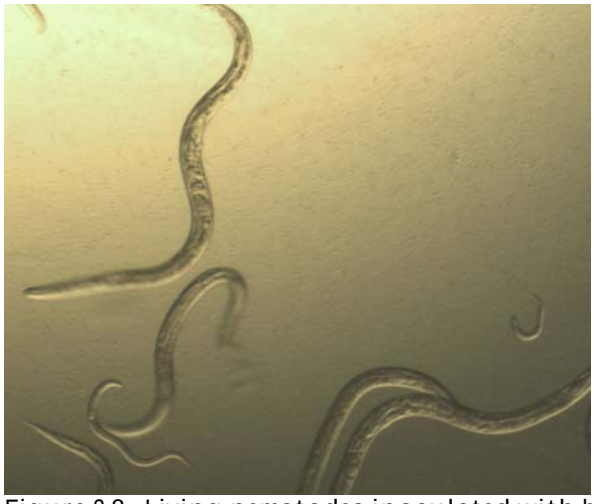


Figure 3.8. Living nematodes inoculated with bacteria

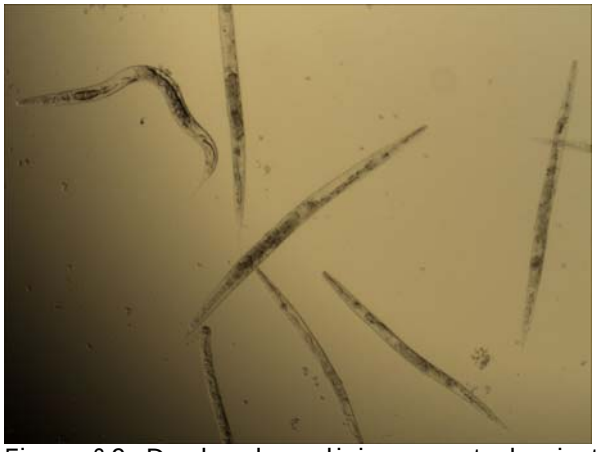


Figure 3.9. Dead and one living nematode – in the left corner

3.6.3 Microbial pathogenicity testing with the *C. elegans* model

After inoculating the nematodes with bacteria, the surviving nematodes were counted at days 1 and 3. The survival is illustrated in Figures 3.10-3.13 in percent survival. In all figures are included the survival of nematodes fed with ***E. coli*** OP50. The pathogenicity was tested with and without horse-blood in the medium. Addition of horse-blood did however not influence pathogenicity of the bacteria to the nematodes. The analysed bacteria are grouped according to their virulence/pathogenicity to the nematodes in four categories (1, 2, 3 and 4), so that 4 has the highest virulence. The least pathogenic bacterium (category 1) was strain no 50 (Bt 600, ***B. thuringiensis*** subsp ***aizawai***), (Figure 3.10). Category 2 contained most of the bacteria: strain bc 11(MADM 1291), strain bc14 (MADM 1561), strain bt48 (Bt407δ PlcR), strain bt52 (***B. thuringiensis*** subsp. ***israelensis***) and strain bt53 (***B. thuringiensis*** subsp. ***kurstaki***), (Figure 3.11). Category 3 bacteria were: Strain bc25 (B-05) and strain bc38 (B4-ac), (Figure 3.12). The bacterium being the most virulent (category 4) was strain bt55 (***B. thuringiensis*** subsp. ***tenebrionis***), (Figure 3.13).

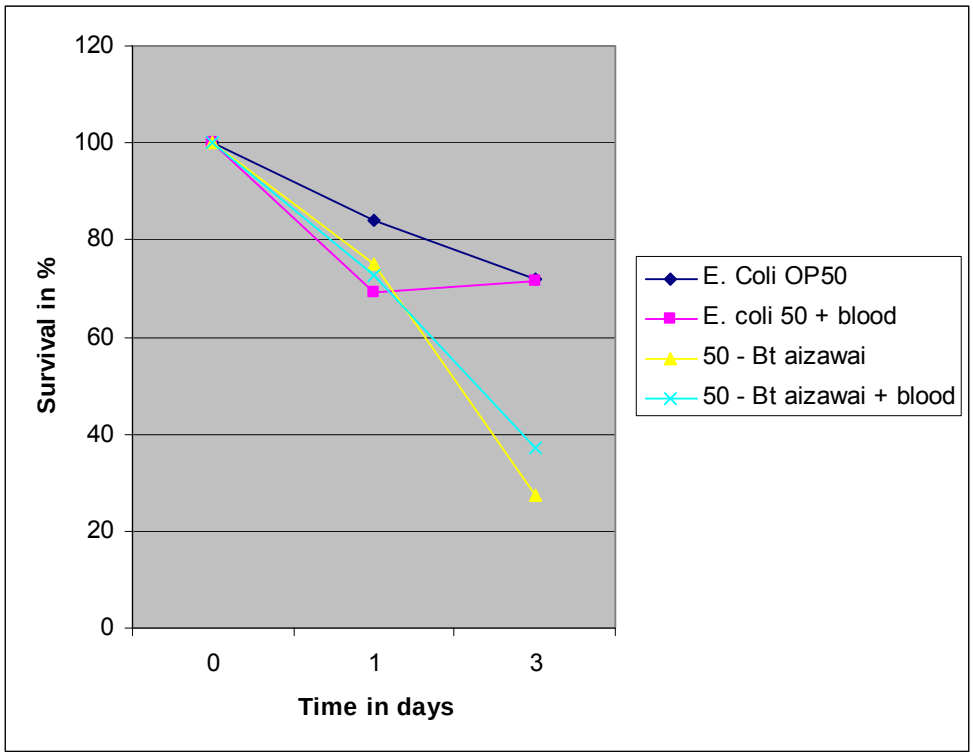


Figure 3.10 . Nematode survival after exposure to a category 1 bacterium.

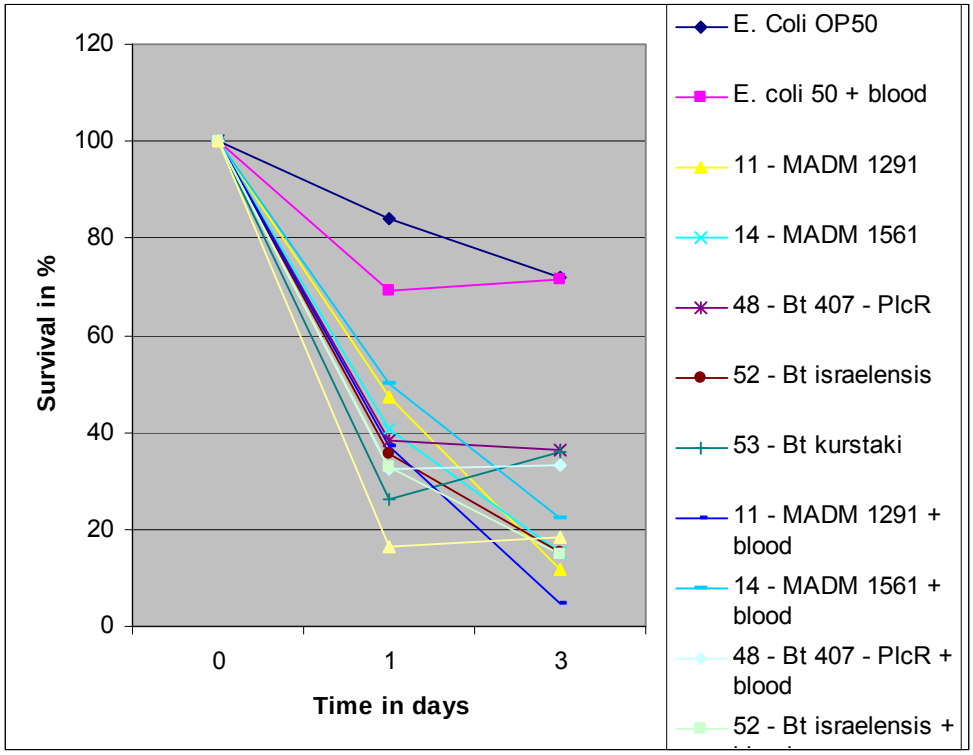


Figure 3.11. Nematode survival after exposure to category 2 bacteria.

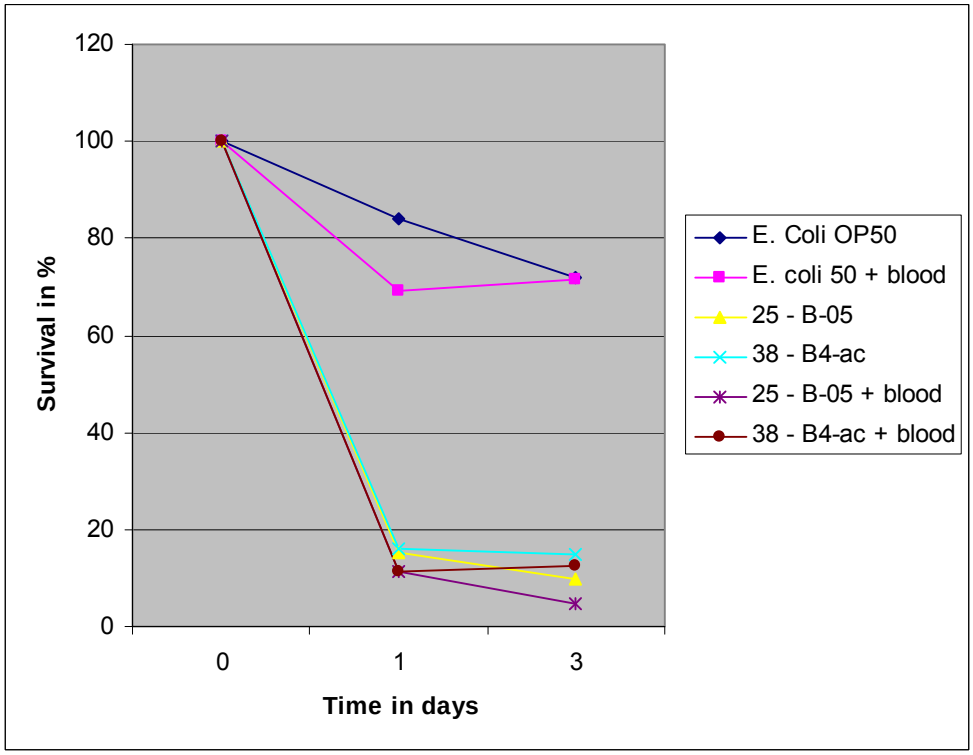


Figure 3.12. Nematode survival after exposure to category 3 bacteria.

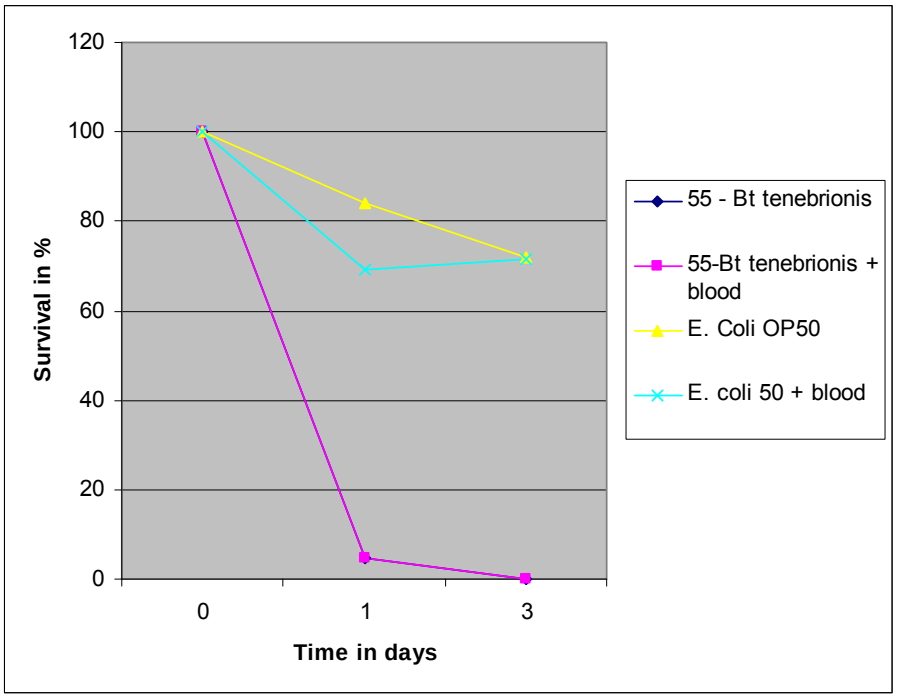


Figure 3.13. Nematode survival after exposure to a category 4 bacterium.

3.6.4 Discussion

The data obtained in the ***C. elegans*** model show clearly that the bacteria have different pathogenicity to the nematodes. It is however also shown that the most virulent bacterium was the beetle product strain, ***B. thuringiensis tenebrionis***. The pathogenicity of this bacterium could however be due to crystal proteins. Attempts to delete the crystal protein encoding plasmid from ***B. thuringiensis tenebrionis*** failed unfortunately, so it was not possible within this project to answer this question (data not shown). The least virulent bacterium was found to be a product strain, ***B. thuringiensis aizawai***, thereby being even less virulent than bt48 (Bt407δ PlcR), the strain with an interrupted PlcR regulator which activates many virulence functions, and as such was expected to be a negative control strain. This presumed negative control strain also grouped together with two of the potential positive control strains and two product strains. If, however the assumption is correct, that PlcR controls the majority of human virulence functions, it could be concluded from these data that that all five strains in category 2 are non-pathogenic to humans.

3.7 Evaluation of *Galleria Mellonella model*

3.7.1 Introduction

As mentioned in the general introduction ***Galleria*** has been used already for determining virulence factors in several pathogens both for insects and humans but principally by infection by injection into the hemocoel, which is to be considered as blood/ lymph of higher animals. With respect to the ***B.cereus*** group bacteria, analysis have been run both by oral infection and by injection and several virulence genes have been indentified, notably those regulated by the PlcR transcriptional regulator, (enterotoxins) HblC, NheB, CytK InhA2. These factors are involved with cytotoxicity and degradation of the instinal cells, and other factors like HlyII and inhA1 and inhA3 (Guillemet

et al, 2010, Cadot et al. 2010) are shown to be important for virulence whn eteh bacteria are injected into the insetc blood system, which might indicate that these factors are involved in resistance to phagocytosis and antimicrobial peptides, which are parts of the immune system similar to human innate immunity. Thus, in this part of the project we will compare the virulence of selected Bc strains for their capacity to kill ***Galleria*** larvae (% mortality) by both routes of infection (oral and injection into the hemocoel) using spores and vegetative bacteria. (sections 3.7.3; 3.7.4; 3.7.5). Before infections studies, we have also considered whether the spores germinate at the same level in all strains in the larval intestine (3.7.2) and we will try to identify whether the genes of interest are expressed in the larval intestine, (called midgut). We are even intending to compare gene expression among strains by the Q-RT-PCR (section 3.7.6.) approach (section 2.2). Meaning that we will recover bacteria from the intestine of the insect larvae and analyse whether the selected virulence genes are expressed when the bacteria are in contact with the insect intestine cells. This point is a challenge by itself since it is difficult to obtain a homogenous population of bacteria ***in vivo.***

3.7.2 In vivo spore germination

Since the infectious stage of Bt and Bc group bacteria can be as both spores and vegetative bacteria and because Bt in biopesticides are mainly found as spores in the commercial formulations and because the Probiotic strains are also supposed to be used as spore preparations, we wanted to analyse the spore germination capacity in the intestine of ***G. mellonella*** larvae. The germination assays have been run on most of the selected strains. Results show (Tabel 3.9) that within Bc and Bt strains there are more or less fast germination. Second the addition of Cry 1C toxin increases germination speed for Bc11, Bc14 and Bt52 but not for Bt53 and Bt48, for which germination is fast and reaches a high level at 3 hours post-infection. Then it is worthy to note that the various strains have their proper germination profile and that all tested strains have the capacity to germinate in the insect.

Tabel 3.9 Percentage of spore germination at 3 and 24 hours post oral infection of *Galleria mellonella*

	3H post infection in living larvae		24H post infection with Cry1C	
Strains	Without Cry1C	With Cry1C	Living larvae	Dead larvae
Bc11 (MADM 1291)	4 ±1	79±10	84±5	96±4
Bc 14 (MADM 1561)	51±5	83±6	ND	99±1
Bc 25 (B-05)	89±5	90±6	ND	99±1
Bc 38 (B-04)	ND	ND	ND	ND
Bc 49	ND	10±5	ND	55±5
Bt 50 (Bta, Agree)	ND	2±1	ND	65±5
Bt 51 (Bta, Xentari)	ND	25±5	ND	66±4
Bt 52 (Bti, Vectobac)	1±1	76±5	31±3	94±5
Bt 53 (Btk, Dipel)	73±10	90±5	68±5	99±1
Bt 55 (Btt, Novodor)	ND	1±1	ND	70±3
Bt407 cry- WT	ND	40±6	ND	80±10
Bt48 (Bt407δ *PlcR*)	93±5	96±3	ND	99±1

3.7.3 Oral infection with spores

Like for the ***in vivo*** spore germination tests there is some variation among the strains but clear cuts cannot be established. Meanwhile, all Bc strains even in association with Cry1C, result in larval mortality, in maximum, between 27% and 37 % (

Figure 3.14), while Bt stains are resulting in higher values running from 50±10 % for Bt407, 63±10% for Bt50 and up to 84±15% for Bt55 and 50±25% for Bt53. As expected the Bt407 PlcR mutant (strain bt48) has low mortality. Cry 1C toxin alone induces 5±5 % mortality and the buffer alone 0 % (data not shown).

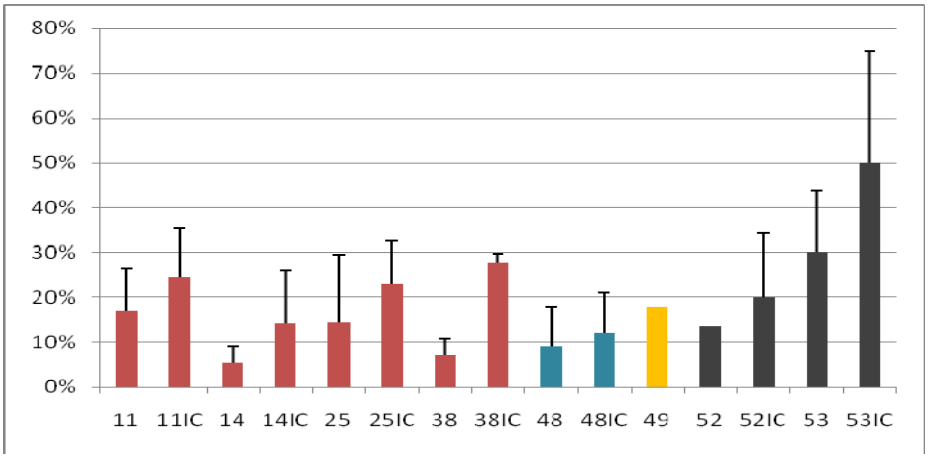


Figure 3.14 Oral infection with spores % Mortality of *G. mellonella* larvae 24 hours post infection with spores (5x10^5/larvae) from various strains ± 3 µg of Cry1C toxin.

3.7.4 Oral infection with vegetative bacteria

To compare with doses used for spore infection assays, all strains were first tested with doses from 1 to 3 x10^6 bacteria (in association with Cry1C). Figure 3.15 shows results from those tests. It seems like we observe less variation among strains with vegetative bacteria compared to spore tests, since all strains, except the ***plcR*** mutant Bt48 (18±3%) and the Bc38 (22±2%), induce mortalities at 24 hours between 39% to 68%. Even the probiotic strain Bc49 induces as high mortality as Bt55 (50±3%). In addition two other Bc strains, Bc11 and Bc25 performed higher mortality than with spores, which is not the case for Bt strains, which had about the same level of virulence for spores as for vegetative bacteria.

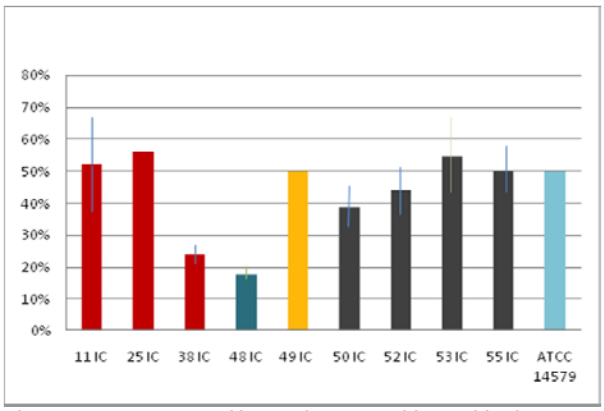


Figure 3.15 Mortality of *G. mellonella* larvae 24 hours post infection with vegetative bacteria 1 to 3 x 10^6 from various strains.

Further tests were run with higher doses (1-2 x10^7) for a couple of strains (Figure 3.16) in order to estimate virulence at high doses but also in order to analyse the number of bacteria present in the midgut at the time points (5 to 6 hours post infection) where mRNA will be extracted for virulence gene analysis. Results indicate a very high mortality for all strains (about 80%) indicating, that all strains, (except as expected for the ***plcR*** mutant (bt48), at high doses, and have fatal impacts on ***G. mellonella*** larvae. The BcATCC14579 strain is used as positive control for a full sequenced ***B.cereus*** strain, it has the same virulence level as BT407 this strain was among those used to develop PCR-primers.

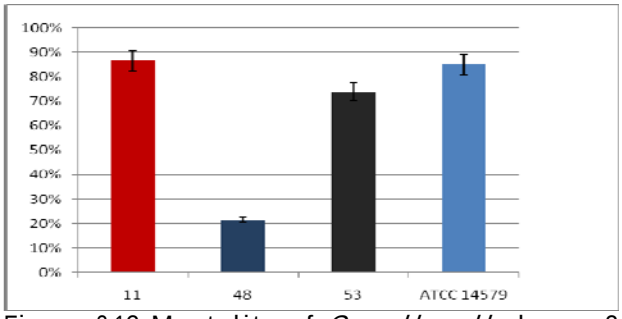


Figure 3.16 Mortality of *G. mellonella* larvae 24 hours post infection with high dose (1-2 10^7) vegetative bacteria associated with Cry1C toxin per larva

3.7.5 Hemocoel infection with spores and vegetative bacteria

In order to compare the infection and virulence level between oral infection and direct injection into the hemocol, infections were performed with several doses of spores and vegetative bacteria. The doses: 1000, 10,000 and 30,000, were run at least 2-3 times and the mortality is shown as percentage plus standard deviation of the mean. The results show that all strains confer a certain level of virulence to ***Galleria*** larvae and that there are variation between mortality obtained with spores and vegetative bacteria. Indeed, the 4 Bc strains are more virulent than Bt at low doses (1000) vegetative bacteria. Figure 3.17) All strains confer high mortality at the highest dose used. For infections with spores (Figure 3.18) there are stronger differences among strains and larval mortality is lower than with vegetative bacteria. : Bt50 and Bt52 only confer low mortality, same level as Bc49 and Bt48, while the Bc strains confers mortality equivalent to the positive control Bt407 and Bt53 and Bt55. Thus our results suggest that at a low dose the Bc strains, which have been involved in human virulence, seem more virulent than Bt strains

following injection with both inocula. It is also remarkable that Bt48 delta ***plcR*** strain is more virulent by injection than by oral infection, which might indicate that PlcR independent factors, like resistance to phagocytosis and antimicrobial peptides are more important for bacterial survival in the hemocoel

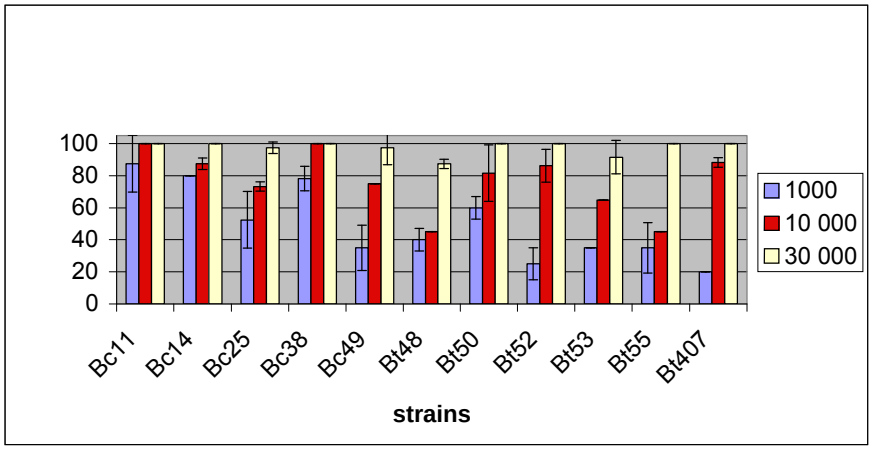


Figure 3.17 Mortality in % at 48H post injection of Bacillus vegetative bacteria (1000, 10,000 and 30,000) into the *Galleria* larvae hemocoel.

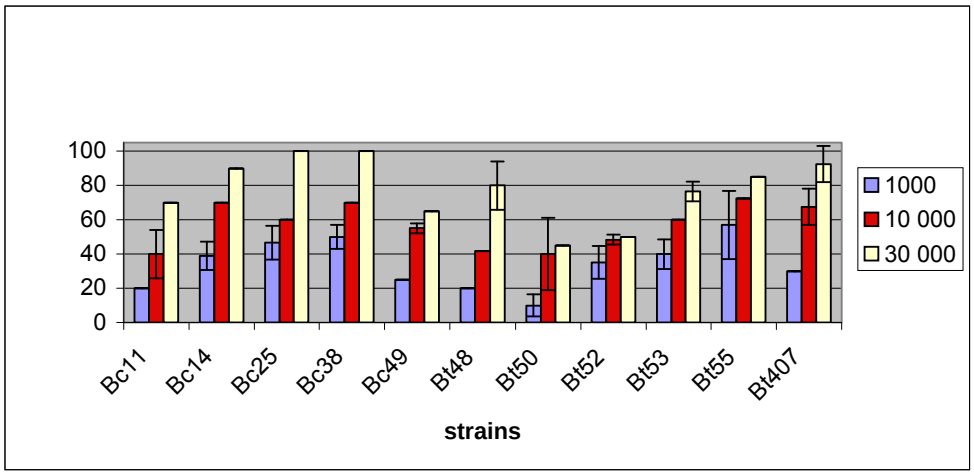


Figure 3.18 Mortality in % at 48H post injection of Bacillus spores (1000, 10,000 and 30,000) into *Galleria* larvae hemocoel .

3.7.6 Q-RT PCR of virulence gene expression in *Galleria* midgut

As mentioned earlier one of the aims to setup RT-PCR gene expression analysis's of virulence genes was not only to study that under ***in vitro*** conditions but more interestingly during infection. With respect to ***G. mellonella*** oral infection we have analysed gene expression at two time point (3 and 6 hours post infection) where the bacteria are still confined in the midgut. Earlier work has shown that the Bt407 strain (both as spores and as vegetative bacteria in association with Cry1C toxin) multiplied in the midgut (Salamitou et al.2000) and our studies have shown that when we infect the larva with about $1x10^7$ bacteria we can recover between $5x10^7$ to $2x10^8$ bacteria in a pool from 5 dissected midgut (2/3 of the intestine). Total RNA extractions were made from several pools of infected larvae following 2 different infections and 2 different extractions. Initial tests at 3 hours had shown that we did not detect expression of virulence genes (***nheB, hblC, cytK***) but only ***plcR*** for Bt53 strain. Thus we decided to focus on infections at 6 hours post infection. It has

been rather complicated to set up this approach ***in vivo***, thus being able to show results with a certain level of reproducibility is almost surprising. The results as shown below in (Tabel 3.10) in which raw data (Ct values) from SYBRgreen Q-PCR experiments performed on total cDNA are shown. Tabel 3.11 presents the relative expression of a selected number of genes (nheB**,** ***hblC*** and c***ytK*** all from the PlcR regulon) by comparison between Bt407 strain and the others strains. Not all 10 selected strains were analysed because of the work load and because these experiments are novel.

Tabel 3.10. Gene transcription (measured in Ct values) during infection of *Galleria mellonella* larvae using primers (Table 2.2). cDNA was prepared from total ANR extracted from insect intestines (midguts) at 6 hours post infection with the various strains. The Gene transcription experiments were performed at INRA using SYBR green.

Genes Strain	*nheB*	*hblC*	*cytK*	*inhA2*	*hlyII*	*tpi*	*rpoB*
Bc11	27,4 24,4	ND	28,8 25,9	36,2	32,5 27,1	21,7 18,6	22,3 19,6
Bt48	34,8 33,5 34,8	32,9 32,3 32,9	34,8 32,8 33,2	34,4 ND	31,6 32,9 32,3	24,8 22,1 22,9	23,5 21,9 23,2
Bt407	25,3 26,9	25,8 27,4	25,3 24,9	28,9 ND	29,1 30,6	20,9 21,2	20,3 22,2
Bc49	28,2 27,5	33,9 33,5	ND ND	32,1	ND ND	22,2 21,4	19,9 20,8
Bt53	25,9 26,7 24,9	28,3 27,9 ND	26,1 25,6 ND	28,1 29,2	28,9 28,7 ND	21,4 21,9 21,5	21,1 21,7 21,5

ND = not detected (if Ct higher than 37). The higher the Ct value the lower the expression. The values are Ct mean (of two replicates) from independent experiments

Interestingly the results show that most of the studied genes are expressed in the midgut of ***Galleria*** and that the expression profile is quite similar to that found ***in vitro*** (see Table 3.4) Meanwhile, the expression level (in quantity higher Ct values) is much lower ***in vivo***, which could be expected since the cDNA from ***in vivo*** extractions is a mixture of cDNA from ***Galleria*** and to a much lower amount, from the bacteria. Thus being able to detect expression is a good point and to measure differences are even more interesting. The results in Tabel 3.10 and Tabel 3.11, indicate that Bt407 strain has the highest ***in vivo*** expression for all genes, besides to that we have also compared expression ***in vitro*** and ***in vivo*** for this strain and its ***plcR*** deleted mutant Bt48, thus we chose to use it as the reference to compare gene expression. First we can conclude that for ***plcR*** mutated Bt407 (Bt48) and the three PlcR regulated genes we observe more expression ***in vivo*** than ***in vitro***, which might indicate that ***in vivo*** we can expect to find other active regulatory factors of the PlcR regulon than ***in vitro,*** where we only find low background expression (100 to 1000 fold lower than in the Bt407 wild type). We observe higher expression for Bt53 and Bc11 than for Bc49 (Probiotic strain) for NheB and CytK toxins genes although the ***nheB*** is well expressed in the Bc49 strain too. For the metalloprotease InhA2 (also part of the PlcR regulon) our results do not permit to make a comparison since the primers are not 100 efficient on all strains. Meanwhile we notice in Tabel 3.10 that this gene is well expressed in both Bt407 and Bt53 with Ct values of 28.9 and 28.1 respectively, while much lower expression is observed in Bc11 and as expected in Bt 48. It was

not possible to compare expression for ***inhA2*** and ***hlyII*** since the primers did not give full efficacy for all analysed strains/genes.

Tabel 3.11 Relative (in %) expression of gene transcription *in vivo* from *Galleria* midgut. Data are based on normalised Ct values from (Table 3.10) for a number of genes using Bt407 gene expression as base (100%) and *rpoB* and *tpi* for normalisation

Gene / Strain		hblC	cytK	nheB
Bt 407	*in vivo*	100	100	100
	in vitro	100	100	100
Bt48 (Bt 407 *plcR*)	*in vivo*	7,9	1	1,6
	in vitro	0,9	0,1	0,5
Bt**53**	*in vivo*	43,1	68,6 [a]	101[a]
Bc 49	*in vivo*	1,2	0	31,1
Bc**11**	*in vivo*	0	13,4	71,5 [a]

a = not significantly different from Bt407. All the other values are significantly different (p-value 0.01, from Bt407 (Quiagen REST Q-RT –PCR software). Yellow background is from *in vitro* expression.

3.7.7 Discussion of the *G. mellonella* model

Altogether, the studies with oral infection with spores show that Bt strains may induce higher mortality to ***Galleria*** than the tested Bc strains although there are large variations among strains. Bt strains Bt53 and Bt55 induce high mortality. It is also interesting that the Bt407 PlcR mutant strains (Bt48) germinate much faster than the parental wild type, (Bt407cry-).One explanation might be that the lack of a functional PlcR regulon can influence the phenotype and quality of the spores resulting in an easier germination capacity. The fast germination of Bt53 (Bt ***kurstaki***) even without Cry1C, may be explained by the natural presence of Cry toxins, generally known to be active towards caterpillars like ***Galleria***, and which then induce changes to the midgut cells resulting in modification of the physiological balance and thus larval sensibility. The role of Bt53 natural Cry toxin is in agreement with the relative lower virulence of Bt53 as vegetative bacteria as compared to spores since in vegetative bacteria the Bt53 cry toxin complex is not present. Since vegetative bacteria resulted in more or less the same level of virulence for all strains, it might indicate that Bc strains are more virulent as vegetative bacteria, than as spores. This might partly be due to the variation in spore germination capacity.

With respect to results from the LD50 values for a few strains (results not shown) virulence levels for the tested strains were not the same as those found with single high doses. This might suggest that to have a real virulence picture of strains, tests of various doses are needed. But this is both time and cost consuming. Then, in some situations high doses alone are acceptable since they can be considered worst-case infections, but it might also be of value to test a low dose, since this might more easily distinguish differences between strains. Similar studies (from our group) using ***Galleria*** to test for virulence

by oral infection with several Bc strains from clinical cases also showed that these strains, except one, was virulent to ***Galleria*** at more or less the same level as the Bt407 and the ATCC14579 strains (Fedhila ***et al.*** 2010), one was even more virulent than average. Thus our results confirm that another number of Bc strains are virulent by oral infection and that the addition of Cry1C helps to increase the induced mortality level but they also seem virulent without Cry1C. This point needs more investigations.

We also tested the virulence of a selected number of strains following injections into the hemocoel, meaning that the bacteria (spores and vegetative) will be in direct contact with the insect blood- lymphe systems without the need to cross intestinal barriers or skin/cuticle. We observe some differences in capacity to induce mortality; the three Bc strains Bc14, Bc25 and Bc38 seems to have a bit higher potential than Bt48, Bc toyoi (Bc49)and Bt50, but in a similar range to Bt407 , Bt53 and Bt55. Analysis for statistical differences have not been run, so we can not strongly conclude from these test. However, Bc strains are the most virulent at low dose as compared to Bt strains, which indicate their virulence potential. As ***Galleria*** is already frequently used to estimate virulence for other bacterial species following injection into the hemocoel (see introduction) it is not a surprise that Bt/Bc strains can induce mortality too. Also, recently 9 Bc strains were tested following injection into the hemocoel (10,000 vegetative bacteria per larva) they showed that 3 strains considered non pathogenic were not virulent to ***Galleria*** (less than 20% mortality) the other reaches levels similar to what we found here, between 50 % to 90 % (Cadot ***et al.,*** 2010). Thus, altogether we can conclude that our results are in the line with these studies and that it is possible to find the potential virulence of a certain strain by using ***Galleria*** as a model but that not all human clinical strains will show up to be virulent in the ***Galleria*** model.

The correlation between virulence and ***in vivo*** virulence gene expression was analysed by RT-PCR and Q-RT-PCR. It was quiet a challenge, and although the results shall be taken with caution especially with respect to quantification it is really exciting that we have been able to show variation in gene expression in bacteria isolated in association with the ***Galleria*** intestinal cell. Our results indicate that there is expression of the considered virulence factors like the enterotoxins (HblC, CytK, NheB). This is the first time that such expressions have been show ***in vivo*** for several genes and in contact with the intestinal environment. Thus we believe these finding are of importance to certify the role of these toxins ***in vivo*** and especially by the oral route of infection, since the Bt48 (plcR) strain is in the low range of virulence following oral infection while it is in the middle range following injection into the hemocoel. Indeed factors like HlyII and metalloproteases InhA1 and InhA3 are shown to be important for infection in the hemocoel (Tran ***et al.*** 2010) while the PlcR regulated InhA 2 protease is more important following oral infection (Fedhila et al. 2002). Thus it is clear that some virulence factors seem to be more important to cross the intestinal barriers and others might be essential to cope with phagocytes and antimicrobial peptides.

3.8 Development of functional mammalian cell models

3.8.1 Introduction

The functional mammalian cell models used in the present work include three different cell lines. Two are of human intestinal origin (HT29-MTX and Caco-2), and one is a macrophage cell line (PoM2) of porcine origin. During culturing, cell lines such as the Caco-2 are able to differentiate into a cell morphology that resembles that of the cells lining the human intestinal tract. To obtain differentiated cells for infection experiments, Caco-2 cells were cultured for 17-18 days and HT29-MTX for 28-29 days. Antibiotics are commonly used to protect mammalian cell lines from infections during maintenance and pre-culturing for experiments. However in the present study, preliminary results showed that even after careful washing of the cell monolayers, trace amounts of antibiotics highly influenced the results (the bacteria were sometimes killed or inhibited). Use of antibiotics during pre-culturing was therefore avoided at all times. Several approaches (model systems) were used in the present work to differentiate the selected bacterial cultures. Initially, the effect of the selected bacteria on the physiology of the Caco-2 monolayer grown on plastic surfaces was investigated by visual inspection using microscopy (section 3.8.2). Good adhesion ability to intestinal cells is considered a potential health promoting trait in probiotic cultures, while it is an indicator of virulence potential in pathogenic bacteria. In comparison invasion of intestinal epithelial cells is considered a pathogenic trait. Adhesion and invasion capability was investigated using the mucus secreting cell-line HT29-MTX and the Caco-2 cell line (section 3.8.3). Cells of the intestine are bound together by tight junctions that create a polarized monolayer. Pathogenic bacteria are able to compromise and destabilize the polarized intestinal epithelial monolayer, leading to disease (e.g. diarrhoea). In comparison probiotic cultures may contribute to further stabilization of the intestinal epithelial monolayer. The supposed effect of the selected bacteria on the integrity of the intestinal epithelial barrier was determined in the present work by use of transepithelial electrical resistance (TEER) measurements of infected polarized HT29-MTX and Caco-2 monolayers grown on filtersupports (section 3.8.4). When the intestinal barrier is compromised, macrophages will be recruited to the site of infection. Macrophages have many functions, one of them is to engulf and kill pathogenic bacteria. However if the macrophages are killed by the bacteria, this indicates that the bacteria are very virulent (Ivec et al. 2007). The effect of the selected bacteria on the viability of the porcine macrophage cell line PoM2 was examined using the MTT-viability assay (section 3.8.5). The reproducibility and robustness of the methods was investigated by using both high and low passage numbers of the cell lines in Appendix 5.

3.8.2 Effect of the bacteria on Caco-2 cell morphology

The experiments in this section were performed to investigate the effect of the selected bacteria on Caco-2 cell physiology as detected by visual inspection by brightfield microscopy. Figure 3.19 shows examples of morphological changes of the Caco-2 monolayer when infected with different strain of ***Bacillus*** spp.. It is seen from the figure that the Caco-2 cells are changed/detached when infected with strain bc11 and bc25 for 4 h. In comparison the monolayer was unchanged as compared to control cells when infected with strain bt50 for 4 h.

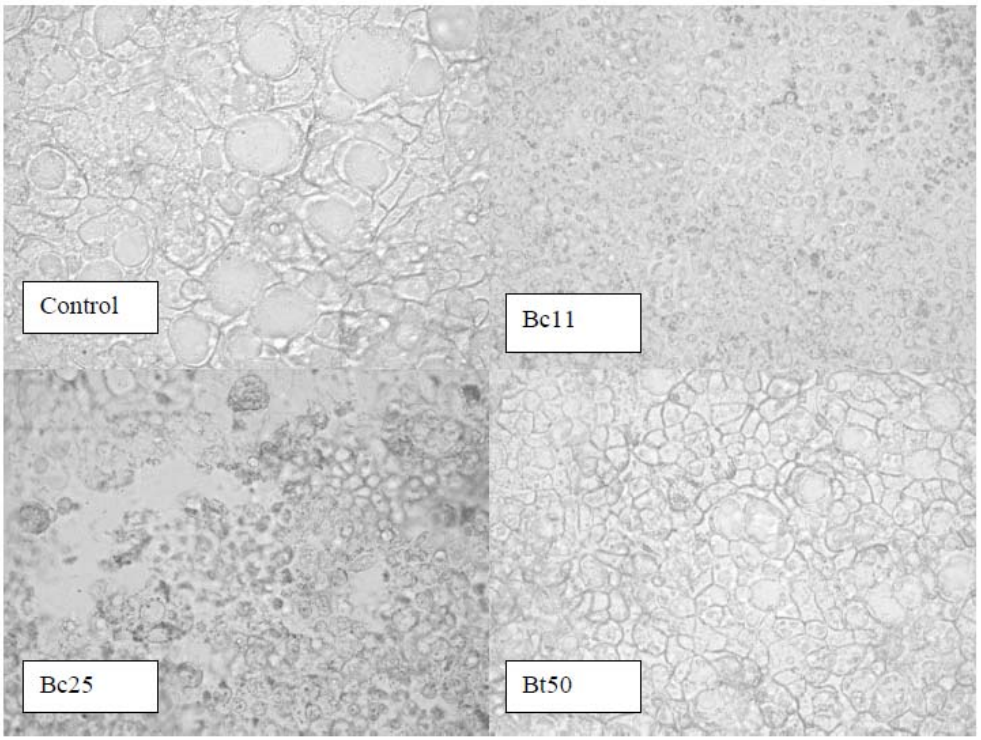


Figure 3.19 Morphological changes to Caco-2 cells infected with various *Bacillus* spp. (vegetative cells) For 4 hours at 37°C, 5% CO_2. The experiment was performed the 11th of June 2009.

From Figure 3.20 it is seen that all the bacteria examined, including the ***B. thuringiensis*** production strains bt50, bt52 and bt53, destroyed (detached) the Caco-2 monolayer within 3-9h depending on the strain and concentration of bacterial cells used. Infection with different doses of bacteria revealed that strains bt50 and bt52 (encircled in red) generally were the least aggressive by being slowest in detaching the Caco-2 monolayer. In comparison ***B. cereus*** strains bc11 (food poisoning) and bc 25 (blood) were generally the most agressive (encircled in grey). Interestingly, bt53 seemed to shift position from being a very aggressive phenotype at high infection doses (positioned together with bc11 and bc25) towards a less aggressive phenotype at the lower infection doses (positioned together with bt50 and bt52).

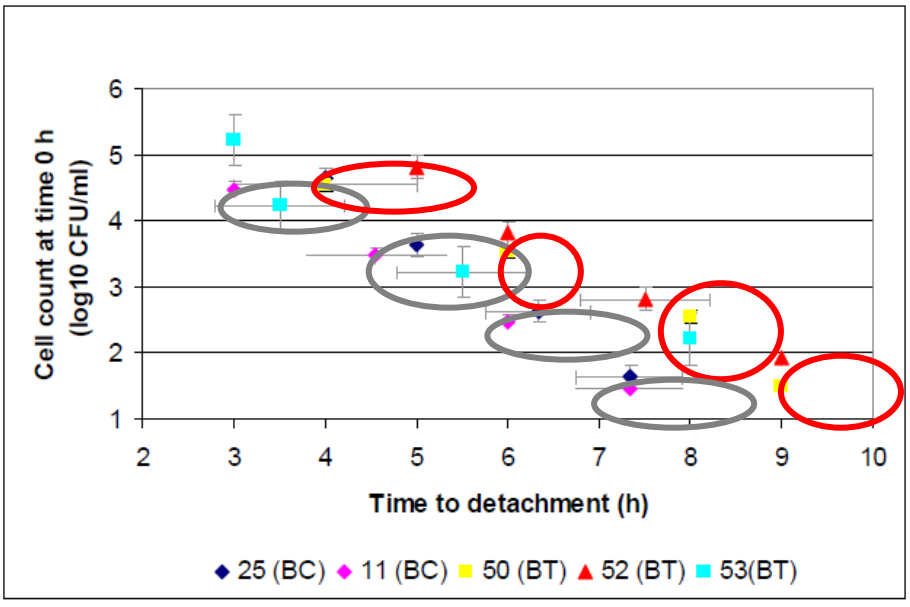


Figure 3.20 Effect of five different bacteria on the physiology of Caco-2 monolayers as observed by bright field microscopy. BC = *B. cereus,* BT = *B. thuringiensis*. The bacteria were added at different concentrations, and the monolayer was observed for a maximum of nine hours. Results are shown as averages of 2-3 independent experiments with standard deviations.

3.8.3 Adhesion and invasion

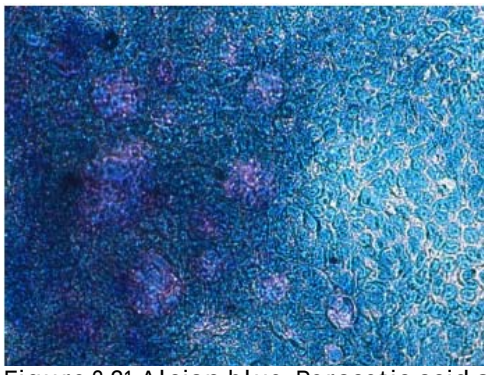


Figure 3.21 Alcian blue, Peracetic acid staining of HT29-MTX cells grown for 29 days. Blue stained areas are acid mucins, and purple stained areas are a mix of acid and neutral mucins.

As described in the introduction, good adhesion ability can be an indicator of probiotic as well as pathogenic potential, depending on the type of bacteria examined. Invasion ability is a pathogenic trait. Adhesion and invasion experiments were performed using vegetative bacterial cells and two different mammalian cell lines, HT29-MTX (grown for 28-29 days) and Caco-2 (grown for 18-19 days). Verification of mucus production by HT29-MTX was obtained by staining with Alcian/PAS (Figure 3.21).
As shown in Figure 3.22, three hours of co-incubation with mucus covered HT29-MTX cells resulted in an increase in bacterial cell counts to between $5.8*10^5$ and $1.2*10^7$ CFU/well. The Bacteria adhered with between $6.4*10^3$ and $2.2*10^6$ CFU/well. Invasion was generally very low (<400 cells/well). Infection with strains bc11, bc38, bt53, bt55 resulted in detachment of the HT29-MTX monolayer as observed by light microscopy.

As shown in Figure 3.23., three hours of co-incubation with Caco-2 cells resulted in an increase in bacterial cell counts to between $1.6*10^6$ and $1.8*10^7$ CFU/well. The Bacteria adhered with approximately 10^4-10^6/well. Similar to with HT29-MTX, invasion by the bacteria was generally very low. Strains bc11, bc25, bc38, bt53, bt55 resulted in detachment of the Caco-2 monolayer, while a weaker effect (rounding of cells) was observed when infection was with strains bt48 and bt50 even though these strains adhered with relatively high numbers as compared to the other bacteria. Strain bt52 did not cause visible damage to the Caco-2 monolayer as observed by light microscopy. Strain bt52 adhered with the lowest number of cells as compared to the other bacteria (Figure 3.23), numbers for bt53 not considered as strong detachment was observed).

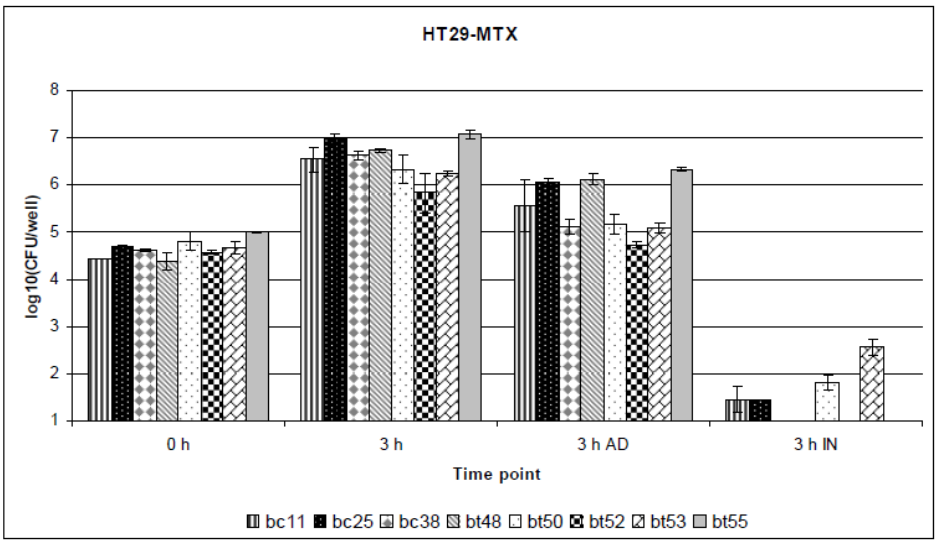


Figure 3.22 HT29-MTX cells (28-29 Days) infected with eight different bacteria (vegetative cells) for 3 hours at 37ºC, 5% CO_2. Data is the average of 2 independent experiments performed in duplicate. Data are shown with standard deviations. Bc = *B. cerues*, Bt = *B. thuringiensis,* AD = Adhesion, IN = Invasion. Invasion was not detected for Bt48 as it was not possible to kill this bacterium with the gentamycin treatment given (200 µµg/ml).

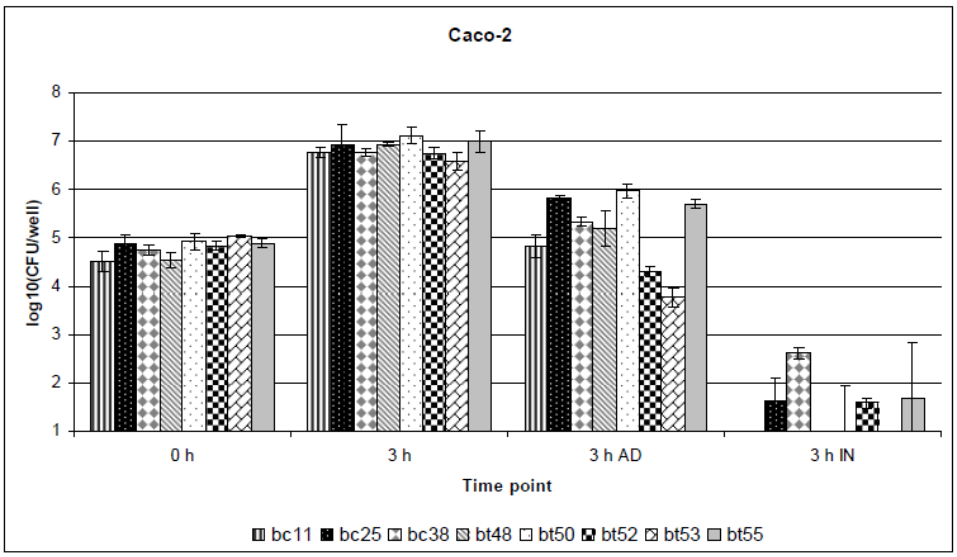


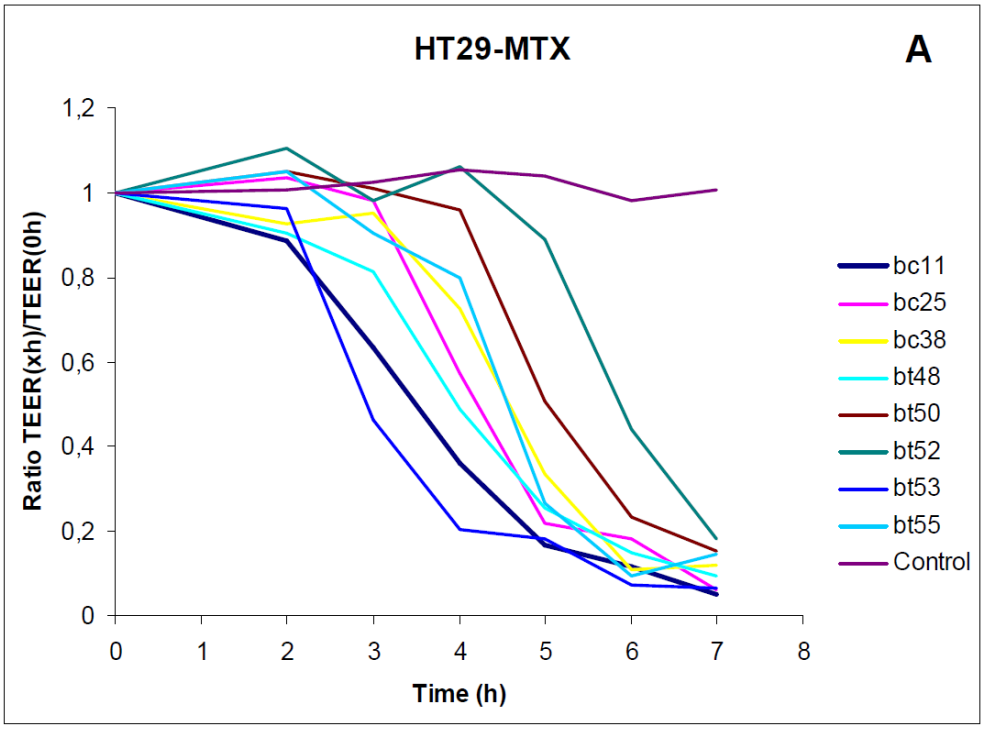
Figure 3.23 Caco-2 cells (17-18 days) infected with various *Bacillus* spp. (vegetative cells) For 3 hours at 37ºC, 5% CO_2. Data is the average of 2-3 independent experiments performed in double. Data are shown with standard deviations

3.8.4 Transepithelial electrical resistance measurents (TEER)

The human gut is lined with a monolayer of polarized intestinal epithelial cells. During infection with a pathogenic microorganism the integrity of the monolayer may be compromised leading to e.g. diarrhea. In our model system, mimicking the polarized intestinal epithelial barrier, transepithelial electrical resistance (TEER) measurements were used to evaluate the integrity of the intestinal polarized epithelial monolayer of Caco-2 or HT29-MTX cells. A decrease in TEER will indicate that the integrity of the epithelial barrier is compromised (weakened) by the bacteria used for infection, whereas

an increase will indicate a strengthening of the barrier. As shown in Figures Figure 3.24 A and B, all of the tested ***Bacillus*** species were able to decrease the TEER to 60% of the initial value within 2½-3 to approximately5- 6 hours depending on the strain. The ability to reduce the TEER decreased in the following order bt53, bc11, bt48, bc25, bc38 = bt55 ($p>0.05$), bt50 ($p>0.05$) and bt52 when HT29-MTX were infected, while the order was bt53, bc25, bc38, bt50 =bt55= bc49, bc11, bt48 and bt52 when Caco-2 cells were infected. Strain bt53 was the most virulent in both models, while the least virulent strain was bt52.

The effect of plcR regulated genes on TEER was demonstrated by comparing the effect of strain bt48 (BT407δplcR) with the wild type of bt48, strain BT407. As shown in Figure 3.25, the curves were similar for bt48 and BT407 indicating that plcR regulated genes does not have an effect on the changes in TEER.



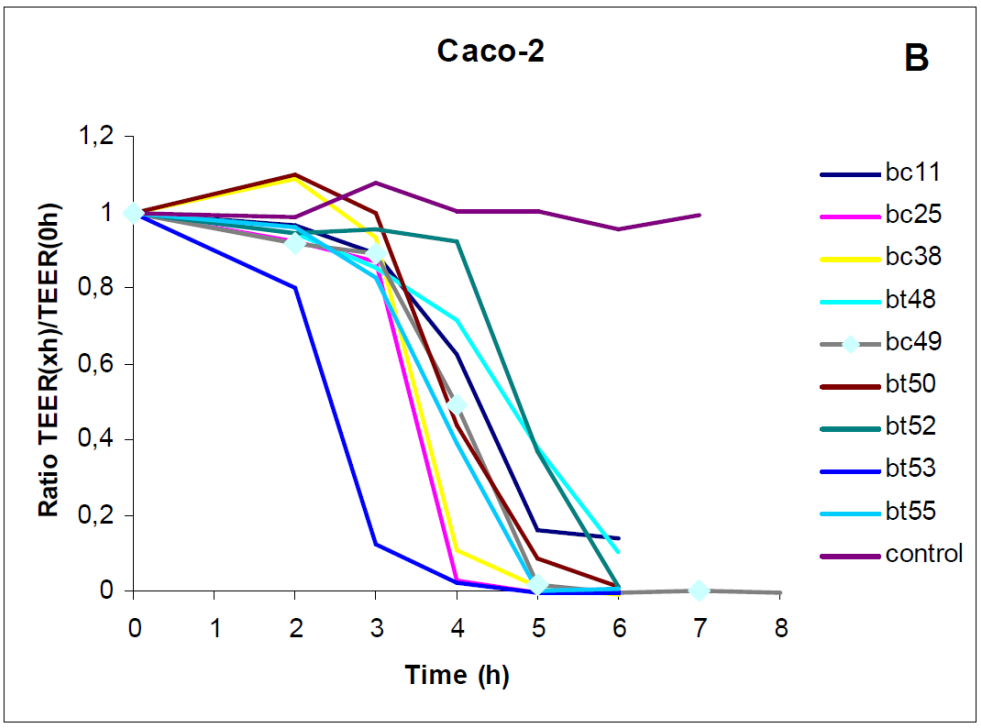


Figure 3.24 TEER of polarized (A) HT29-MTX and B) Caco-2 monolayers exposed to various *Bacillus* spp. at a concentration of $1*10^4$ CFU/well (strains bc25, bc38, bt50, bt52, bt53, bt55) AND $3*10^3$ CFU/well (Strains bc11, bt48). TEER ($\Omega*cm_2$) is expressed as the ratio of TEER at time t in relation to the initial value (at time zero [t0]) for each series. Strains bc11, bc25, bc38 and Bc49 are *B. cereus,* bt48, bt50, bt52, bt53, bt55 are *B. thuringiensis*. Results are averages of at least 2 experiments performed in triplicate. Standard deviations are not shown (for standard deviations, See TEER Appendice 5 TEER) For strains bt48 and bt52 the HT29-MTX assay was a sinlge determination performed in triplicate.

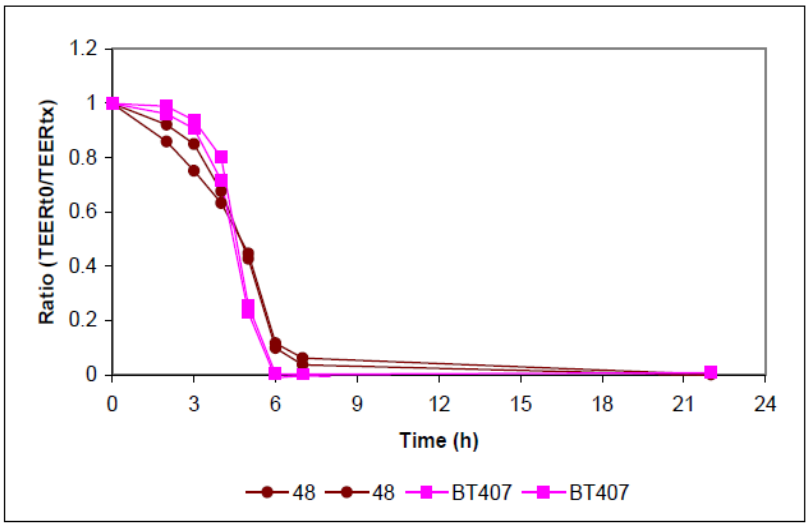


Figure 3.25 The Effect of plcR regulated genes on TEER of Caco-2 cells as demonstrated by comparison of the wild type strain BT407 and The plcR deletion mutant of BT407 (strain bt48). The data are averages of results for three wells performed in duplicate on 2 different days

3.8.5 Mitochondrial activity of PoM2 exposed to Bacillus spp.

Macrophages are recruited to sites of infection. One role (of many) of macrophages is to engulf and kill pathogenic bacteria. If the bacteria kill the macrophages it is a sign of virulence. Mitochondrial function can be used as an indicator of viability. Results for the mitochondrial function of PoM2 monocyte/machrophages determined 22h post infection with various ***Bacillus*** spp. by use of the MTT- assay is shown in Table 3.12. The results in table 5, show the effect of the moiety of infection (ratio of bacteria to POM2 cells), the effect of killing the bacteria 1 hour post infection as compared to after 22 h with gentamicin, and finally the effect of using different passages of the POM2 cell line (the assay was performed in 2009 and repeated in 2010 using POM2 cells of different passages).

As shown in Table 3.12, in most instances, even though the bacteria were added at very low numbers (moiety of infection of 1:600) the macrophages were not able to engulf and kill them. Instead the macrophages were killed (<10% dehydrogenase activity).When the moiety of infection was 1:6, the results were similar to those obtained with a moiety of infection of 1:600.

As shown in Table 3.12, if gentamicin was added 1 h post infection to kill the bacteria, the macrophage survival was much higher for most of the bacterial strains tested as compared to when gentamicin was added 22 h post infection.

Table 3.12 Mitochondrial function of monocyte/macrophage cells, i.e. PoM2 exposed to various Bacillus spp. Gentamicin was either added either after 22 or 1 h of infection. The mitochondrial function (dehydrogenase activity) was measured using the MTT-assay and is used as an index of surviving cells (percentages). The assay was repeated three times (in triplicate) in 2009 and two times (in triplicate) in 2010 in order to evaluate the reproducibility of the assay. *Single determination in triplicate"

Strain	Activity (%) Moiety of infection (1:600) 100 µg gentamicin/ml at 22 h of infection PASSAGE 44	Activity (%) Moiety of infection (1:6) 100 µg gentamicin/ml at 22 h of infection PASSAGE 44	Activity (%) Moiety of infection (1:6) 100 µg gentamicin/ml at 22 h of infection PASSAGE 61-66	Activity (%) Moiety of infection (1:6) 100 µg gentamicin/ml at 1 h of infection PASSAGE 44	Activity (%) Moiety of infection (1:6) 100 µg gentamicin/ml at 1 h of infection PASSAGE 61-66
bc11	12±7	9±6	70±20	54±8	50±38
bc25	2±2	1±1	18±12	110±21	75±34
bc38	5±3	7±5	7±5	105±8	65±41
bt48	42± 29	55±39	54±23	53±10	59±23
BT407	nd	nd	16±12	nd	49±53
bc49	nd	nd	172±19	nd	96±26
bt50	26±46	9±10	21±16	102±0	87±18
bt52	27±24	16±10	39±24	102±0	96±18
bt53	6±6	10±8	16±13	14±7	22±20
bt55	10±9	10±14	10±8	21±9	49±36
ATCC14579*	nd	nd	1±1	nd	82±5

As shown in Table Table 3.12, except for strain bc11, the results were similar when exposing the bacteria to early and later passages of POM2 cells, when gentamicin was added 22 h post infection. However if gentamicin was added 1 h post infection, the average values for the percentage activity were lower for the later passages of POM2 as compared to the earlier passages when infected with strains bc25 and bc38. Infection with the other strains resulted in similar % activities for the early and late POM2 cell passages.

Bc49, ***B. cereus*** var. ***toyoi*** was least detrimental to the POM2 cells, whereas ***B. thuringiensis*** strain bt53 seemed to be the most virulent strain (gentamicin added at 1 and 22 h post infection). ***B. cereus*** var toyoi (bc49) used as a probiotic for swine positively stimulated POM2 (a porcine cell line) the most as compared to the other strains tested (gentamicin added at 22 h). The effect of the PlcR regulator on macrophage viability was demonstrated by use of strains Bt48 (Bt407δplcR) and Bt407. Infection with strain bt48 (BT407δplcR) and Bt407 resulted in average percentage POM2 activities of 54 and 16%, respectively when gentamicin was added 22h post infection. These results indicate that genes regulated by plcR are involved in macrophage killings.

3.8.6 RT-PCR

Virulence gene expression of the ***Bacillus*** spp. strains exposed to Caco-2 monolayers was attempted detected by use of Real time PCR (RT-PCR). RT-PCR results from a preliminary experiment based on cDNA produced from RNA from samples from a TEER experiment is shown in Table 3.13 It is seen that all genes are expressed (CT values between 23 and 29) by strain bt53 at the time of transfer to the apical side of the Caco-2 cells (MEM time 0 h). Further all genes are expressed at 2 h of the TEER experiment in sample A (CT-values 22-26), while in sample B the genes are not highly expressed (CT values 31-37). Expression of the 16S gene is not stable throughout the experiment (CT values ranging between 24 and 33) and is therefore not suitable as a reference gene.

Table 3.13 Average CT values obtained for strain Bt53 for 7 different genes during a TEER experiment lasting 2 hours. The initial bacterial load was $4*10^5$ CFU per well. And the TEER was 5% of original TEER at 2 hours. The experiment is a preliminary experiment. (Slope values of standard curves were between -3.6 and -3.1).

Timepoint	*plcR*	*nheB*	*cytK*	*hblC*	*inhA2*	*ilsA*	16S
LB-broth (0h)	23.1	25.3	27.1	25.0	29.1	32.7	24.5
MEM (0h)	25.2	23.0	27.5	26.4	26.3	27.5	29.1
TEER 2 h A	24.6	21.5	25.1	25.8	26.3	23.2	24.0
TEER 2 h B	37.1	30.7	36.9	38.0	36.5	36.3	32.9

Gene transcriptions at 0h, 3h and 4 h of infection of Caco-2 cells were attempted obtained for most of the ***Bacillus*** spp. isolates/strains examined in the present work. At 3 and 4 h the samples were divided into non adherent and adherent bacterial cells. Generally the bacterial cell counts were very low at the time of sampling (see Table 3.14), making it difficult to obtain enough mRNA to get detectable expression in the RT-PCR assays. Further the RNA from the Caco-2 cells which was extracted together with the bacterial RNA was probably very dominating compared to that from the bacteria, making it

even more difficult. Due to the sensitivity of the Caco-2 cells to the ***Bacillus*** (fast detachment of Caco-2 cells at high ***Bacillus*** cell counts) it was not possible to increase the initial bacterial cell counts for infection in order to get more mRNA. We also attempted to use alternative methods for gene extractions: e.g. by increasing the adhesion area (to obtain more bacteria to extract from), as well as by trying to disrupt the Caco-2, but not the bacterial cells before collecting the samples for RNA extraction (by adding a specific stop solution). However, none of these alternative methods gave better results (results not shown). Below are the results for the RT-PCR assays where we were able to obtain gene expressions (Table 3.14).

As shown in Table 3.14, it was not possible to detect gene expressions from the adhering Bc11 cells at 3 h and 4 of infection, however the non adherent Bc11 cells (floating above the Caco-2 cells) expressed the virulence gene ***cytK*** at 3 and 4 h of infection (combined from different experiments). Non adherent Bt50 cells expressed the virulence associated genes ***cytK, ilsA, nheB***, while the gene expression levels of the adherent cells were too low to detect. Strain Bt52 (non adherent cells) expressed the virulence genes ***cytK*** and ***nheB*** even though no visible change could be observed to the Caco-2 cells. Strain Bt53 (non adherent cells) expressed all the virulence associated genes except for ***hlyII*** and ***inhA2*** at 3 h of infection (Caco-2 cells strongly detached).

Table 3.14 Gene transcriptions of various *Bacillus cereus* and *Bacillus thuringiensis* strains sampled at 0, 3 and 4 h of infection of differentiated Caco-2 cells (17-18 days) at 37°C. Gene transcriptions (CT-values, indicated as either present (+) or absent (-)) were detected by use of SYBRG technology, using the Applied Biosystems FAST7500 RT-PCR machine. Each gene was determined in triplicate and melting curve analysis of the PCR-products was used for verifications. Changes to the Caco-2 monolayer was observed by bright field microscopy at 3 and 4 hours

				Virulence genes							House keeping genes	
Date	Strain/sample	Cell count	Caco-2	*plcR*	*nheB*	*hblC*	*cytK*	*ilsA*	*inhA2*	*hlyII*	*tpi*	*pta*
	Bc11/0h		none	-	(+)	-	+	(+)	-		+	+
	Bc11/3h		detach	-	-	-	+	-	+		+	-
	Bc11/3h Ad			-/-	-/-	-/-	-/-	-/-	-/-		-/-	-/-
9/3-10	Bc11/3h	$2,3*10^6$	detach				-				-	-
	Bc11/3h Ad	$1,7*10^2$					-				-	-
	Bc 11/4h	$9,5*10^6$	strong detach				+				+	+
	Bc 11/4 Ad	$9,7*10^4$					-				-	-
9/3-10**	Bt48/3h	$2,8*10^6$	none				?				?	+
	Bt48/3h Ad	$6,5*10^3$					-				?	-
	Bt48/4h	$1,3*10^7$					-				-	-
	Bt48/4h Ad	$6,5*10^5$					-				+	+
8/10-09	Bt50/0h	$8.5*10^4$	none	+	+	+	+	-	(+?)	-	+	(+)
	Bt50/3h	$1.4*10^7$		+	+	-	+	+	(+?)	-	+	
	Bt50/3h AD	$9.6*10^5$		(+?)	(+?)	-	+	-	?	-	+	
22/10-09	Bt52/0h	$6.9*10^4$	none		-		-				(+)	-
	Bt52/3h	$5.7*10^6$			+		+				+	+
	Bt52/3h AD	$2.1*10^4$		-	+	-	(+)	-			+	
7 and 8/10-09	Bt53/0h	$1.1*10^5$	none	+	+	+	+	+	-	-	+	-
	Bt53/3h	$4.1*10^6$	Strong detach	+/nd	+/+	+/(+)	+/+	+/nd	(+?)/nd	-/nd	+/+	(+)/+
	Bt53/3h AD	$6.4*10^3$		-	-	-	-	-	-	-	(+)	-
8/10-09	Bt55/0h	$7.9*10^4$	none	+/+	+/+	+/+	+/+	+/-		+/+	+/+	(+)/-
	Bt55/3h	$1.1*10^7$	detach	-/-	-/+	-/-	-/-	-/-		-/-	-/-	-/-
	Bt55/3h AD	$5.2*10^5$		-/-	-/-	-/nd	-/nd	-/-		-/-	-/-	-/-

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3.8.7 Discussion of the Functional mammalian cell models

3.8.7.1 The effect of bacteria on Caco-2 cell morphology

The results for the effect of bacterial cell density on time for Caco-2 cell detachment indicate that if the tested ***Bacillus*** spp. is present in high enough numbers in the intestine they may be equally able to cause damage to intestinal epithelial cells (diarrhoea). However, if the bacteria are present in low numbers (as is probably more realistic) some of them may be less able to compete (perhaps with the other intestinal microflora) because they are slower, resulting in an inability to cause damage (diarrhoea). Generally the most virulent strains were ***B. cereus*** strain bc11 (diarrheal isolate) and ***B. thuringiensis*** bt53 (***B thuringiensis*** subsp. ***kurstaki,*** DIPEL), and the least virulent were bt50 and bt52 (***B. thuringiensis*** subsp**.** ***azaiwai*** (Agree) and ***B. thuringiensis*** subsp. ***israelensis*** (Vectobac)) in this assay. Interestingly strain bt53 shifted virulence potential at the very low infection dose, to a less virulent phenotype as compared to the other tested strains. The results show that it is very important to include the effect of dosage, when evaluating virulence potentials. Based on the results obtained, and in order to be able to differentiate the ***Bacillus*** spp. according to virulence potential, it was decided to use low bacterial cell densities in all of the functional mammalian cell assays.

3.8.7.2 Adhesion and Invasion/Microscopy assay and gene expression during infection

In the present study, there seemed to be no correlation between the number of adherent bacteria and morphological changes to the mucin covered HT29-MTX monolayer. As indicated earlier, the mucosal layer is the first line of defence towards pathogens of the gastrointestinal tract. According to Moncada et al (2003), the survival of pathogens in the gastrointestinal tract lies in their ability to colonize the mucus layer. According to Belley et al (1999) adhesion of a pathogen to the mucin layer can result in four types of interactions: 1) elimination of the adhered pathogen through sloughing and peristalsis, 2) colonization but the pathogen is denied access to the underlying epithelial layer, 3) colonization and expression of virulence factors, and 4) the mucus layer is penetrated and the pathogen invades and gets access to the epithelial cells. Results obtained in the present work showed that ***B. cereus*** strains bc11 and bc38 (food poisoning strains), ***B. thuringiensis*** bt55 (***B thuringiensis*** subsp. ***tenebrionis,*** NOVODOR) and especially ***B. thuringiensis*** bt53 (***B thuringiensis*** subsp. ***kurstaki,*** DIPEL) caused morphological changes to the HT29-MTX monolayer within the observation time. The results indicated that these particular strains produce enzymes that cause a relatively rapid degradation of the mucin layer. This ability may be an important indicator of virulence potential that indicates a potential for the more serious interactions (types 3 or 4) with the epithelial layer as suggested by Belley et al. (1999). While there was no clear correlation between the number of adhering cells and cell destruction in the HT29-MTX assay, there seemed to be a correlation between number of adhering cells and damage to the Caco-2 cells (no gel forming mucus). Thus, the strain that were least effective in adhering (bt52,) to the Caco-2 cell line was also the least destructive to the Caco-2 monolayer as observed by microscopy**.** The results indicate that the direct contact between bacteria and epithelial cells may increase the virulence. The results support the suggestion by Andersson et al. (1998) that adhesion capability of ***B. cereus*** (observed with Caco-2 cells) could be a virulence factor (more bacteria to produce virulence factors in close proximity to the cells). The results indicate that several of the strains may be virulent once the mucin

layer is compromised. The bacteria that were most virulent in both assays (Caco-2 and HT29-MTX) include strains bc11, bc38, bt53 and bt55. Strains bc11 and bc38 are both isolated from food poisoning outbreaks, while bt53 and bt55 are used as biopesticides.

While some ***B. thuringiensis*** strains produce parasporal toxins active against insects, then there are also some ***B. thuringiensis*** strains that produces cancer cell killing peptides (parasporins) during sporulation (Yasutake et al. ,2005; Yamshita et al., 2000). In the present study spores were not produced during the adhesion/invasion experiments (results not shown), and the virulence of bt53 and bt55 to the cancer cell lines was therefore probably not due to crystal toxin production (or production of any parasporins).

Regarding the ability of the bacteria to invade, our results for invasion capability to Caco-2 and HT29-MTX cells are similar to those obtained by Ramaro et al, (2006), who found that a ***B. thuringiensis*** and a ***B. cereus*** strain were poorly or not invasive to HeLa cells. In comparison, in the work by (Minnard et al., 2004) it was observed that 5 different ***B. cereus*** strains were invasive (between 1.72-4.86 $\log_{10}$ CFU/ml) to Caco-2 cells.

It was not easy to obtain gene expression data for the infection experiments (Caco-2 cell line) due to the low bacterial cell counts at the time of sampling. Furthermore the primers used for the detections may not have been equally efficient for all strains (e.g the nheB and inhA primers for the Bc11 strain), resulting in absence of gene expressions. These factors contribute to the fact that we are not able to reach any definite conclusions on the effect of virulence gene expression on the infection of mammalian intestinal cell lines. We did however observe that the virulence associated gene ***cytK*** was expressed by most of the ***Bacillus*** spp. during infection. The fact that we were able to detect expression of this gene may indicate that it is highly expressed during infection, and that this gene could be important for virulence of the ***Bacillus cereus*** group. One of the most virulent strains, the production strain bt53 was found to express all the tested virulence genes during infection of the Caco-2 cells, while one of the less virulent production strains bt50 also expressed all the genes except for ***hblC.*** This work is the first to detect virulence gene expressions of ***Bacillus cereus*** group species (by RT-PCR) during infection of an intestinal mammalian cell model.

3.8.7.3 Transepithelial electrical resistance measurements (TEER)

Transepithelial electrical resistance measurements have been widely used to estimate the effect of various microorganisms on the integrity of the epithelial barrier in ***in vitro*** model systems of the intestine. To our knowledge, no studies have been performed on the effect of ***B. cereus*** or ***B. thuringiensis*** on the TEER (intestinal barrier integrity) using ***in vitro*** intestinal cell models. In the present work two different mammalian intestinal cell lines were used (Caco-2 and HT29-MTX), and we observed that the tested ***Bacillus cereus*** group species strains decreased the TEER in a strain dependent manner. A recent work on the effect of ***B. cereus*** on TEER of an ***in vitro*** blood retina barrier was performed by (Moyer et al. 2008). In the work by Moyer at al. (2008) the results obtained for a plcR deficient mutant of ***B. cereus*** and its wild type, were similar to those obtained for the plcR deficient mutant of ***B. thuringiensis*** and its wildtype in the present study for the intestinal cell line Caco-2. Both the plcR mutant and the wild type caused a rapid decline in TEER (eventually followed by cell death), with the plcR deficient mutant decreasing the TEER slightly slower than the wild type (Moyer et al., 2008).

Our results confirm the suggestion of Moyers et al. (2008) that the decrease in TEER is likely to occur independently of plcR regulated toxins. Moyer et al (2008) showed that expression of the tight junction proteins ZO-1 and occludin (important for mammalian cell polarisation, i.e. a high TEER) decreased while the retinal cells were still viable; indicating that infection with ***B. cereus*** decreasing the TEER was not the result of the observed cell death. This may also be true for the infection of the intestinal cell lines in the present work, but needs to be investigated. In the work by (Minnaard et al. 2004) it was shown that strains of ***B. cereus*** caused alteration of the F-actin cytoskeleton (disassembly) in a strain and dose dependent manner. Further that the alteration was suggested to be a result of bacterial eukaryote contact (nothing happened when bacterial cell supernatant was added), Minnaard et al (2004). For another pathogen, ***Vibrio cholerae*** it has been shown that the production of hemaglutamin/protease resulted in perturbation of barrier function of kidney epithelial cells through affecting tight junctions and the F-actin cytoskeleton. In the assays performed in the present work ***B. thuringiensis*** strain bt53 showed to decrease the TEER the fastest, while ***B. thuringiensis*** bt52 decreased TEER the slowest. Based on the results obtained in the present work, we suggest that it is the speed for permeabilisation of the intestinal barrier that indicates the virulence potential. The results from the HT29-MTX assay are comparable to results from the other assays (detachment, adhesion/invasion), whereas the results from the Caco-2 assay deviates with regard to the intermediate virulent strains.

3.8.7.4 Mitochondrial activity of PoM2 exposed to Bacillus spp.

The mitochondrial function of PoM2 monocyte/machrophage cells was determined 22h post infection with various ***Bacillus*** spp. by use of the MTT-assay. It should be noted that, the system is static, and that the nutrients may be depleted during the 22 h because the bacteria grow, thus the macrophages may die from nutrient depletion (or from toxins/enzymes produced by the bacteria). Adding gentamicin 1 h post infection in order to kill the bacteria to prevent their growth may prevent an excessive enzyme/toxin production, as well as nutrient depletion. This method may provide a truer model system (mimicking a semi-dynamic system).

4 Comparison, discussion and conclusions on the evaluation potential of the models in relation to risk assessment

The overall aim of the project was to investigate the potential of three different models (***C. elegans, G. mellonella,*** and functional mammalian cell models) for evaluating the virulence of microbial pest control agents. In this work, ***Bacillus thuringiensis*** was used as a model organism; in particular we used the strains from the products Agree, Vectobac, Dipel and Novodor, representing four different subspecies of ***B. thuringiensis***, used as larvicides on various insect pests. Further, it was the aim of the project to compare these models to the models in current use for human risk assessment of microbial pest control agents. We will in this chapter first compare and discus the models and thereafter discus and conclude on their applicability in relation to risk assesmment of microbial pest control agents.

4.1 Comparison and overall discussion of the models.

Three different approaches were studied for the assesment of the ptotential virulence of bacterial plant protection agents, these approaches included the nematoide ***C. elegans***, the wax moth G. mellonella and functionel mammalian cell models, further were the ability of the bacteria to survive stomach conditions and to grow at mammal body temperatures and above that investigated.

The potential virulence and factors of importance for virulence was investigated by a comparative approach, where the four different ***B. thuringiensis*** strains were compared to carefully selected ***B. cereus*** and ***B. thuringiensis*** strains, which could be considered as positive and negative controls in relation to pathogenicity in and via the human gastro-intestinal tract. The approach used for the selection of the appropriate comparators existing of comparison to other strains and their origin by MLST and the ability to grow at 43°C, has generated a selection which according to the obtained results seems to be suitable for the approach. This comparative approach, using a broad array of strains consisting of strains from microbial pest control agents, assumed pathogenic strains (isolates related to food poisonings and somatic cases) and probiotic or strains attenuated in their virulence potential, and comparing them in the three different modelssystems is evidently the strength of this project.

In the present work we were aware of the fact that several factors will influence the virulence potential/the ability to cause gastro-intestinal disease. The basis for a spore-forming bacterium to cause gastro-intestinal diseases, as diarrhea, is illustrated in Figure 4.1.

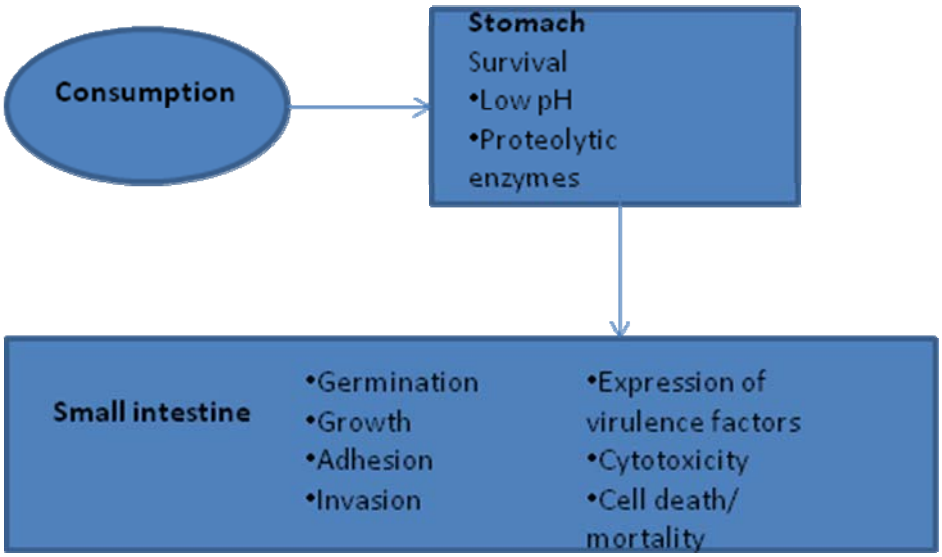

Figure 4.1. Some of the basics for spore-forming bacteria to cause gastro-intestinal diseases.

The first assumption is indeed that the spore and or vegetative cell need to be consumed in some kind of food, the next is that it is able to survive the acidic conditions and the proteolytic enzymes in the stomach, and enter the small intestine and here survive the activity of e.g. bile acids and pancreatic enzymes. In the small intestine the spore has to germinate, the resulting vegetative cell has to multiply and express virulence factors in concentrations, which are able to damage the cells and eventually cause damage leading to cell death. In this project we have studied models for the survival of spores/vegetative cells in conditions resembling the stomach (low pH and pepsin) and the small intestine (bile acids), and germination was studied in ***G. mellonella*** larvae. ~~A~~dhesion, invasion and effect on the integrity of the intestinal epithelial barrier was studied in different mammalian cell-models, the expression of selected virulence factors ***in vitro*** and ***in situ*** in ***G. mellonella*** and the Caco-2 intestinal cell model. Cytotoxicity was studied with three different mammalian cell lines and the virulence in two different invertebrate models. Our results are summarized in Table 4.1 and Table 4.2. Table 4.1 summarizes data which characterize the strains, as growth at different temperatures, survival at simulated stomach and gut conditions, and presence/expression of different enterotoxin genes; while Table 4.2 summarizes the results from the different virulence models. On the basis of all the results shown in Table 4.1and Table 4.2 a cluster analysis was performed to make a direct comparison between all the results possible; the assumption of this analysis is that all results are considered equal counting with the same weight and independent. The result of this analysis is shown as a dendrogram in. It appears from Figure 4.2, that the strains is divided into two main clusters, one existing of the Bt50 and Bt52 together with the two control strains (Bt48 and Bc49), the remaining strains are divided into two subclusters existing of B38, Bc14 and Bc25; and Bt53, Bc11 and Bt55, respectively.

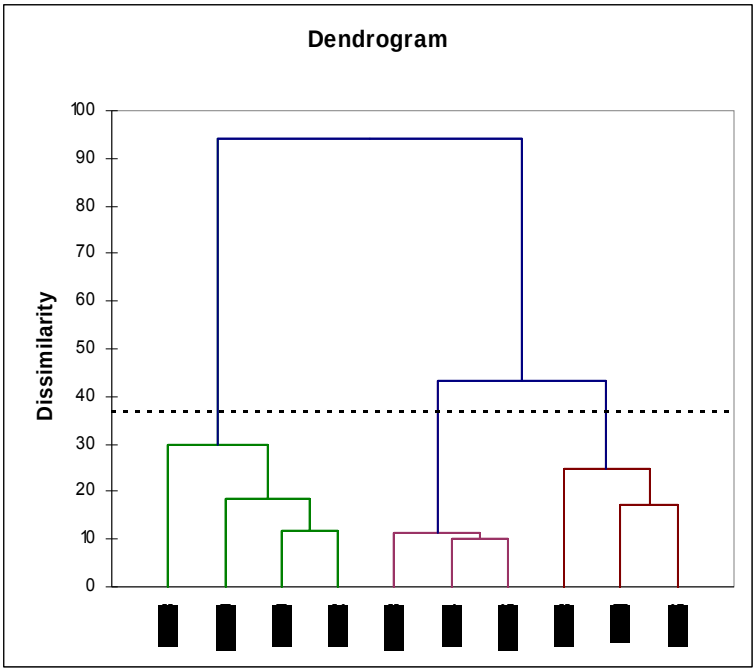

Figure 4.2. Dendrogram based on a cluster-analysis of the results summarized in Table 4.1 and Table 4.2. The cluster- analysis is based on agglomerative hierachial clustering with the dissimilarities estimated as Euclidian distances and Wards method used for the agglomeration. The establishment of statistical significant different classes is based on variance decomposition analysis. The analysis was performed with the help of the statistical package XLSTAT 2010, 5.07.

It appears from these two tables and the cluster analysis, if the four strains from the microbial pest control products (Bt50, Bt52, Bt 53, Bt 55) are compared mutually and with the four strains from contaminated food, blood and a gastrointestinal case (Bc11, Bc14, Bc25, Bc38) and the negative control (Bt48) and the strain from a probiotic (Bc49), that:

- Bt50 and Bt52 the ***B. thuringiensis aizawai*** and ***israelensis*** strains from Agree and Vectobac behave in many ways similar in the different models. They have limited growth at 43°C, survives well at the stomach conditions, affects to different extent the Caco-cells, and to a lesser extent the HT-29 MTX cells, and has a limited effect on the PoM2 cells after 22 hours (if gentamicin is added 1 h post infection), they have some, but low, effects on ***Galleria*** and the nematode ***C. elegans***. From an overall point of view are these two strains more comparable with the negative control strains than with the positive controls. They seem therefore to have a pathogenic potential which are lower, than the strains from pathogenic cases, especially the two strains isolated from blood and a gastrointestinal case (Bc25 and Bc38).
- Bt53 and Bt55 the ***B. thuringiensis kurstaki*** and ***tenebrionis*** strains from Dipel and Novodor behave in many ways also similarly in the models. They grow at 43°C, are affected by the stomach conditions, they negatively affect all of the mammalian cell lines, and ***Galleria***, while different effects on the nematode ***C. elegans*** are observed, as Bt55 has

a considerable negative effect on the nematodes, while Bt53 has a much lesser pronounced effect. From an overall point of view are these two strains more comparable with the strains from the pathogenic cases (food poisoning and somatic), than with the negative controls. They seem therefore to have a pathogenic potential which does not differ from the potential of the strains from the pathogenic cases. Their main difference from the “non pathogenic” strains (bt48, bc49) is that they are affected by the acidic stomach conditions.

Oral pathogenecity of ***B. thuringiensis*** have previously been investigated by the use of rodents, notably rats. In such studies has the microorganism been given as an oral dosage, often in high densities, the effects on the rats as survival followed through app. three weeks, follwed by post mortem examination after this time period (Bishop et al., (1999), Wilcks et al. (2006)). Further has a number of microbial pest control agents, including pesticides based on ***B. thuringiensis***, been assesed for oral toxicity by corresponding procedures, in connection with applications for approval of the specific plant protection product. In general no adverse effects have been observed in these studiesThis is in contradiction to the results presented here, where pronounced effects of bacteria from two plant protection products on ***C. elegans***, ***Galeria*** and on different mammal cells have been observed. This might be caused by substantial differences in the sensitivity for potential virulence factors involved in oral pathogenecity between the rodents and the “models” studied in this project,. Based on experience from experimental analysis it has been concluded that rodents are unsuitable for testing the safety to humans of oral exposure (Bishop et al., (1999), Wilcks et al. (2006a,b)). Thus the approaches analysed in this project seems to be mmore sensitive and discriminatory than the rodent-models often used in this connection and generally used in the risk assesmment of microbial pest control agents.

However, it is in this connection important to remember that a potentially pathogenic bacterial strain not necessarily will cause illness, if it is consumed by a human. This will be dependent on several factors, such as the total number and density of the spores consumed, the food items they are consumed in connection with, the fate and behaviour of the bacterium within the human gastro-intestinal system and the health status of the consumer. All these factors were not studied as parts of this project.

Table 4.1 Characterization of studied *B. cereus* and *B. thuringiensis* strains: *in vitro* growth at different temperatures and in stimulated stomach conditions and presence/expression of enterotoxin genes.

Strains	N° in the project	Bc11	Bc14	Bc25	Bc38	Bt48	Bc49	Bt50	Bt52	Bt53	Bt55	Bt407 Cry-
	Original name	MADM12 91	MAD M1561	B-05	B4-ac	(Bt407 Cry- ΔplcR)	*Toyoi*	*Aisawai*	*isralensis*	*kurstaki*	*tenebrionis*	
	Cluster (Guinebretières,)	No fit	III	IV	IV	III (?)	III	IV	(IV)	IV	(IV)	III(?)
	Origins/function	Contaminated food		Blood	Gastro intestin	Neg control	Pro- biotic	Product strains				Positive control
								Agree	Vectbac	Dipel	Novodor	
GROWTH												
Temperature tolerance (growth at)	43°C micro aerob	1	5	5	5	1		1	1	4	3	nd
	30°C micro aerob	2	4	5	5	3		3	3	5	4	nd
	10°C micro aerob	5	5	5	5	5		5	3	5	5	nd
Stomach (survival)	Acid pH 2	5	5	5	5	5		5	5	**2**	**3**	nd
	Bile salt (0.3g/L)	4	3	4	4	5		4	4	4	4	nd
Growth rates in LB-broth at 37C	**µmax (doublings/h)**	3.7	5.3	3.5	3.1	2.5		2.2	2.7	1.4	2.8	nd

ENTEROTOXINS												
Strains		Bc11	Bc14	Bc25	Bc38	Bt48	Bc49	Bt50	Bt52	Bt53	Bt55	Bt407
Presence and expression of « enterotoxins »	Enterotoxins	NheB = N				HblC=H		CytK = C		HlyII=h		
		N H C h	N H C h	N H C h	N H C h	N H C h	N H C h	N H C h	N H C h	N H C h	N H C h	N H C h
	Presence	+ - + +	+ - + +	+ + + -	+ + + -	+ + + +	+ + + -	+ + + -	+ + + +	+ + + +	+ + + +	+ + + +
	Expression *in vitro*	+ - + +	nd	nd	nd	+ + + +	+ + +	nd	+ + + +	+ - - -	nd	+ + + +
	Expression *in vivo Gm*	+ - + +	nd	nd	nd	low +	+ + nd	nd	nd	+ + + +	nd	+ + + +
	Expression *in vivo Caco2*	+ - + +	+ - + +	nd	nd	low +	+ + nd	nd	+ + + +	nd	nd	+ + + +

Values : 5 is highest growth , 1 is lowest 0 = no effect; + = presence, - =absence of genes or expression, nd= not determined

Table 4.2 Summarized results from the studied virulence models exposed to *B. cereus* (Bc) and *B. thuringiensis* (Bt) strains

Strains	N° in the project		Bc11	Bc14	Bc25	Bc38	Bt48	Bc49	Bt50	Bt52	Bt53	Bt55	Bt407 Cry-
	Original name		MADM1 291	MADM1561	B-05	B4-ac	(Bt407 Cry- ΔplcR)	*Toyoi*	*Aisawai*	*israelensis*	*kurstaki*	*tenebrionis*	
	Cluster following (Guinebretières et al. 2008, 2010))		No fit	III	IV	IV	III (?)	III	IV	(IV)	IV	(IV)	III (?)
	Origins/function		Contaminated food		Blood	Gastro intestin	Neg control	Pro- biotic	Product strains				Pos control
									Agree	Vectbac	Dipel	Novodor	
CELL LINES													
Caco2 Intestinal (Human)	TEER		3	nd	4	4	3	4	4	3	5	4	3
	Adhesion		4	nd	5	4	4	nd	5	3	4	5	nd
	Detachment/cytotox		4	nd	4	4	2	nd	2	0	5	4	nd
HT-29 MTX Intestinal Mucus (human)	TEER		4	nd	3	3	4	nd	3	2	5	3	nd
	Adhesion		5	nd	5	4	5	nd	4	3	4	5	nd
	Invasion		1	nd	1	0	0	nd	1	0	2	0	nd
	Detachment		4	nd	0	4	0	nd	0	0	5	4	nd
PoM2 Macrophages (porcine)	Cytotox/mitochondial activity Gentamycin after 22 h		2-5	nd	5	5	3	0	4-5	4-5	5	5	5
PoM2 Macrophages (porcine)	Cytotox/mitochondial activity Gentamycin after 1 h		3	nd	0-2	0-2	3	1	0-1	0-1	4-5	3-4	3
INVERTEBRATE MODEL													
Galleria (Gm) insects larvae mortality at 37°C	*In vivo* Spore germination (3H)		4	4	5	4	5	nd	1	3	5	2	3
	Oral Cry1C+	spores	3	3	3	4	1	3	nd	3	5	4	4
		vegetative	5	5	5	4	2	nd	nd	4	5	5	5
	Hemocoel spores $1x10^4$		3	5	4	5	3	4	2	3	4	3	4
	Hemocoel vegetative $1x10^3$		5	5	3	4	2	2	2	2	2	2	2
	Hemocoel vegetative $1x10^4$		5	5	4	5	2	4	4	3	2	3	5
C.elegans nematode	Virulence /mortality		2	2	3	3	2	nd	1	2	2	5	nd

Values : 5 is highest viruelnce ; 1 is lowest; 0= no effect; nd= not determined

4.2 Conclusions with regard to risk assesment

With regard to the use of the results from the project in relation to risk assessment of microbial pest control agents we conclude, that:

- Generally the models show good discriminatory power, which is superior to those known from animal models. Therefore these models may provide a good alternative to already existing models.
- Studies on the pathogenic potential of strains used as microbial pest control agents should be carried out by a comparative approach, where the strain are compared to other strains including strains which could be regarded as positive and negative controls Based on the tested strains we would suggest Bc11 and Bc38 as positive controls (most virulent) and Bt48 or the probiotic strain Bc49 as negative controls, at least in respect to ***in vitro*** and ***in vivo*** infection models.
- Evaluation of a biological pest control agent cannot be performed by use of a single assay, due to the complexity of factors affecting the virulence potential. For example the production strains Bt53 and bt55 showed the highest virulence potential in most of the models. However at the same time these particular strains were less likely to survive passage of the stomach acidity (which will probably depend on the food matrix) as compared to some of the less virulent strains. Thus important initial factors to be analyzed, include the ability of the strain to grow at human/mammalian body temperature (37°C) and its ability to survive the conditions in the stomach (low pH).
- The functional mammalian assays are very sensitive and their use is dependent on the availability of the assays and expertise in relation to their use and the interpretation of the results. The choice of a certain functional mammalian assays to use in the risk assessment will be dependent of the availability of models and expertise in relation to the specific strain to be analysed. The three cell lines and approaches tested in this project are all relevant, but from a practical point of view (reproductibility and the less subjective), it might be that cytotoxicity test would the best choice.
- The ***Galleria*** and ***C. elegans*** models seem both to be simple and effective for studies on virulence. These models are quite easy to perform and analyse, however they are also dependent on expertise in relation to their use and the interpretation of the results.

For strains which have a potential for causing illness, it is important to perform an exposure analysis for humans. This analysis should take the use of the microbial pest control agents and knowledge about residuals as the starting point.

The conclusion from the obtained results are that we have shown a correlation between the models, meaning that single cell models (cell cultures) and whole animals models (***Galleria*** and nematode) generate similar results related to virulence potential. Interestingly the tested biocontrol ***B. thuringiensis*** strains

fall into two groups of virulence levels. A level which was also found in pulmonary infections of mice, showing that Bt ***kurstaki*** (Bt53) was significantly more virulent than Bt ***israelensis*** (Bt52) (Hernandez et al.,1999). Thus we can conclude that with at least these two strains a relationship between the results obtained with mice and the models tested in this project exist, although the routes of infection were quite different. This might suggest that the use of higher animal models are not needed in routine but can be replaced by several and less ethical and costly approaches as those used in this project. So we porpose to use a tiered approach for the human risk assesment of microbial pest control agents, starting with simple cell- models, using invertebates as a next step and finally to include rodents, notably to investigate clearance time and the occurrence in different tissues.

5 Perspectives

The models evaluated in the present work show good promise as alternatives to the existing rodent models, by providing a better discriminatory power. In the present work we evaluated the effect of oral exposure (all three models), and to some extent the effect if the bacteria would enter the blood stream (***G. mellonella***). Thus we investigated primarily oral exposure; the usefulness of our models and other simple models for the study of other routes of exposure needs to be investigated.

Our results are in line with a very recent review on toxin production and food safety implications of ***B. cereus*** (Ceuppens et al., 2011). They conclude" Toxin expression is subject to complex regultation which is still far from completely elucidated. Beside the ingfluence of numerous environmental parameters on toxin expression there is also huge strian dependent variability under identical circumstances. The diffence between strains with high and low toxin expression remains obscure, despite numerous worthy efforts to comprehernd the ***B. cereus*** genome and its variability. Future research should consist of experiments conducteds in relevant food matrices or intestinal environments and take into account that toxin production is not always proportional to growth." These general conclusions apply also to studies with ***B. thurnigiensis*** strains from microbial pest control agents.

The conclusions of the present work are based on single culture (bacterial) interactions with the hosts (mammalian cell models, ***G. mellonella, C. elegans***) and we do not know what would happen in the more competitive environment of the gastrointestinal tract where many other bacteria are present at high densities, in total 10^{12}./g. Although in the case of ***Galleria*** there is a natural intestinal flora composed of mainly Enterococcus species (Jarosz, 1979). Further, we conclude that use of a combination of several models is necessary in order to cover the complexity of factors affecting the virulence potential. E.g. it is important to know the ability to survive the first barriers of the gastro-intestinal tract (acid and bile) as well as the effects on the intestinal epithelium. All in all, we suggest that further supportive studies could include a full scale experimental model resembling the human gastro-intestinal tract, consisting of different adjacent compartments: 1) saliva amylase, 2) hydrochloric acid + enzymes, 3) bile, 4) model intestine with an intact human intestinal microbiota to confirm and add to our results obtained with the various models. We believe that the models tested in this work are more simple and easy to handle (allows investigation of a high number of strains), than a full scale gastro-intestinal model, which therefore should not be considered as an alternative.

Further we also realise the importance of exposure. Since all the mesophilic ***B. cereus*** group species seem to be able to potentially cause damage/disease it is important to know more about human exposure for these bacteria and their infective dose, especially for the strains used in microbial pest control agents. Exposure analysis needs to be based on knowledge about their use, fate in the environment and in food.

In the current risk assessment scheme, waivers for oral exposure studies can be accepted if the organism of interest does not grow at human body temperatures, does not produce any known human pathogenic toxins and/or no exposure to consumers is anticipated. So one way to get forward is maybe to expand the range of arguments where waivers for animal studies can be accepted. Besides the temperature, the ability to survive under low pH and bile acid conditions could be a first easy experiment to perform and assess. If the strain is very sensitive to both low pH and bile acids, the strain will have hard conditions to survive to the intestine and cause disease, and thereby oral studies could be waived.

5.1 Research perspectives

Although the models investigated in the present work have been applied to various pathogenic viruses and fungi by others (e.g. Botic et al., 2007; Ivec et al., 2007; Frank and Hostetter, 2007), the models applicability for viruses and fungi used as microbial pest control agents need to be confirmed and tested in practice. Thus to be sure about the possibility to use the here tested virulence models more research and specific projects are needed. In accordance with the above conclusions, more research are needed to confirm the usefulness of the models with other Bt/Bc strains and the addition of more less time consuming ***in vitro*** approaches mimicking the passage of stomach and intestine are needed. In the aim to propose new guidelines it is also important to consider in details the correlation between dose and the speed (time) of activity. Another aspect considered in this project was the presence and expression of the enterotoxins (Hbl, Nhe, CytK) and the recently proposed toxin HlyII in the tested strains. These toxins are so far considered as indicators for risk of at least Bc/Bt related intestinal diseases and we found that these genes where present in most of the strains although Hbl was absent in two Bc strains associated with contaminated food and HlyII was absent in the two Bc strains associated with human infections which also turned out to be virulent. Meanwhile more research is needed to determine a larger panel of PCR primers to detect the specific genes, in which small variation in the sequence might results in faults conclusions. Also our studies and results related to expression of virulence factors measured by RT-Q-PCR ***in vivo*** merits more attention from a research point of view. Indeed, it was interesting to observe the modulation of expression virulence factors occurred even within a specific regulon (PlcR), while comparing ***in vitro*** and ***in vivo*** conditions, indicating that the presence of a gene is not necessary conducting to expression. Thus to consider the variation in expression, more research on ***in vitro*** system micking ***in situ*** intestinal conditions using relative Q-RT PCR could be of interest as recently shown by Cadot ***et al.*** 2010 for HlyII and two metalloproteases. Finally the use of antibodies to detect for production/ quantification of virulence factors also needs more research and development, since the actual detecting kits are more or less efficient and are only detecting one compound in each of the Hbl and Nhe toxin complexes.

6 References

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7 Vocabulary and abreviations

Table 7.1

Word/abbreviation	Explanation
Aerobic conditions	Oxygen rich
ANOVA	analysis of variance
BCET-RPLA toxin detection kit	Commercial kit to detect subunit L2 from HBL
BHI	Brain Heart Infusion (growth media)
BHIG	BHI supplemented with 1% glucose
Caco-2	A human colon adenocarcinoma cell line
cDNA	Complementary DNA. cDNA is single-stranded DNA made from a messenger RNA template
CFU:	Colony forming units
cytK	Cytotoxin K
DMEM	Dulbecco´s modified eagle medium
DMSO:	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH
EFSA	European Food Safety Authority
FBS	Fetal Bovine Serum
Galleria mellonella :	A moth called the “greater wax moth” which is a pest of honey combs. The larval form is used for infection in this study.
HBL	Haemolysin BL (*B. cereus* enterotoxin)
hblA	Hemolysin A, component of tripartite enterotoxin in *B. cereus*
hlyII	hemolysin
HT29-MTX	Mucus secreting cell line of human carcinogenic intestinal origin, is used to study the effect on physical barrier (mucus)
ilsA	Iron-regulated leucine rich surface protein
InhA2	Zinc metalloprotease
LB	Luria-Bertani (growth media)
LD50	Bacterial dose which kills 50 %
MEM	Minimal essential medium
Micro aerobic conditions	Oxygen poor
MLST	Multi locus sequencing typing
MQ-water	Milli-Q water
MRD	Maximum Recovery Diluent
mRNA	messenger RNA
MTT	Methylthiazolyldiphenyl-tetrazolium bromide
NEAA	non essential amino acids
NGM	Nematode Growth Medium

nheB	Non haemolytic enterotoxin B, component of tripartite enterotoxin in *B. cereus*
OD	Optical density
PAS	Peracetic acid
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PI	Propidium iodide
PoM2	PoM2 cells is a monocyte/macrophage cell line established from the peripheral blood of a pig
Probiotic bacterium	Beneficial bacterium, like lactic acid bacteria, which act in the gut and increases the host health
Q-PCR	Quantitative PCR
Q-RT PCR	Quantitative reverse transcriptase polymerase chain reaction. Serve to quantify gene expression. Is based on extraction of mRNA, copied to cDNA .by reverse transcriptase and which is used for relative quantification of geneexpression
RNA	RiboNucleic Acid
RPM:	Rotations per minute
RT-PCR	Reverse transcriptase PCR
RTV	Real time viewing
SGF	Simulated gastric fluid
SYBR Green	SYBR Green is a fluorescent dye that binds to double stranded DNA
TEER	Trans epithelial electrical resistance

8 Appendices

Appendice 1

TABLE 8.1 Initial strain list with potentially positive controls

Consecutive strain number	Internal DMU strain number	Original Strain name	Origin	Reference or source
1	Bt1258	F284/78	Pork pie 1978	Fletcher and Logan, 1999
2	Bt1259	(F4370/75	Grilled Chicken. Canada 1975	Guinebretière et al, 2002
3	Bt1186	(NVH 391-98)	Produce CytK	Fagerlund et al. BMC Mocrobiol.(43) :1-8
4	Bt1260	(1651-00)	Caramel pudding, 2000	INRA
5	Bt1261	98hmpl63	Salsifis FR 2000	INRA
6	Bt1262	NVH 0075-95	Gastro-intestinal	INRA
7	Bt1263	NVH80	Gastro-intestinal	INRA
8	Bt1264	LMG 17605	Fast-food 1977	
9	Bt1265	LMG 17604	Chinese pancake 1976	Guinebretière et al, 2002
10	Bt 667	MADM 1282	Vanilla profiterole	Marilena de Muro, Brazil
bc 11	Bt 676	MADM 1291	Birthday cake	Marilena de Muro, Brazil
12	Bt 689	MADM 1552	Boiled potatoes	Marilena de Muro, Brazil
13	Bt 695	MADM 1558	Polenta	Marilena de Muro, Brazil
bc14	Bt 698	MADM 1561	Cooked chicken	Marilena de Muro, Brazil
15	Bt 713	MADM 1576	Uncooked chicken	Marilena de Muro, Brazil
16	Bt 721	MADM 1584	Boiled rice	Marilena de Muro, Brazil
17	Bt 729	MADM 1592	Savoury crêpe	Marilena de Muro, Brazil
18	Bt 730	MADM 1593	Uncooked chicken	Marilena de Muro, Brazil
19	Bt 731	MADM 1594	Boiled potatoes	Marilena de Muro, Brazil
20	Bt 747	MADM 157/1	Boiled rice	Marilena de Muro, Brazil
21	Bt 1198	B-01	Blood, Patient 4	Gaur et al., 2001
22	Bt 1199	B-02	Blood, Patient 5	Gaur et al., 2001
23	Bt 1200	B-03	Blood, Patient 6	Gaur et al., 2001
24	Bt 1201	B-04	Blood, Patient 7	Gaur et al., 2001
bc25	Bt 1202	B-05	Blood, Patient 8	Gaur et al., 2001

26	Bt 1203	B-06	Blood, Patient 9	Gaur et al., 2001
27	Bt 1204	B-07	Blood, Patient 10	Gaur et al., 2001
28	Bt 1205	B-08	Blood, Patient 1	Gaur et al., 2001
29	Bt 1207	B-10	Blood, Patient 11	Gaur et al., 2001
30	Bt 1208	B-11	Blood, Patient 12	Gaur et al., 2001
31	Bt 1210	B-13	Blood, Patient 2	Gaur et al., 2001
32	Bt 1211	B-14	Blood, Patient 3	Gaur et al., 2001
33	Bt 1215	B-18	Blood, Bacteraemia	St Jude
34	Bt 1224	B-27	Blood, Bacteraemia	St Jude
35	Bt 1227	B-30	Blood, Bacteraemia	St Jude
36	Bt 1228	B-31	Blood, Bacteraemia	St Jude
37	Bt 1239	B-42	Blood, Sepsis, Haemolysis	St Jude
bc38	Bt 642	B4-ac	Gastro-intestinal	Agata et al., 1995
39	Bt 156	ATCC 10987	Genetic similarity to pathogens	ATCC
40	Bt 160	ATCC 14579	*B. cereus* Type strain	ATCC
41	Bt 163	F4810/72	Emetic	Turnbull et al., 1979
42	Bt 164	F837/76	Not Clinical, but high HBL expression	Beecher et al., 1995
43	Bt 165	F2038/78	Clinical isolate	Fletcher and Logan, 1999
44	Bt 169	ATCC 7064	Blood	ATCC
45	Bt 627	F4433/73	Gastro-intestinal	Fermanian et al., 1997
46	Bt 983	Nc 7401	Emetic	Agata et al., 1994

St Jude): St. Jude Children´s Research Hospital, Mail stop 600, 332 N Lauderdale St. Memphis, TN 38105. **ATCC**): American Type Culture Collection, www.atcc.org

Table 8.2 Initial strain list with potentially negative controls

Consecutive strain numbers	Internal DMU strain number	Original Strain name	Origin	Reference or source
47	Bt 651	BCIP 5832	Probiotic	From Bactisubtil, Mazza et al., 19994
bt48	Bt 959	Bt 407 PlcR	Inactivated PlcR regulator	Salamitou et al., 2000
bc49	Bt 1254	*B. cereus* var. *toyoi* (CNCM I-1012/NCIMB 40112)	Probiotic	A free sample from Rubinum, Spain

Table 8.3 Initial product strain list

Consecutive strain numbers	Internal DMU strain number	Product name	Serotype	Original Strain name	Reference or source
bt50	600	Agree 50WP	*aizawai*	GC-91	Certis
51	591	Xentari	*aizawai*	ABTS-1857	Valent BioSciences
bt52	1253	Vectobac-12AS	*israelensis*	AM 65-52	A free sample from Borregaard, Bioplant, DK
bt53	1255	Dipel	*kurstaki*	ABTS-351	Valent BioSciences
54	1191	Gl. HD-1 standard	*kurstaki*	HD-1	From Christina Nielsen-LeRoux
bt55	1256	Novodor	*tenebrionis*	NB-176	Free sample from "Andermatt-Biocontrol AG", Switzerland

Appendice 2

Growth at 30 and 43°C in liquid and solid media.

The results for qrowth in liquid media are listed in Table 8.4, and as it can be observed, do most of the strains grow well at both temperatures and under both oxygen conditions. In general, the strains used in plant protection products, grow less well at 43°C and under micro aerobic conditions, which could indicate that the commercial strains are less well adapted to survive in the human body. Results for growth on solid media is shown in Table 8.5, in general there is a good agreement between the results from the growth studies in liquid and solid media. The strains growing less well in liquid media also give smaller colonies on solid media. Some strains (e.g. 43, 47 and 52) do not grow up at all on solid media, whereas in liquid media growth, although poor, is observed. This could be due to differences in media used, and in general bacteria grow better in liquid than on solid media. The results from the temperature screening, where together with the MLST data and the history of the strains, used to select the ten strains used in further experiments.

Overall conclusion: In general, the strains used in plant protection products, grow less well at 43°C and under micro aerobic conditions, which could indicate that the commercial strains are less well adapted to survive in the human body.

Table 8.4 Growth of 55 strains at 30 and 43°C under aerobic and micro aerobic conditions in broth. Growth was scored after 24 hrs incubation.

Strain	43°C aerobic	43°C micro aerobic	30°C aerobic	30°C micro aerobic
F284/78 (1)	2/3	1/1	1/2	3/3
F4370/75 (2)	2/2	1/1	1/1	1/3
NVH 391-98 (3)	2/2	3/2	2/2	2/3
1651-00 (4)	2/2	2/1	1/2	2/2
98hmpl63 (5)	2/2	2/3	1/2	2/3
NVH 0075-95 (6)	3/3	2/3	2/2	2/2
NVH80 (7)	3/3	2/3	2/3	2/2
LMG 17605 (8)	2/3	2/3	2/2	3/3
LMG 17604 (9)	2/1	(1)/1	2/2	2/2
MADM 1282 (10)	3/3	3/3	2/3	2/2
MADM 1291 (11)	1/2	(1)/1	2/2	1/3
MADM 1552 (12)	1/2	3/3	1/2	3/3
MADM 1558 (13)	2/2	3/3	2/3	3/2
MADM 1561 (14)	2/2	3/3	3/3	3/2
MADM 1576 (15)	2/2	3/3	1/2	3/3
MADM 1584 (16)	2/2	3/3	1/2	3/3
MADM 1592 (17)	2/2	2/2	1/2	3/3
MADM 1593 (18)	2/2	3/3	1/2	3/3
MADM 1594 (19)	2/2	3/3	1/2	2/3
MADM 157/1 (20)	3/3	3/3	1/2	2/3
B-01 (21)	3/3	3/3	2/3	3/3
B-02 (22)	3/2	3/3	2/2	3/3
B-03 (23)	3/2	3/2	2/3	3/2
B-04 (24)	3/3	3/3	1/2	3/3
B-05 (25)	2/2	3/3	1/2	3/3
B-06 (26)	3/3	3/2	3/3	1/2

B-07 (27)	3/2	3/2	2/3	2/2
B-08 (28)	1/1	1/(1)	1/1	1/1
B-10 (29)	2/2	3/2	1/2	2/3
B-11 (30)	2/3	2/2	3/3	2/2
B-13 (31)	3/3	3/3	3/3	1/3
B-14 (32)	3/3	3/3	1/2	3/3
B-18 (33)	2/2	1/2	1/2	3/1
B-27 (34)	2/2	1/2	1/2	1/1
B-30 (35)	1/2	1/1	1/2	(1)/(1)
B-31 (36)	3/2	2/1	1/1	3/1
B-42 (37)	3/3	2/3	3/3	1/3
B4-ac (38)	1/2	1/3	1/3	3/3
ATCC 10987 (39)	3/3	2/3	1/2	3/3
ATCC 14579 (40)	2/3	2/3	1/2	2/3
F4810/72 (41)	3/2	2/1	3/1	3/2
F837/76 (42)	1/1	2/2	1/1	3/2
F2038/78 (43)	(1)/1)	1/-	1/1	1/3
ATCC 7064 (44)	1/1	1/1	1/1	1/1
F4433/73 (45)	2/2	2/2	1/1	3/1
Nc 7401/2455 (46)	3/3	2/3	3/3	3/3
BCIP 5832 (47)	1/(1)	-/-	1/1	1/1
Bt 407 ΔplcR (48)	2/1	-/1	3/3	2/3
Agree 50 WP (50)	2/2	-/2	2/1	1/3
Xentari (51)	1/2	1/(1)	1/2	2/3
Vectobac 12AS (52)	(1)/3	1/-	1/2	2/3
Dipel (53)	3/3	3/1	3/2	3/3
Gl.HD1 standard (54)	2/2	-/(1)	1/(1)	-/-
Novodor (55)	1/2	2/1	1/3	2/3
Negative control	-/-	-/-	-/-	-/-

* No growth: -; poor growth: 1; normal growth: 2; high growth: 3. Food borne isolates: yellow; Disease causing isolates: red; Commercial strains: green; Probiotic strains: blue. The experiment was performed twice.

Table 8.5 Sizes of bacterial colonies grown on solid substrate

Consecutive strain numbers	Internal DMU strain number	43°C incubator			30°C incubator			30°C §
		19/8	20/8	21/8	19/8	20/8	21/8	22/8
1	Bt1186	9	16	23	+	9	12	
2	Bt1259	1	1	2	+	8	12	
3	Bt1186	8	13	17	+	12	18	
4	Bt1260	8	13	17	+	12	17	
5	Bt1261	11	19	27	+	13	21	
6	Bt1262	10	16	23	+	8	11	
7	Bt1263	9	15	19	+	10	14	
8	Bt1264	10	17	24	+	14	21	
9	Bt1265	8	12	20	+	12	19	
10	Bt667	7	13	18	+	9	12	
bc 11	Bt676	9	19	38	+	17	25	
12	Bt689	9	18	26	+	13	18	
13	Bt695	8	14	24	+	13	21	
bc14	Bt698	11	22	42	+	18	27	
15	Bt713	13	28	35	+	16	27	
16	Bt721	9	16	24	+	17	24	
17	Bt729	8	15	22	+	7	11	
18	Bt730	12	22	31	+	10	15	
19	Bt731	9	16	21	+	14	19	

Consecutive strain numbers	Internal DMU strain number	43°C incubator			30°C incubator			30°C §
		19/8	20/8	21/8	19/8	20/8	21/8	22/8
20	Bt747	10	17	24	+	16	23	
21	Bt1198	15	26	39	+	17	26	
22	Bt1199	13	24	34	+	14	23	
23	Bt1200	14	26	36	+	17	26	
24	Bt1201	17	30	45	+	17	25	
bc25	Bt1202	15	26	37	+	18	26	
26	Bt1203	7	12	17	+	8	11	
27	Bt1204	14	24	36	+	11	18	
28	Bt1205	13	26	35	+	18	26	
29	Bt1207	7	13	16	+	7	11	
30	Bt1208	7	13	18	+	10	18	
31	Bt1210	6	11	15	+	9	11	
32	Bt1211	8	18	28	+	19	27	
33	Bt1215	11	21	29	+	18	26	
34	Bt1224	10	19	27	+	18	27	
35	Bt1227	10	18	27	+	24	39	
36	Bt1228	7	14	20	+	8	12	
37	Bt1239	7	12	17	+	7	11	
bc38	Bt642	11	21	27	+	14	22	
39	Bt156	8	15	23	+	11	15	
40	Bt160	7	16	22	+	9	14	
41	Bt163	12	21	29	+	11	15	
42	Bt164	2	6	11	+	6	-	
43	Bt165	0	0	0	+	9	12	5
44	Bt169	9	16	24	+	24	32	
45	Bt627	9	17	23	+	11	17	
46	Bt983	12	21	29	+	20	33	
47	Bt651	0	0	0	+	18	25	6
bt48	Bt959	2	5	10	+	11	16	
bc49	Bt1254	11	20	26	+	10	14	
bt50	Bt600	0	7	12	+	18	25	
51	Bt591	0	2	7	+	12	17	
bt52	Bt1253	0	0	0	+	15	22	6
bt53	Bt1255	7	15	24	+	17	25	
54	Bt1191	1	7	13	+	12	18	
bt55	Bt1256	0	0	0	+	11	17	7

-: No measurement available
+: Growth observed
§: Where no growth was observed after 3 days at 43°C, Petri-dishes were incubated at 30°C for 24 hours as an inoculation control
Green: Colony is larger than 25 mm
Yellow: Strains having larger colonies at 43°C than at 30°C
Blue: Strains having smaller colonies at 43°C than at 30°C
Pink: Strains having similar colonies at 43°C and at 30°C

Appendice 3

Sequence types

MLST analysis

The seven household genes to be analysed

glpF (glycerol uptake facilitator protein),
gmk (guanylate kinase, putative),
ilvD (dihydroxy-acid dehydratase),
pta (phosphate acetyltransferase),
pur (phosphoribosylaminoimidazolecarboxamide),
pycA (pyruvate carboxylase)
tpi (triosephosphate isomerase)

Column 1: Consecutive strain number according to Tabel 3.1, Tabel 3.2, and Tabel 3.3

Column 2: Internal DMU strain number according to Tabel 3.1, Tabel 3.2, and Tabel 3.3

Column 3-9: Assignment of sequence number to the seven household genes, according to http://blast.ncbi.nlm.nih.gov/Blast.cgi.

Column 10: Assignement af the strain to a Sequence Type (ST), using http://blast.ncbi.nlm.nih.gov/Blast.cgi. ST number in italics means either that the sequence combination is not found in the database, or that the amount of sequence data are incomplete. ST numbers in bold indicate that the combination of sequences is known in the database

no	DMU name	The seven household genes							ST	Related strains
		glpF	gmk	ilvD	pta	pur	pycA	tpi		
1	Bt1258	10	-	15	-	-	16	7	*13*	

id	isolate	variety serovar	country	year	source	glp	gmk	ilv	pta	pur	pyc	tpi	ST
65	T04b001	kenyae	Kenya	1962	.	10	8	15	12	2	16	7	13
66	T04b012	kenyae	Bulgaria	1974	.	10	8	15	12	2	16	7	13
67	T04b054	kenyae	Iraq	1986	.	10	8	15	12	2	16	7	13
68	T04b060	kenyae	Iraq	1987	.	10	8	15	12	2	16	7	13
69	T04b073	kenyae	Chile	1993	soil	10	8	15	12	2	16	7	13

2	Bt1259	48	-	33	-	44	31	51	*440*	

id	isolate	variety serovar	country	year	source	glp	gmk	ilv	pta	pur	pyc	tpi	ST
797	R11	cereus	.	.	Vomit + diarrhoea	48	30	47	61	44	31	51	440

3	Bt 1186	-	8/32	8	11	11	12	7	*4*	

id	isolate	variety serovar	country	year	source	glp	gmk	ilv	pta	pur	pyc	tpi	ST
23	ATCC 14579	cereus	USA	1916	.	13	8	8	11	11	12	7	4
281	B548	cereus	.	.	.	13	8	8	11	11	12	7	4
799	R25	mycoides	.	.	.	13	8	8	11	11	12	7	4

4	Bt1260	40	(28)	14	12	2	50	7	**98**	

id	isolate	variety serovar	country	year	source	glp	gmk	ilv	pta	pur	pyc	tpi	ST
158	2002734358	cereus	USA	1981	Faeces diarrhoea	40	28	14	12	2	50	7	98

5	Bt1261	13	8	8	11	4	12	53	**395**	

id	isolate	variety	country	year	source	glp	gmk	ilv	pta	pur	pyc	tpi	ST

6	Bt1262	3	2	31	5	16	3	4	**26**	

		serovar											
667	F99/5941	cereus	.	1999	environmental	13	8	8	11	4	12	53	395

id	isolate	variety serovar	country	year	source	glp	gmk	ilv	pta	pur	pyc	tpi	ST
8	S73	cereus	.	1972	vomit	3	2	31	5	16	3	4	26
9	S74	cereus	.	1977	rice	3	2	31	5	16	3	4	26
15	S710	cereus	.	.	.	3	2	31	5	16	3	4	26
31	F3080 B/87	cereus	UK	1987	poultry	3	2	31	5	16	3	4	26
32	F3942/87	cereus	UK	1987	rice	3	2	31	5	16	3	4	26
33	F 4810/72	cereus	USA	1972	vomit	3	2	31	5	16	3	4	26
214	2002734478	cereus	USA	2004	blood	3	2	31	5	16	3	4	26
285	F95/9130	cereus	UK	1995	environmental	3	2	31	5	16	3	4	26
544	NC7401	cereus	Japan	1974	faeces	3	2	31	5	16	3	4	26
558	BC_7	cereus	Italy	2006	other food	3	2	31	5	16	3	4	26
596	G0501	cereus	Japan	2005	rice	3	2	31	5	16	3	4	26
597	NC1247	cereus	Japan	1996	rice	3	2	31	5	16	3	4	26
598	NC1291	cereus	Japan	1999	milk products	3	2	31	5	16	3	4	26
599	NC1363	cereus	Japan	2001	faeces	3	2	31	5	16	3	4	26
600	NC-G15	cereus	Japan	1995	faeces	3	2	31	5	16	3	4	26
601	NC-I55	cereus	Japan	1980	faeces	3	2	31	5	16	3	4	26
602	NC92	cereus	Japan	1978	faeces	3	2	31	5	16	3	4	26

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										604 NC444 cereus Japan 1982 environmental 3 2 31 5 16 3 4 26 605 NC524 cereus Japan 1983 environmental 3 2 31 5 16 3 4 26 606 NC529 cereus Japan 1983 environmental 3 2 31 5 16 3 4 26 607 NC541 cereus Japan 1983 environmental 3 2 31 5 16 3 4 26 608 NC604 cereus Japan 1990 environmental 3 2 31 5 16 3 4 26 609 NC622 cereus Japan 1990 environmental 3 2 31 5 16 3 4 26 610 NC1237 cereus Japan 1996 rice 3 2 31 5 16 3 4 26 611 NC1239 cereus Japan 1996 rice 3 2 31 5 16 3 4 26 639 CBC_99014 cereus Japan . . 3 2 31 5 16 3 4 26 640 CBC_04001 cereus Japan . . 3 2 31 5 16 3 4 26
7	Bt1263	19	2	31	5	19	3	91	*205*	id isolate variety serovar country year source glp gmk ilv pta pur pyc tpi ST 578 BC_28 cereus Italy 2006 other food 19 2 21 5 19 3 2 205 728 KW2 . . . . 19 2 21 5 19 3 2 205
8	Bt1264	24	12	33	37	48	31	19	**104**	id isolate variety serovar country year source glp gmk ilv pta pur pyc tpi ST 175 BGSC 4Y1 tochigiensis Japan . soil 24 12 33 37 48 31 19 104
9	Bt1265	6	4	42	4	16	6	(3)	**92**	Food poisoning outbreak id isolate variety serovar country year source glp gmk ilv pta pur pyc tpi ST 151 2002734349 cereus USA 1981 faeces 6 4 42 4 16 6 3 92

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<tr><td>10</td><td>Bt 667</td><td>3</td><td>2</td><td>63</td><td>5</td><td>36</td><td>3</td><td>55</td><td>164</td><td>
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<tr><td>48</td><td>m1552</td><td>cereus</td><td>Brazil</td><td>1987</td><td>other food</td><td>40</td><td>9</td><td>14</td><td>26</td><td>37</td><td>36</td><td>7</td><td>66</td></tr>
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<tr><td>728</td><td>KW2</td><td>.</td><td>.</td><td>.</td><td>.</td><td>19</td><td>2</td><td>21</td><td>5</td><td>19</td><td>3</td><td>2</td><td>205</td></tr>
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15	Bt 713	29	2	24	5	32	3	22	**47**
16	Bt 721	40	9	14	12	12	14	7	*18 138 156*

228	2002734506	cereus	USA	1997	other food	67	2	63	5	36	3	4	144
282	F00/3086	cereus	UK	2000	. neutropenia	67	2	63	5	36	3	4	144
612	NC1274	cereus	India	1997	vomit	67	2	63	5	36	3	4	144
613	NC-std	cereus	UK	.	rice	67	2	63	5	36	3	4	144
614	NC526	cereus	Japan	1983	environmental	67	2	63	5	36	3	4	144
615	NC610	cereus	Japan	1990	environmental	67	2	63	5	36	3	4	144

id	isolate	variety serovar	country	year	source	glp	gmk	ilv	pta	pur	pyc	tpi	ST
50	m1576	cereus	Brazil	1987	poultry	29	2	24	5	32	3	22	47

id	isolate	variety serovar	country	year	source	glp	gmk	ilv	pta	pur	pyc	tpi	ST
104	T13028	pakistani	Chile	1993	soil	11	9	14	12	12	14	7	18
133	172560_W	cereus	UK	2003	wound	11	9	14	12	12	14	7	18
265	180215.F	cereus	UK	2004	.	11	9	14	12	12	14	7	18
279	101937.Q	cereus	.	2004	.	11	9	14	12	12	14	7	18
283	F95/2410	cereus	UK	1995	.	11	9	14	12	12	14	7	18
533	C3g6	.	UK	2003	leaves	11	9	14	12	12	14	7	18
643	140936.S	cereus	UK	2004	.	11	9	14	12	12	14	7	18

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id	isolate	variety	country	year	source	glp	gmk	ilv	pta	pur	pyc	tpi	ST

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384</td><td>
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<tr><td>7</td><td>F4431/3</td><td>cereus</td><td>.</td><td>1973</td><td>rice</td><td>19</td><td>2</td><td>18</td><td>17</td><td>19</td><td>3</td><td>2</td><td>28</td></tr>
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<tr><td>290</td><td>153804.Y</td><td>cereus</td><td>UK</td><td>2004</td><td>.</td><td>19</td><td>2</td><td>59</td><td>17</td><td>19</td><td>3</td><td>2</td><td>182</td></tr>
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<tr><td>648</td><td>154145.Q</td><td>cereus</td><td>UK</td><td>2004</td><td>.</td><td>19</td><td>2</td><td>3</td><td>17</td><td>19</td><td>3</td><td>2</td><td>384</td></tr>
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/ 66</td><td>Som nr 12</td></tr>
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21	Bt 1198	12	8/32	8/67/9	11	11	12	7		Ligner nok mest nr 3, men sekvenserne er ikke helt gode nok
22	Bt 1199	117	9	14	18	20	14	7		Kun 4 af 7 har match. Men i alle de nærtliggende ST´er der kun ét tilfælge med et isolat fundet i lunge --- og det er ukendt om det er patogent. desuden er der en del Bt´er
23	Bt 1200	6	4	41	5	43	46	3	**90**	

id	isolate	variety serovar	country	year	source	glp	gmk	ilv	pta	pur	pyc	tpi	ST
149	2002734384	cereus	USA	2000	Blood Human septicaemia	6	4	41	5	43	46	3	90
287	143298.B	cereus	UK	2004	.Human	6	4	41	5	43	46	3	90

24	Bt 1201	(51)	(2)	122	5	-	3	91	*90*	4 matches

id	isolate	variety serovar	country	year	source	glp	gmk	ilv	pta	pur	pyc	tpi	ST
149	2002734384	cereus	USA	2000	blood	6	4	41	5	43	46	3	90
287	143298.B	cereus	UK	2004	.	6	4	41	5	43	46	3	90

25	Bt 1202	11	9	14	12	12	36	7	*18*	6 matches

id	isolate	variety serovar	country	year	source	glp	gmk	ilv	pta	pur	pyc	tpi	ST
104	T13028	pakistani	Chile	1993	soil	11	9	14	12	12	14	7	18
133	172560_W	cereus	UK	2003	Burn wound infection	11	9	14	12	12	14	7	18
265	180215.F	cereus	UK	2004	human	11	9	14	12	12	14	7	18
279	101937.Q	cereus	.	2004	.human	11	9	14	12	12	14	7	18
283	F95/2410	cereus	UK	1995	.human cellulitis	11	9	14	12	12	14	7	18

26	Bt 1203	6	4	53	4	16	6	3	**127**	
27	Bt 1204	5	4	3	4	15	6	16	**32**	SE NR 39
28	Bt 1205	12	8	8	7	9	12	7	*184*	6 matches
29	Bt 1207	24	30	(33)	37	44	31	5/45	*123*	5 mathces

id	isolate	variety serovar	country	year	source	glp	gmk	ilv	pta	pur	pyc	tpi	ST
533	C3g6	.	UK	2003	leaves	11	9	14	12	12	14	7	18
643	140936.S	cereus	UK	2004	human	11	9	14	12	12	14	7	18

id	isolate	variety serovar	country	year	source	glp	gmk	ilv	pta	pur	pyc	tpi	ST
207	MM_4	cereus	USA	2003	other food	6	4	53	4	16	6	3	127

id	isolate	variety serovar	country	year	source	glp	gmk	ilv	pta	pur	pyc	tpi	ST
16	ATCC 10987	cereus	Canada	1930	milk products	5	4	3	4	15	6	16	32
205	MM_2	cereus	USA	2003	other food	5	4	3	4	15	6	16	32
791	R1	cereus	.	.	.	5	4	3	4	15	6	16	32

id	isolate	variety serovar	country	year	source	glp	gmk	ilv	pta	pur	pyc	tpi	ST
292	156429.N	cereus	UK	2004	.	12	8	8	14	9	12	7	184

id	isolate	variety serovar	country	year	source	glp	gmk	ilv	pta	pur	pyc	tpi	ST
201	2002734363	cereus	USA	1972	Faeces diarrhoea	48	30	33	37	44	48	45	123
202	2002734366	cereus	USA	1972	other food	48	30	33	37	44	48	45	123

4 mathces

id	isolate	variety serovar	country	year	source	glp	gmk	ilv	pta	pur	pyc	tpi	ST
552	BC_1	cereus	Italy	2006	other food	48	30	33	37	44	103	5	369
574	BC_24	cereus	Italy	2006	other food	48	30	33	37	44	103	5	369

30	Bt 1208	6	4	41	5	43	46	3	**90**

id	isolate	variety serovar	country	year	source	glp	gmk	ilv	pta	pur	pyc	tpi	ST
149	2002734384	cereus	USA	2000	Blood Human septicaemia	6	4	41	5	43	46	3	90
287	143298.B	cereus	UK	2004	.Human	6	4	41	5	43	46	3	90

31	Bt 1210	6	4	53	4	16	6	3	**127**

id	isolate	variety serovar	country	year	source	glp	gmk	ilv	pta	pur	pyc	tpi	ST
207	MM_4	cereus	USA	2003	other food	6	4	53	4	16	6	3	127

Appendice 3

32	Bt 1211	12	8	13	10	2	17	7	*381*

id	isolate	variety serovar	country	year	source	glp	gmk	ilv	pta	pur	pyc	tpi	ST
637	CBC_01002	cereus	Japan	.	Human Diarhoea	33	8	13	10	2	17	7	381

33	Bt 1215	37	9	14	12	12	14	7	**138**

id	isolate	variety serovar	country	year	source	glp	gmk	ilv	pta	pur	pyc	tpi	ST
220	2002734371	cereus	USA	1978	lung	37	9	14	12	12	14	7	138

34	Bt 1224	96	8	36	30	41	17	(53)	
35	Bt 1227	-	8	8	7	9	12	7	*142 155 184*

Only 4 matches --- new ST-type

id	isolate	variety serovar	country	year	source	glp	gmk	ilv	pta	pur	pyc	tpi	ST
225	2003018578	cereus	USA	2003	blood	13	8	8	11	9	12	7	142
226	2003018577	cereus	USA	2003	blood	13	8	8	11	9	12	7	142
232	000-843-51	cereus	Japan	2004	blood	13	8	8	11	9	12	7	142
286	134070.J	cereus	UK	2004	.	13	8	8	11	9	12	7	142
288	201183.D	cereus	UK	2003	.	13	8	8	11	9	12	7	142
560	BC_9	cereus	Italy	2006	other food	13	8	8	11	9	12	7	142
567	BC_17	cereus	Italy	2006	other food	13	8	8	11	9	12	7	142
579	BC_29	cereus	Italy	2006	other food	13	8	8	11	9	12	7	142
581	BC_32	cereus	Italy	2006	other food	13	8	8	11	9	12	7	142
619	B-4ac	cereus	Belgium	.	faeces	13	8	8	11	9	12	7	142
622	NC1225	cereus	Japan	1996	faeces	13	8	8	11	9	12	7	142
623	NC1292	cereus	Japan	1999	rice	13	8	8	11	9	12	7	142
629	NC499	cereus	Japan	1983	other food	13	8	8	11	9	12	7	142
636	CBC_99006	cereus	Japan	.	.	13	8	8	11	9	12	7	142
638	CBC_03004	cereus	Japan	.	.	13	8	8	11	9	12	7	142

id	isolate	variety serovar	country	year	source	glp	gmk	ilv	pta	pur	pyc	tpi	ST
241	NC1269	cereus	India	1991	milk products	13	8	8	14	9	12	7	155

36	Bt 1228	6	4	41	5/11/4	43	46	3	**90**

id	isolate	variety serovar	country	year	source	glp	gmk	ilv	pta	pur	pyc	tpi	ST
149	2002734384	cereus	USA	2000	blood	6	4	41	5	43	46	3	90
287	143298.B	cereus	UK	2004	.	6	4	41	5	43	46	3	90

37	Bt 1239	6	4	53	4	16	6	3	**127**

id	isolate	variety serovar	country	year	source	glp	gmk	ilv	pta	pur	pyc	tpi	ST
207	MM_4	cereus	USA	2003	other food	6	4	53	4	16	6	3	127

38	Bt 642	13	8/32	8	11	9	12	7	**142**

id	isolate	variety serovar	country	year	source	glp	gmk	ilv	pta	pur	pyc	tpi	ST
225	2003018578	cereus	USA	2003	blood	13	8	8	11	9	12	7	142
226	2003018577	cereus	USA	2003	blood	13	8	8	11	9	12	7	142
232	000-843-51	cereus	Japan	2004	blood	13	8	8	11	9	12	7	142
286	134070.J	cereus	UK	2004	.	13	8	8	11	9	12	7	142
288	201183.D	cereus	UK	2003	.	13	8	8	11	9	12	7	142
560	BC_9	cereus	Italy	2006	other food	13	8	8	11	9	12	7	142
567	BC_17	cereus	Italy	2006	other	13	8	8	11	9	12	7	142

												food											
										579	BC_29	cereus	Italy	2006	other food	13	8	8	11	9	12	7	142
										581	BC_32	cereus	Italy	2006	other food	13	8	8	11	9	12	7	142
										619	B-4ac	cereus	Belgium	.	faeces	13	8	8	11	9	12	7	142
										622	NC1225	cereus	Japan	1996	faeces	13	8	8	11	9	12	7	142
										623	NC1292	cereus	Japan	1999	rice	13	8	8	11	9	12	7	142
										629	NC499	cereus	Japan	1983	other food	13	8	8	11	9	12	7	142
										636	CBC_99006	cereus	Japan	.	.	13	8	8	11	9	12	7	142
										638	CBC_03004	cereus	Japan	.	.	13	8	8	11	9	12	7	142
39	Bt 156	5	4	3	4	15	(6)	16	**32**	**id**	**isolate**	**variety serovar**	**country**	**year**	**source**	**glp**	**gmk**	**ilv**	**pta**	**pur**	**pyc**	**tpi**	**ST**
										16	ATCC 10987	cereus	Canada	1930	milk products	5	4	3	4	15	6	16	32
										205	MM_2	cereus	USA	2003	other food	5	4	3	4	15	6	16	32
										791	R1	cereus	.	.	.	5	4	3	4	15	6	16	32
30	Bt 160	13	8	8	11	11	12	7	**4**	**id**	**isolate**	**variety serovar**	**country**	**year**	**source**	**glp**	**gmk**	**ilv**	**pta**	**pur**	**pyc**	**tpi**	**ST**
										23	ATCC 14579	cereus	USA	1916	.	13	8	8	11	11	12	7	4

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<tr><td>799</td><td>R25</td><td>mycoides</td><td>.</td><td>.</td><td>.</td><td>13</td><td>8</td><td>8</td><td>11</td><td>11</td><td>12</td><td>7</td><td>4</td></tr>
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<tr><td>797</td><td>R11</td><td>cereus</td><td>.</td><td>.</td><td>vomit</td><td>48</td><td>30</td><td>47</td><td>61</td><td>44</td><td>31</td><td>51</td><td>440</td></tr>
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id	isolate	variety serovar	country	year	source	glp	gmk	ilv	pta	pur	pyc	tpi	ST
798	R12	cereus	.	.	other food DIARREa	48	30	129	61	44	31	51	441

46	Bt 983	3	2	31	5	16	3	4	**26**

id	isolate	variety serovar	country	year	source	glp	gmk	ilv	pta	pur	pyc	tpi	ST
8	S73	cereus	.	1972	vomit	3	2	31	5	16	3	4	26
9	S74	cereus	.	1977	rice	3	2	31	5	16	3	4	26
15	S710	cereus	.	.	.	3	2	31	5	16	3	4	26
31	F3080 B/87	cereus	UK	1987	poultry	3	2	31	5	16	3	4	26
32	F3942/87	cereus	UK	1987	rice	3	2	31	5	16	3	4	26
33	F 4810/72	cereus	USA	1972	vomit	3	2	31	5	16	3	4	26
214	2002734478	cereus	USA	2004	blood	3	2	31	5	16	3	4	26
285	F95/9130	cereus	UK	1995	environmental	3	2	31	5	16	3	4	26
544	NC7401	cereus	Japan	1974	faeces	3	2	31	5	16	3	4	26
558	BC_7	cereus	Italy	2006	other food	3	2	31	5	16	3	4	26
596	G0501	cereus	Japan	2005	rice	3	2	31	5	16	3	4	26
597	NC1247	cereus	Japan	1996	rice	3	2	31	5	16	3	4	26
598	NC1291	cereus	Japan	1999	milk products	3	2	31	5	16	3	4	26
599	NC1363	cereus	Japan	2001	faeces	3	2	31	5	16	3	4	26
600	NC-G15	cereus	Japan	1995	faeces	3	2	31	5	16	3	4	26
601	NC-I55	cereus	Japan	1980	faeces	3	2	31	5	16	3	4	26
602	NC92	cereus	Japan	1978	faeces	3	2	31	5	16	3	4	26

										603	NC199	cereus	Japan	1979	environmental	3	2	31	5	16	3	4	26
										604	NC444	cereus	Japan	1982	environmental	3	2	31	5	16	3	4	26
										605	NC524	cereus	Japan	1983	environmental	3	2	31	5	16	3	4	26
										606	NC529	cereus	Japan	1983	environmental	3	2	31	5	16	3	4	26
										607	NC541	cereus	Japan	1983	environmental	3	2	31	5	16	3	4	26
										608	NC604	cereus	Japan	1990	environmental	3	2	31	5	16	3	4	26
										609	NC622	cereus	Japan	1990	environmental	3	2	31	5	16	3	4	26
										610	NC1237	cereus	Japan	1996	rice	3	2	31	5	16	3	4	26
										611	NC1239	cereus	Japan	1996	rice	3	2	31	5	16	3	4	26
										639	CBC_99014	cereus	Japan	.	.	3	2	31	5	16	3	4	26
										640	CBC_04001	cereus	Japan	.	.	3	2	31	5	16	3	4	26
										641	CBC_04003	cereus	Japan	.	.	3	2	31	5	16	3	4	26
47	Bt 651	43	26	8	42	39	41	30	*111*	**id**	**isolate**	**variety serovar**	**country**	**year**	**source**	**glp**	**gmk**	**ilv**	**pta**	**pur**	**pyc**	**tpi**	**ST**
										150	2002734347	cereus	USA	1982	blood	43	26	35	42	39	41	30	111
										184	BGSC 6A7	cereus	.	.	.	43	26	35	42	39	41	30	111
										196	2004721594	cereus	USA	2004	hair clippers	43	26	35	42	39	41	30	111
										197	2004721597	cereus	USA	2004	hair clippers	43	26	35	42	39	41	30	111
										379	B4o5	.	UK	2003	leaves	43	26	35	42	39	41	30	111
										380	B4o11	.	UK	2003	leaves	43	26	35	42	39	41	30	111
										383	C3i2	.	UK	2003	leaves	43	26	35	42	39	41	30	111

										384	C3i3	.	UK	2003	leaves	43	26	35	42	39	41	30	111
										386	C3i6	.	UK	2003	leaves	43	26	35	42	39	41	30	111
										387	C3i8	.	UK	2003	leaves	43	26	35	42	39	41	30	111
										388	C3m2	.	UK	2003	leaves	43	26	35	42	39	41	30	111
										390	C3m4	.	UK	2003	leaves	43	26	35	42	39	41	30	111
										395	C3o5	.	UK	2003	leaves	43	26	35	42	39	41	30	111
										404	C3o14	.	UK	2003	leaves	43	26	35	42	39	41	30	111
										409	C3o19	.	UK	2003	leaves	43	26	35	42	39	41	30	111
										429	C3o40	.	UK	2003	leaves	43	26	35	42	39	41	30	111
										438	C3o41	.	UK	2003	leaves	43	26	35	42	39	41	30	111
										453	A3s11	.	UK	2003	soil	43	26	35	42	39	41	30	111
										454	A3s2	.	UK	2003	soil	43	26	35	42	39	41	30	111
										483	E2i4	.	UK	2003	leaves	43	26	35	42	39	41	30	111
										484	E2i6	.	UK	2003	leaves	43	26	35	42	39	41	30	111
										487	E2m2	.	UK	2003	leaves	43	26	35	42	39	41	30	111
										490	E2o1	.	UK	2003	leaves	43	26	35	42	39	41	30	111
										492	E2o4	.	UK	2003	leaves	43	26	35	42	39	41	30	111
										493	E2o6	.	UK	2003	leaves	43	26	35	42	39	41	30	111
										494	E2o7	.	UK	2003	leaves	43	26	35	42	39	41	30	111
										495	E2o8	.	UK	2003	leaves	43	26	35	42	39	41	30	111
										497	E2o10	.	UK	2003	leaves	43	26	35	42	39	41	30	111
										500	E2s1	.	UK	2003	soil	43	26	35	42	39	41	30	111

										502	E2s4	.	UK	2003	soil	43	26	35	42	39	41	30	111
										503	E2s6	.	UK	2003	soil	43	26	35	42	39	41	30	111
										505	E2s8	.	UK	2003	soil	43	26	35	42	39	41	30	111
										506	A4s2	.	UK	2003	soil	43	26	35	42	39	41	30	111
										508	A4s5	.	UK	2003	soil	43	26	35	42	39	41	30	111
										512	B4s2	.	UK	2003	soil	43	26	35	42	39	41	30	111
										513	B4s3	.	UK	2003	soil	43	26	35	42	39	41	30	111
										514	B4s5	.	UK	2003	soil	43	26	35	42	39	41	30	111
										517	B4g1	.	UK	2003	leaves	43	26	35	42	39	41	30	111
										518	B4g2	.	UK	2003	leaves	43	26	35	42	39	41	30	111
										520	B4g10	.	UK	2003	leaves	43	26	35	42	39	41	30	111
										521	C3s1	.	UK	2003	soil	43	26	35	42	39	41	30	111
										522	C3s2	.	UK	2003	soil	43	26	35	42	39	41	30	111
										523	C3s3	.	UK	2003	soil	43	26	35	42	39	41	30	111
										524	C3s4	.	UK	2003	soil	43	26	35	42	39	41	30	111
										529	C3g1	.	UK	2003	leaves	43	26	35	42	39	41	30	111
										531	C3g3	.	UK	2003	leaves	43	26	35	42	39	41	30	111
										532	C3g5	.	UK	2003	leaves	43	26	35	42	39	41	30	111
										625	NC390	cereus	Japan	1982	faeces	43	26	35	42	39	41	30	111
48	Bt 959	15	6	-	8	3	7	14	*10*	**id**	**isolate**	**variety serovar**	**country**	**year**	**source**	**glp**	**gmk**	**ilv**	**pta**	**pur**	**pyc**	**tpi**	**ST**
										51	T01001	thuringiensis	Canada	1958	.	15	6	10	8	3	7	14	10

49	Bt 1254	19/65	1	52	1	1	37	24	145	
50	Bt 600	9	8/32	16	13	2	16	9	15	

id	isolate	variety serovar	country	year	source	glp	gmk	ilv	pta	pur	pyc	tpi	ST
52	T01015	thuringiensis	Bulgaria	1962	.	15	6	10	8	3	7	14	10
53	T01022	thuringiensis	USA	1964	other	15	6	10	8	3	7	14	10
55	T01326	thuringiensis	Chile	1994	soil	15	6	10	8	3	7	14	10
125	T01058	thuringiensis	Switzerland	1964	.	15	6	10	8	3	7	14	10
186	DSM 2046	thuringiensis	.	.	.	15	6	10	8	3	7	14	10
261	BGSC 4A1	thuringiensis	.	1915	.	15	6	10	8	3	7	14	10
315	HD-1	kurstaki	USA	1970	other	15	6	10	8	3	7	14	10
793	R3	thuringiensis	.	.	.	15	6	10	8	3	7	14	10
794	R4	thuringiensis	.	.	.	15	6	10	8	3	7	14	10
806	R108	thuringiensis	.	.	other	15	6	10	8	3	7	14	10

id	isolate	variety serovar	country	year	source	glp	gmk	ilv	pta	pur	pyc	tpi	ST
229	2002734520	cereus	USA	1975	blood	65	1	52	1	1	37	24	145
284	F95/8201	cereus	UK	1995	.	65	1	52	1	1	37	24	145

id	isolate	variety serovar	country	year	source	glp	gmk	ilv	pta	pur	pyc	tpi	ST
80	T07033	aizawai	Japan	1975	.	9	8	16	13	2	16	9	15
81	T07058	aizawai	France	1983	.	9	8	16	13	2	16	9	15
83	T07180	aizawai	Spain	1992	.	9	8	16	13	2	16	9	15
576	BC_26	cereus	Italy	2006	other food	9	8	16	13	2	16	9	15

51	Bt 591	9	8	16	13	2	16	9	**15**
52	Bt 1253	15	7	7	2	4	16	13	*9*
53	Bt 1255	7	8	16	13	43	16	7	*8*

As Bt600 above

id	isolate	variety serovar	country	year	source	glp	gmk	ilv	pta	pur	pyc	tpi	ST
5	169-S-5	thuringiensis	UK	2005	leaves	15	7	7	2	5	16	13	9

id	isolate	variety serovar	country	year	source	glp	gmk	ilv	pta	pur	pyc	tpi	ST
1	S57	cereus	USA	.	mud	7	8	16	13	2	16	7	8
2	S58	cereus	USA	.	faeces	7	8	16	13	2	16	7	8
3	S59	cereus	USA	.	mud	7	8	16	13	2	16	7	8
56	T03a001	kurstaki	France	1962	.	7	8	16	13	2	16	7	8
57	T03a075	kurstaki	Iraq	1976	.	7	8	16	13	2	16	7	8
58	T03a172	kurstaki	Pakistan	1982	.	7	8	16	13	2	16	7	8
59	T03a287	kurstaki	Kenya	1988	.	7	8	16	13	2	16	7	8
60	T03a361	kurstaki	Australia	1990	.	7	8	16	13	2	16	7	8
300	164-S-1	thuringiensis	UK	2005	leaves	7	8	16	13	2	16	7	8
316	248-S-1	thuringiensis	UK	2005	leaves	7	8	16	13	2	16	7	8
392	C3o2	.	UK	2003	leaves	7	8	16	13	2	16	7	8
393	C3o3	.	UK	2003	leaves	7	8	16	13	2	16	7	8
394	C3o4	.	UK	2003	leaves	7	8	16	13	2	16	7	8
396	C3o6	.	UK	2003	leaves	7	8	16	13	2	16	7	8
398	C3o8	.	UK	2003	leaves	7	8	16	13	2	16	7	8
399	C3o9	.	UK	2003	leaves	7	8	16	13	2	16	7	8

400	C3o10	.	UK	2003	leaves	7	8	16	13	2	16	7	8
401	C3o11	.	UK	2003	leaves	7	8	16	13	2	16	7	8
402	C3o12	.	UK	2003	leaves	7	8	16	13	2	16	7	8
403	C3o13	.	UK	2003	leaves	7	8	16	13	2	16	7	8
405	C3o15	.	UK	2003	leaves	7	8	16	13	2	16	7	8
406	C3o16	.	UK	2003	leaves	7	8	16	13	2	16	7	8
407	C3o17	.	UK	2003	leaves	7	8	16	13	2	16	7	8
408	C3o18	.	UK	2003	leaves	7	8	16	13	2	16	7	8
410	C3o20	.	UK	2003	leaves	7	8	16	13	2	16	7	8
411	C3o21	.	UK	2003	leaves	7	8	16	13	2	16	7	8
412	C3o22	.	UK	2003	leaves	7	8	16	13	2	16	7	8
414	C3o24	.	UK	2003	leaves	7	8	16	13	2	16	7	8
415	C3o25	.	UK	2003	leaves	7	8	16	13	2	16	7	8
417	C3o27	.	UK	2003	leaves	7	8	16	13	2	16	7	8
418	C3o28	.	UK	2003	leaves	7	8	16	13	2	16	7	8
419	C3o29	.	UK	2003	leaves	7	8	16	13	2	16	7	8
421	C3o32	.	UK	2003	leaves	7	8	16	13	2	16	7	8
423	C3o34	.	UK	2003	leaves	7	8	16	13	2	16	7	8
424	C3o35	.	UK	2003	leaves	7	8	16	13	2	16	7	8
425	C3o36	.	UK	2003	leaves	7	8	16	13	2	16	7	8
426	C3o37	.	UK	2003	leaves	7	8	16	13	2	16	7	8
427	C3o38	.	UK	2003	leaves	7	8	16	13	2	16	7	8

										430 C3o42 . UK 2003 leaves 7 8 16 13 2 16 7 8 474 E5m5 . UK 2003 leaves 7 8 16 13 2 16 7 8 478 E5g1 . UK 2003 leaves 7 8 16 13 2 16 7 8 479 E5s9 . UK 2003 soil 7 8 16 13 2 16 7 8 481 DiPel . . . . 7 8 16 13 2 16 7 8 498 E2o20 . UK 2003 leaves 7 8 16 13 2 16 7 8 536 7_1_0 . UK 2005 leaves 7 8 16 13 2 16 7 8 537 6_2_Y . UK 2005 leaves 7 8 16 13 2 16 7 8 676 D10-18.A.i . UK 2006 soil 7 8 16 13 2 16 7 8 697 ABTS 351 kurstaki USA 2008 other 7 8 16 13 2 16 7 8 698 ABTS 2279 kurstaki USA 2008 other 7 8 16 13 2 16 7 8 699 ABTS 2423 kurstaki USA 2008 other 7 8 16 13 2 16 7 8
54	Bt 1191	7	8/32	16	13	2	16	7	**8**	As above
55	Bt 1256	93	6	29	91	4	50	39	*206*	id isolate variety serovar country year source glp gmk ilv pta pur pyc tpi ST 729 KW3 . . . . 93 6 29 91 4 82 39 206

Appendice 4

Primer design

Virulence gene primer designs for Q-RT PCR have been done on alignments of gene sequences from8 ***B. cereus*** and ***B. thuringiensis*** sequenced genomes via Blast. The program Primer Express (Applied Biosystems) was used to determine the best conserved regions. The primers are of 20 to 30 base pairs and give amplicons from 65-85 bp and are listed in Table 2.2. Primers for reference genes for Q-RT-PCR were designed using the CLS main workbench software version 5 (CLC Bio, Aarhus, Denmark), or the Primer Express vs. 3.0 software (Applied Biosystems, Foster City, CA) based on DNA sequences obtained in the present project (BMH/DMU). The reference primer sequence including the ***rpoB*** primer sequences provided by Christina Nielsen Leroux, INRA are shown in table Table 2.2

The virulence gene primers were initially evaluated by conventional PCR for strains bc11, bc25, bc38, bt48, bc49, bt50, bt52, bt53 and bt55. The PCR protocol used was 1 cycle at 95°C for 2 min, 40 cycles at 95°C for 1 min, 60°C for 30 s, 72°C for 1 min, and 1 cycle at 72°C for 10 min. The reference gene primers were also initially evaluated by conventional PCR for strains bc 11, bc25, bc38, bt48, bc49, bt50, bt52, bt53, bt55. The PCR protocol used was 1 cycle at 94°C for 1 min, 40 cycles at 95°C for 30 s, 58°C for 30 s, 72°C for 30 s, and 1 cycle at 72°C for 5 min. DNA for the PCR was extracted according to the protocol by Hansen and Hendriksen (2001).

Results are shown in Table 8.6. Not all of the tested bacteria gave positive PCR products. Strain bc 11 was PCR negative for ***hblC, inhA2*** and ***plcR***, strain bc14 was PCR negative for ***cytK, hblC*** and ***hlyII,*** strain bc25, bc38 and bt53 were PCR negative for ***hlyII,*** strain bc49 old was PCR negative for ***cytK, hblC*** and ***hlyII,*** while bc49 new was PCR positive (detected by RT-PCR) for all the tested virulence genes except for ***hlyII.*** The applicability of the primers to the projects bacterial strains was also evaluated by producing standard curves based on genomic DNA. Results are shown in Appendix 5 and areas marked in grey indicate primer/strain combinations which are acceptable (slope values between -3.6 and -3.1).

In order to be able to quantify expression or at least the relative expression of the genes using the SYBR green Quantitaive PCR it is important to analyse the performance of the primers. This was done with individual genes on genomic DNA (Table 8.7) and on pools of cDNA from representative strains where the genes were shown to be expressed in LB broth (Table 3.4).

Table 8.6 Presence of target genes in selected *Bacillus strains*. Test of qRT-PCR primers by conventional PCR by KU-LIFE using genomic DNA as template.

	Gene (primers)								
Genes/ strains	*plcR*	*nheB*	*hblC*	*cytKA*	*cytKB*	*hlyII*	*ilsA*	*inhA2*	16SrRNA
Bc11*	-	((+))	-	+	+	+	+	-	+
Bc14*	+	+	-	-	-	-	+	+	+
Bc25	+	+	+	+	+	-	+	+	+
Bc38	+	+	+	+	+	-	+	+	+
Bt48*	-	+	+	+	+	+	+	+	+
Bt407	+	+	+	+	+	+	+	+	+
Bc49** new	+	+	+	+	nd	-	+	+	
Bt50	+	+	+	+	+	-	+	+	+
Bt52	+	+	+	+	+	+	+	+	+
Bt53	+	+	+	+	+	-	+	+	+
Bt55	+	+	+	+	+	+	+	+	+

*The plcR gene was also negative when tested with an alternative primer pair (Fricker et al., 2008). Similarly when tested with alternative primers strain bc 11 was PCR negative for hblC and NheA strain bc14 was negative for *hblC* and *cytk* (Guinebretiere et al., 2002; Hansen and Hendriksen 2001). These experiments have not been described in materials and methods.

**Detected by gene transcription assays (RT-PCR) at later stages of this project

***This strain was not tested by PCR, the primers were evaluated by using primer blast on the genome sequence (Genbank accession no. NC010184). A (+) indicates that the primers did not show 100% identity.

Table 8.7 Slope and r-values of standard curves obtained with genomic DNA using Power SYBRgreen technology, on a FAST7500 real time PCR machine. An acceptable slope is between -3.6 and -3.1.

	inhA2		*hblC*		*nheB*		*cytK*		*plcR*		*16S rRNA*		*rpoB*		*tpi (1)*		*pur*		*glp*	
strain	Slope	R-value	Slope	R-value	Slope	R-value	Slope	R-value	Slope	R-value	Slope	R-value	Slope	R-value	Slope	R-value	Slope	R-value	Slope	R-value
bc 11*	-3.28	0.96	-	-	-2.99	0.98	-3.35		-	-	-3.71	1.00	-3.5		-3.53	0.99	-3.85	0.985	-3.33	0.93
bc14	-3.45	0.98	-	-	-3.5	1.00	-	-	-3.69		-3.66	1.00								
bc25	-3.43	0.99	-3.55	1.00	-3.47	1.00	-3.42		-3.76		-3.67	1.00								
bc38	-3.31	1.00	-3.60	1.00	-3.41	1.00	-3.42		-3.48		-3.69	1.00								
bt48	-3.47	1.00	-3.52	0.995	-3.59	1.00			nd	nd										
bt50	-2.87	0.98	-3.96	1.00	-2.3	0.97	-3.89	1.00					-3.78	0.98	-3.57	0.99	-3.57	0.99	-3.70	0.99
bt53**	-3.25	0.99	-3.37	0.98	-3.58	1.00	-3.10	0.98	-3.22	0.99										

*NheA slope for bc 11 is uncertain, **Performed on cDNA from strain bt53 grown in LB-broth culture, OD650 = appr. 1

Appendice 5

TEER

The importance of cell passage and repeatability between experiments were examined. The reproducibility of the results using different Caco-2 cell passages and Caco-2 cells cultured at different time points from different freeze tubes (FR1 and FR2) are shown in detail for strain bt48 (BT407δplcR) in Figure 8.1. For strain bt48 the results were highly consistent regardless of freeze tube and passages investigated. As shown in Figure 8.2 and Figure 8.3, results obtained with HT29-MTX and Caco-2 cells were fairly consistent between experiments (different passages of cell lines) for almost all strains. The exceptions were strains bc11, bc49 and bt50 which showed some deviation in one or both assays

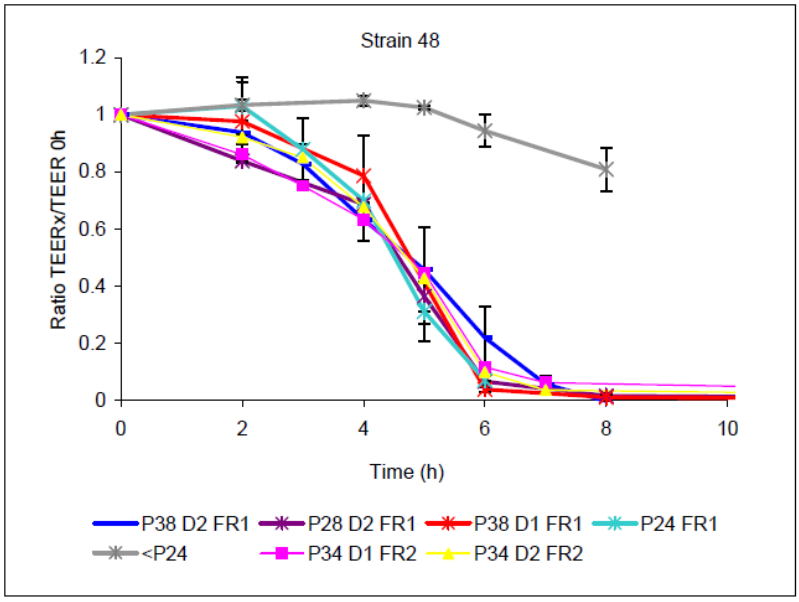

Figure 8.1. An example of TEER results obtained for a single strain using different passages (P) and freeze tubes (FR1 and FR2, cultured at different time points) of Caco-2. The results were obtained between June 2009 and February 2010.

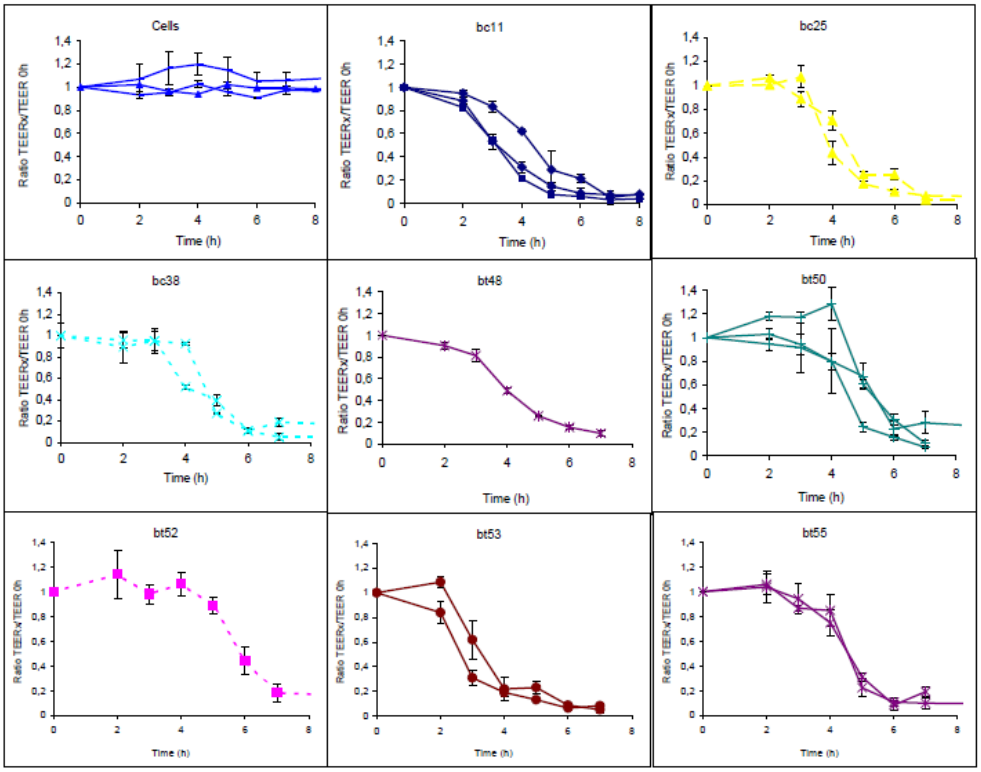

Figure 8.2. TEER of polarized HT29-MTX monolayers exposed to various *Bacillus* spp. at a concentration of approximately $1*10^4$ CFU/well (strains bc25, bc38, bt50, bt52, bt53, bt55) AND $3*10^3$ CFU/well (Strains bc11, bt48). TEER ($\Omega*cm_2$) is expressed as the ratio of TEER at time t in relation to the initial value (at time zero [t0]) for each series. Strains bc11, bc25, bc38 and bc49 are *B. cereus,* bt48, bt50, bt52, bt53, bt55 are *B. thuringiensis*. Results are averages of at least 2 experiments (bt48 and bt52 only 1 experiment) performed in triplicate and are shown with Standard deviations.

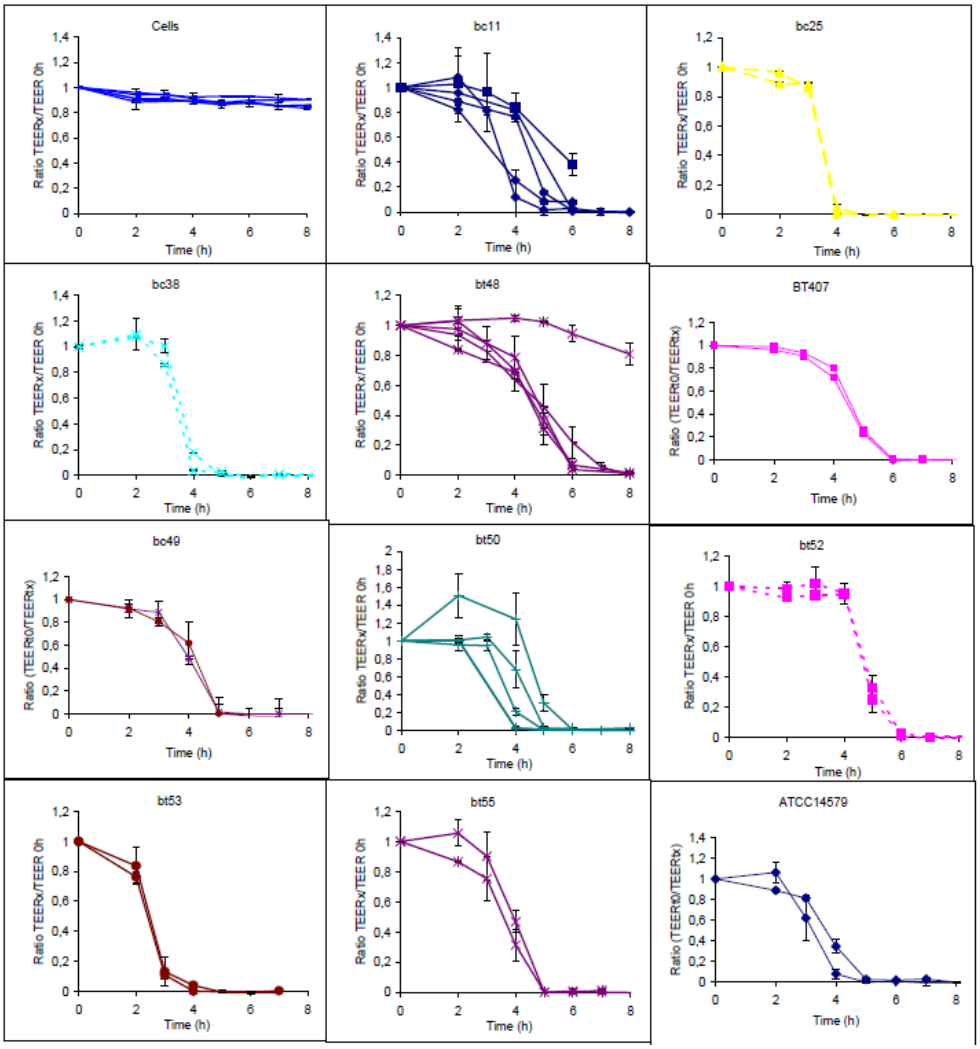

Figure 8.3. TEER of polarized Caco-2 monolayers exposed to various Bacillus spp. at a concentration of approximately $1*10^4$ CFU/well (strains bc25, bc38, bt50, bt52, bt53, bt55) AND $3*10^3$ CFU/well (Strains bc11, bt48 and bc49). TEER ($\Omega*cm_2$) is expressed as the ratio of TEER at time t in relation to the initial value (at time zero [t0]) for each series. Strains bc11, bc25, bc38 and bc49 are *B. cereus,* bt48, bt50, bt52, bt53, bt55 are *B. thuringiensis*. Results are averages of at least 2 experiments performed in triplicate and are shown with Standard deviations.