Bacillus cereus ATCC 14579: Physiological and genetic responses to bile, bile salts and mucin

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Thesis for the degree of Master of Science in Molecular Biology
Department of Molecular Biosciences, University of Oslo
December 2005

ACKNOWLEDGMENTS

This work was performed at the Department of Molecular Biosciences, Oslo University, from January 2005 to December 2005 under supervision of 1. amanuensis William Davis.

First, I would like to thank my supervisor, 1. amanuensis William Davies, for excellent guidance in the laboratory, theoretical matters and in the writing process. Thank you for letting my take part in the design of this project and for giving my opportunities to try out different methods in the laboratory. Thanks for sending me to the proteomics course in York.

Special thanks to my co-students, Ine Robertsen and Solveig Sirnes for superb cooperation and good company in the laboratory.

A special thank also to Solveig Ravnum for excellent cooperation with the microarray-project, and thanks to all other people at "Kolstø group" at the institute of pharmacy for letting me participate in the journal club. Thanks also to Karoline Fægri and Endre Anderssen from "FUGEs mikromatriseplattform" in Trondheim for letting me use the script for microarray-analysis.

I would also like to express gratitude to Professor Reidun Sirevåg, and her students; Elisabeth, Ingunn, Lise, and, Jan for good company and scientific discussions. Thanks to Bård Mathiesen and Turid Berdal Gangnæs for good technical assistance.

And finally my deepest thanks to my wife, Marte, for good support and understanding during this work, and for being the one you are.

Oslo, December 2005

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ABSTRACT

The aim of this thesis was to investigate the *in vitro* physiological and genetic responses of *Bacillus cereus* ATCC 14579 to bile, bile salts and mucin, substances which would be encountered in the gastrointestinal tract.

B. cereus was grown in media containing bile/bile salts and growth only occurred at low concentrations (no growth observed on LB-agar plates with 0.02 % bile salts). Preincubation with bile salts at non-lethal concentrations did not increase the levels of bile salts tolerance.

Cultures of *B. cereus* were grown to the mid-exponential growth phase and shifted to media containing bile (0.03 %), bile salts (0.005 %) and mucin (0.25 %). Global expression patterns (shifts to bile and bile salts containing media) were determined by hybridization of total RNA to microarrays. The expression of selected genes, fibronectin binding protein and two internalin genes, in shift experiments to bile, bile salts and mucin containing media were investigated by RT-PCR. The microarray data indicated a general stress response to bile and bile salts. The RT-PCR indicated a down-regulation of a putative virulence factor, internalin, in bile salts cultures.

Proteins were extracted from the shift experiments (not mucin) 30 minutes after the shift in a LiCl-buffer and analyzed by 2-D protein electrophoresis. Protein spots showing differential expression were excised, trypsin digested and analysed by MALDI-TOF. Protein identification by protein mass fingerprinting showed that dihydrolipoamide dehydrogenase was up-regulated in both shift experimets. Enolase was up-regulated in bile cultures, but down-regulated in bile salts cultures

All off the findings in this study indicate that *B. cereus* ATCC 14579 vegetative cells would not survive in the intestinal environment. Down-regulation of virulence factors and induction of what seem to be a *secondary* stress response indicates that *B. cereus* ATCC 14579 does not use bile as a factor for sensing the intestinal environment.

ABBREVIATIONS

#	number(s)
~	approximately
°C	degrees Celcius
1-D	one-dimensional
2-D(E)	two-dimensional (electrophoresis)
A	absorption
ATCC	American Type Culture Collection
bp	base pair(s)
CBB	Coomassie Brillant Blau R-250
cDNA	complementary DNA
cfu	colony forming units
Chaps	3-[(3-Cholamidopropyl)dimethylammonio]propanesulfonic acid
cm	centimeter
Ct	crossing threshold
Da	Dalton
DEPC	diethyl pyrocarbonate
dH2O	destilled water
dhg	dehydrogenase
dhla	dihydrolipoamide dehydrogenase
DNA	deoxynucleic acid
dNTP	Deoxyribonucleotide triphosphate
ds	double stranded
DTT	(R,R)-Dithiothreitol
e.g.	exempli gratia (for example)
EDTA	ethylenediaminetetraacetic acid
EDTA	ethylenediaminetetraacetic acid
EMBOSS	The European Molecular Biology Open Software Suite
fbp	primer name for fibronectin binding protein (BC3873)
g	gravitational force
g	gram
gap	primer name for glyceraldehyde-3-phosphate dhg (BC5141)
h	hour(s)
i. e.	id est (that is)
IEF	isoelectric focusing
intA	primer name for internalin A (BC0552)
intB	primer name for internalin A (BC1331)
IPG	Immobilized pH gradients
IR-media	Ine Robertsen media
kb	kilobasepair(s)
kDa	kiloDalton
KEGG	Kyoto Encyclopeida of Genes and Genomes (Japan)
kg	kilogram
L or l	liter
LB	Luria Bertani
M	molar (mol/liter)
MALDI–TOF	Matrix Assisted Laser Desorption Ionization – Time of Flight
mg	milligram (10^{-3} g)
MIC	minimum inhibitory concentration (50 % reduction of growth)
min	minute(s)
ml	milliliter (10^{-3} l)

mM	millimolar (millimol/liter)
mm	millimeter (10^{-6} meter)
µm	micrometer (10^{-6} meter)
µg	micro gram (10^{-6} g)
µl	micro liter (10^{-6} liter)
µM	micromolar (µmol/l)
MOPS	3-N-morpholino propansulfonic acid
mOsm	milliosmol
mRNA	messenger RNA
MS	Mass spectrometry
NCBI	National Center for Biotechnology Information (USA)
NCBInr	NCBI non-redundant protein database
NL	Non-linear
nm	nanometer (10^{-9} meter)
ON	Over night
PAGE	Polycrylamide-gel electrophresis
PCR	Polymerase chain reaction
pmol	picomol (10^{-12} mol)
R^2	Correlation coefficient
rcf	relative centrifugal force
RFU (rfu)	relative fluorescence unit(s)
RNA	ribonucleic acid
rpm	revolutions per minutes
rRNA	ribosomal RNA
RT	room temperature
RT (PCR)	reverse transcriptase (PCR)
SDS	Sodium dodecyl sulfate
sec	second(s)
spp	species
st.dev	standard deviation
TAE	Tris acetic acid EDTA
TCA	Tricarboxylic acid (cycle), Krebs cycle
TEMED	N,N,N',N'-tetramethylethylenediamine
T_m	melting temperature
Tris	2-Amino-2-(hydroxymethyl)-1,3-propanediol
TSB	Tryptic soy broth
U	unit
V	Volt
v/v	volume/volume
w/v	weight/volume
X	degree of concentration / concentration factor
x or *	multiplied

CONTENTS

1 INTRODUCTION

1.1 *Bacillus cereus*

1.1 *Bacillus cereus*

1.1.1 The *Bacillus* genus

The *bacillus* genus is a diverse group of gram positive endospore-forming rod shaped bacteria. They are aerobic or facultative anaerobic and found ubiquitously in the environment commonly isolated from soil, water, dust and air (Reviewed by Drobniewski, 1993). The vegetative cells range from approximately 0.5 by 1.2 to 2.5 by 10 μm in length and most *Bacillus* species grow optimally at temperatures from 25 °C to 37 °C. Several thermophilic and psychrophilic species exists which can grow at temperature up to 75 °C and down to 3 °C (Drobniewski, 1993). Many *bacilli* produce extracellular hydrolytic enzymes capable of breaking down polymers such as polysaccharides, proteins or peptides and nucleic acids permitting the bacteria to use the monomers/oligomers as carbons sources and electron donors (Maidgan, 2003). Some species in the bacilli genus are responsible for the production of several antibiotics, such as bacitracin, polymyxin and cirulin. (Maidgan, 2003) Traditionally they are classified as low GC gram positive bacteria, however their GC level range from 32 % to 69 %. (Drobniewski, 1993; Maidgan, 2003)

1.1.2 The *Bacillus cereus* group

The *Bacillus cereus* group (or *Bacillus cereus sensu latu*) contains *Bacillus cereus*, *Bacillus anthracis*, *Bacillus thuringiensis*, *Bacillus weihenstephanensis*, *Bacillus mycoides* and *Bacillus pseudomycoides* (Drobniewski, 1993; Lechner *et al.*, 1998;

Nakamura, 1998). These six species are phenotypically and genetically closely related, but with regard to pathogenicity they differ; *Bacillus cereus* – food poisoning, systemic infections, *Bacillus thuringiensis* – insect pathogen and *Bacillus anthracis* – the causative agent of anthrax.

Bacillus thuringiensis has the ability to produce insecticidal crystal toxins with different specificity towards different insect larvae (*lepidoptera*, *choleoptera*, *diptera*) inside the bacteria cell during sporulation (reviewed by Schnepf *et al.*, 1998). When insect larvae ingest spores and their inclusion bodies the spores gain access to haemolymph, a source of nutrients suitable for germination and growth, resulting in the death of the larvae (Aronson, 1993). The crystal toxin genes (*cry*) are most frequently encoded on extracromosal plasmids (Gonzalez & Carlton, 1984; Sekar & Carlton, 1985). The insecticidal property has made *Bacillus thuringiensis* the most widely used biopesticide in the world, it has been in use for several decades (Hofte & Whiteley, 1989) and is therefore of immense value to the agricultural industry.

Bacillus anthracis is widely known as the causative agent of the potentially lethal disease anthrax. It is described as non-haemolytic, encapsulated and non-motile, (Reviewed by Mock & Fouet, 2001). The most lethal form of anthrax is caused by the inhalation of spores, which are taken up by alveolar macrophages. The spores are transported to lymph nodes where germination occurs. The bacteria then replicates to high numbers in the blood, ultimately leading to the death of the host (Reviewed by Abrami *et al.*, 2005). The genes encoding the lethal effect are located on two plasmids: The anthrax toxin subunits (lethal factor, LF; edema factor, EF and protective antigen, PA) are located on a plasmid; pXO1. The genes (*cap*BCAD) encoding the poly-D-glutamate capsule, in which also are implicated in virulence, is located on pXO2 (reviewed by Abrami *et al.*, 2005). The capsule is not *necessary* for uptake to macrophages, however Δ*cap*BCAD mutants are highly attenuated for inhalation anthrax (Drysdale *et al.*, 2005).

Bacillus mycoides is easily distinguished from the other members of the *Bacillus cereus* group by its rhizoid colony shape made by curving filaments of bacteria cells and its lack of motility (Priest, 1993). *Bacillus pseudomycoides* resembles *Bacillus mycoides*, but lack the long filaments of bacteria cells, hence the name *pseudes*; false,

and *mycoides*; fungus-like. It is distinguished from *Bacillus mycoides* and *Bacillus cereus* by differences in cell membrane fatty acid composition. *Bacillus pseudomycoides* was recognized as a species in 1998, and phylogenetically positioned as a member of the *Bacillus cereus* group (Nakamura, 1998).

Bacillus weihenstephanensis is distinguished from *Bacillus cereus* by its psychrophilic abilities. It is capable of growth below 7 °C, but not at 43 °C. The DNA sequence show high similarity to *B. cereus*, but have differences, especially in cold shock genes. *Bacillus weihenstephanensis* was recognized as a species of the *Bacillus cereus* group in 1998 (Lechner *et al.*, 1998). It is not known whether it is capable of causing food poisoning like *Bacillus cereus*, but in (2002) Stenfors, Mayr et al. indicated that many *B. weihenstephanensis* strains have the genetic makeup for producing essential pathogenicity factors, and that some do so under laboratory conditions. However, not all psychrotolerant strains of the *B. cereus* group are necessary *B. weihenstephanensis* (Stenfors & Granum, 2001)

The close relationship between members of the *Bacillus cereus* group has been investigated by different molecular methods: (1) rRNA studies (Ash *et al.*, 1991; Ash & Collins, 1992; Bavykin *et al.*, 2004), (2) multilocus enzyme electrophoresis (Carlson *et al.*, 1994; Helgason *et al.*, 2000), (3) multilocus sequence typing (Helgason *et al.*, 2004; Ko *et al.*, 2004; Priest *et al.*, 2004) and (4) rep-PCR fingerprinting (Cherif *et al.*, 2003). The consensus of this work seem to be that the species in the *Bacillus cereus* group are so closely related that they all belong to the same species. It also seems that it is rather the plasmid they harbor, than the chromosome, which contributes to the pathogenic specificity. Therefore, strains of *Bacillus anthracis* that have lost their plasmids have been isolated and characterized as *Bacillus cereus* (Turnbull *et al.*, 1992). Similarly *Bacillus cereus* strains that receive plasmid(s) encoding crystal toxins (from *thuringiensis*) or capsule formation (from *anthracis*) would be identified as *Bacillus thuringiensis* (Gonzalez *et al.*, 1982) or *Bacillus anthracis* (Green *et al.*, 1985), respectively. Bacteria of the *Bacillus cereus* group do have the ability to take up the pBtoxis plasmid of *Bacillus thuringiensis* by conjugation (Hu *et al.*, 2005) and transformation (Gonzalez *et al.*, 1982), and that these recipients were active against insect targets (Hu *et al.*, 2005).

The species status of the members of the *Bacillus cereus* group is therefore questionable. Ash, Farrow et al. (1991), Helgason, Okstad et al. (2000) and Kolsto, Lereclus et al. (2002) have proposed that members of the *Bacillus cereus* group should be considered belonging to the same species, and the different strains should be classified as variants of *Bacillus cereus* (e.g. *Bacillus cereus* var. *anthracis*.)

1.1.3 *Bacillus cereus*

Bacillus cereus is a motile, endospore forming, aerobe or facultative anaerobe gram positive bacterium that occurs ubiquitously in soil and in many raw processed foods such as rice, milk and diary products, vegetables and spices (Choma *et al.*, 2000; Christiansson *et al.*, 1999; Guinebretiere *et al.*, 2003). The spores of *Bacillus cereus* can tolerate harsh physical and chemical conditions, and will also survive pasteurization. Consequently they will germinate in processed foods when temperature and other conditions are desirable for vegetative growth, and competitive flora is absent.

To date four genomes of *B. cereus* strains are fully sequenced; ATCC 14579, ATCC 10987, E33L (ZK), and G9241 (Brettin *et al.*, 2005; Ivanova *et al.*, 2003; Rasko *et al.*, 2004). The latter is available in contigs at NCBI. *B. cereus* G9241 is known for causing anthrax-like disease (Rasko *et al.*, 2005). December this year the fifth genome, *Bacillus cereus* NVH391-98, was published as a draft sequence by the Doe Joint Genome Institute (USA). This strain was involved in a serious food poison outbreak in France 1998 (Lund *et al.*, 2000). The sequenced genome consists of approximately 5.5 Mb, and ~5000 protein coding sequences. Sequence comparisons with *B. subtilis* and other *B. cereus* group members reveals that while *B. subtils* has many enzymes for degradation of polysaccharides, members of the *B. cereus* group have fewer enzymes for polysaccharide degradation, but are well equipped with enzymes for degradation of polypeptides and proteins (Anderson *et al.*, 2005; Ivanova *et al.*, 2003; Rasko *et al.*, 2004; Rasko *et al.*, 2005). It was therefore proposed by Jensen *et al*. (2003) that all species in the *B. cereus* group are most likely symbionts or parasites of animals hosts rather than strict soil bacteria. This is recognized by comparisons of *B. cereus* group extracellular proteomes which reveals that many of the secreted proteins are involved in degradation of proteins (Gohar *et al.*, 2005).

Bacillus cereus has many extracellular proteins putatively involved in pathogenesis. Many of these are under control of the pleiotropic regulator *plcR* (Agaisse *et al.*, 1999; Salamitou *et al.*, 2000) and includes proteins like collagenases, phospholipases, haemolysins, proteases and enterotoxins (Gohar *et al.*, 2002). Genome analysis has indicated that the *plcR*-regulon may be involved in the regulation of 4 other transcription factors, metabolic enzymes, motility, sporulation, surface proteins, capsule formation and more (Ivanova *et al.*, 2003). *In vitro plcR* reaches expression maximum at the beginning of the stationary phase, and is regulated in a quorum sensing manner (Slamti & Lereclus, 2002). Except for the emetic and entereo-toxins, it is not known how these degradative enzymes are implicated in pathogenesis, or what types of injury they can inflict or cause, in man or insects.

Bacillus cereus is a commensialist/parasite of insects and an opportunistic pathogen, capable of causing several infections, in man. Infections caused by *Bacillus cereus* have been recorded since the beginning of the last century, and fall mainly into six categories (reviewed by Drobniewski, 1993): (1) local infections, particularly in sites of burns, injures, or the eye; (2) bacteremea and septicaemia; (3) infections of the central nervous system; (4) respiratory infections; (5) endocarditis and pericarditis; and (6) two kind of food poisoning syndromes. Usually infections occur in immuno-compromised persons, after traumatic injury like penetration wounds or burns sites, or in neonates (Reviewed by Drobniewski, 1993; and Kotiranta *et al.*, 2000) *Bacillus cereus* is the second most frequent cause of keratitis (Drobniewski, 1993) and *Bacillus cereus* eye infections are often severe and in a number of cases leads to blindness, (reviewed by Hemady *et al.*, 1990).

However, *Bacillus cereus* is most known for its food poisoning abilities. Generally it causes one of two types of syndromes; (1) nausea and vomiting, caused by an emetic toxin; or (2) diarrhea caused by at least one of three enterotoxins. Both types of syndromes are usually mild and last for less than 24 hours. Consequently food poisoning caused by *Bacillus cereus* is underreported (Granum & Lund, 1997). Some strains, however, cause rather severe infections and can in the worst case have fatal outcome. In France 1998 an outbreak of the diarrheal type led to the death of three

persons (Lund *et al.*, 2000), and in 2004 a young girl died after infection with an emetic strain (Dierick *et al.*, 2005).

The emetic syndrome is caused by a heat- acid- and protease stable emetic toxin that is produced in the food, named cereulide. The toxin does not lose activity after treatment by heat (126 °C, 90 min), cold (4 °C, 2 months), extreme pH (pH 2 and 11, 90 min) or by proteases (trypsin and pepsin) (Melling & Capel, 1978). Ingestion of living bacteria is therefore not necessary for symptoms, which usually last for 1 to 5 hours (Mortimer & McCann, 1974). The cereulide is a cyclic dodecadepsipeptide that consists of a 36-memberd ring with ester and amide bonds one after each other which is non-ribosomally synthesized (Agata *et al.*, 1994). It functions as K^+-specific ionophore that induces swelling of mitochondira in livercells, and thereby inducing cell death in the liver and vomiting (Agata *et al.*, 1995; Mikkola *et al.*, 1999). The genetic determinants of cereulide has recently been documented to be located on a plasmid (Hoton *et al.*, 2005)

The symptoms of the diarrheal type of food-poisoning is described as abdominal pain, cramps and watery diarrhea and are usually experienced 8 – 16 hours after ingestion of contaminated food and usually lasts for 12 – 24 hours (Granum, 1994) The symptoms are caused by at least one of two enterotoxins; HBL, hemolysin BL and/or NHE, non-hemolytic enterotoxin (Granum *et al.*, 1993; Lund & Granum, 1996; Lund & Granum, 1997). In addition a third toxin, cyt K, that was involved in a more severe case of food-poisoning causing necrotic enteritis has been described (Hardy *et al.*, 2001; Lund *et al.*, 2000). All these enterotoxins are regulated by the *plcR*-regulon (Gohar *et al.*, 2002). The enterotoxins are heat-liable and production of toxins in the small intestine is necessary for causing symptoms (Granum, 1994; Lund & Granum, 1997).

Except for the emetic- and enterotoxins, the molecular mechanism of pathogenesis in these infections is poorly understood. Little is also known of the ability of *B. cereus* to survive and eventually colonize the intestine. *B. cereus* is present and asymptotic in faeces of 14 % to 43 % of investigated individuals (Ghosh, 1978; Turnbull & Kramer, 1985), which implies an ability to colonize the GI-tract, but it is not known whether these bacteria are probiotic or just “passing through”. To the best of my knowledge

very little is known about the genetic mechanisms for colonization of *B. cereus* in the GI-tract.

Bacillus cereus ATCC 14579 is the reference (type) strain of *Bacilus cereus*. It is an environmental isolate and harbors the three enterotoxins, but not the emetic toxin (Ivanova *et al.*, 2003).

1.2 The Gastrointestinal tract

1.2.1 The human gastrointestinal tract

When food and bacteria are ingested they enter the oral cavity and reach GI-tract where the oral cavity ends. The first part, the esophagus, reaches from the oral cavity to the stomach. The food and bacteria enter the stomach and are subjected to low pH (~2) and digestive enzymes and muscle contractions. As foods leave the stomach it enters the small intestine, which is divided in three sections; the duodenum, jejunum and ileum. Bile and pancreatic enzymes are secreted into the duodenum and most of the digestion occurs in the small intestine. When digested foods leave the small intestine they enter the large intestine where water and electrolytes are re-absorbed.

Mucus

The inner layer of GI-tract is covered with mucus which acts as a lubricant for foods and as a protective barrier between the endothelial cells and the environment. In addition constitutes an important part of the non-specific defence system where it functions as a trap/barrier for particles and microorganisms. The mucus layer contains mostly glycoproteins and proteoglycans, in which various types of mucin are the major components. Mucin is a glycoprotein that contains over 80 % carbohydrate and is present both in soluble form in the outer level, and strongly attached to the endothelial cells (Feldman *et al.*, 1998). In addition lipids, proteins and nucleic acids are also found in the mucus-layer (Reviewed by Allen *et al.*, 1993). The composition and abundance of the mucus is subjected to variation in all parts of the GI-tract, but it makes 200 to 600 µm thick continuous gel throughout the GI-tract. (Allen *et al.*, 1993; Feldman *et al.*, 1998)

On the other side of the endothelial cells is the basal lamina which is a wide layer of extracellular matrix. The basal lamina consists of fibrous proteins such as collagen, fibronectin, laminin and elastin (Alberts *et al.*, 2002).

Bile

Bile acid and *bile salt* are generally synonymous terms; *bile acid* refers to the molecule as acid, e. g. cholic acid, and *bile salt* refer to the salt of the same molecule, e. g. natrium-cholate. In this thesis the terms bile acids and bile salts are used interchangeably.

Bile is a yellow/green aqueous solution that is secreted into the upper duodenum from the bile duct. It is synthesized in the liver, and partially stored and concentrated in the gallbladder. Its major organic constituents are bile acids (salts) cholesterols, phospholipids and the pigment biliverdin (Dawson, 1998). In addition is immunoglobin A and mucus secreted into the bile (Hofmann, 1999). Inorganic ions such as Na^+, Cl^-, and K^+ are also found and the osmolarity is generally isotonic with plasma (Erlinger, 1994) . A section of the constituents and properties of bile is listed in table 1.1. The concentration of bile acids (salts) can be up to 8 % in the gallbladder and ranging from 0.2 % to 2 % in the small intestine (Dawson, 1998).

Table 1.1 Major constituents and properties of human hepatic bile. Data collected from (Erlinger, 1994)

Constituent/property	
Sodium (mmol/l)	~145
Potassium (mmol/l)	~5
Chloride (mmol/l)	~110
Bile salts (mmol/l)	3 - 45
Cholesterol (mmol/l)	2 - 8
Phospholipids (mmol/l)	0.3 - 11
pH	7.5–8.0
Osmolarity (mOsm/kg)	~300

There are a number of different bile acids, but the most abundant are; cholic acid, deoxycolic acid, chenodeoxycholic acid and lithocolic acid, which constitute approximately 95 % of the bile acids in human bile (Carey & Duane, 1994). Both cholic acid and chenodeoxycholic acid are conjugated to either glycine or taurine by peptide bonds before secretion. The intestinal flora modify these bile acids by

cleaveage of the peptide bond to form unconjugated bile acids (Carey & Duane, 1994) These reactions are usually carried out by bacterial enzymes called bile salt hydrolases (Christiaens *et al.*, 1992; Desmet *et al.*, 1995; Grill *et al.*, 1995). 7α-dehydroxyalation which converts cholic acid to deoxycholic acid and chenodeoxycholic acid to lithocholic acid is also a process performed by the microflora in the intestine and seems to be limited to anaerobic bacteria such as *Eubacterium* and *Clostridium* (Doerner *et al.*, 1997; Wells & Hylemon, 2000) The structures of these bile acids is shown in figure 1.1

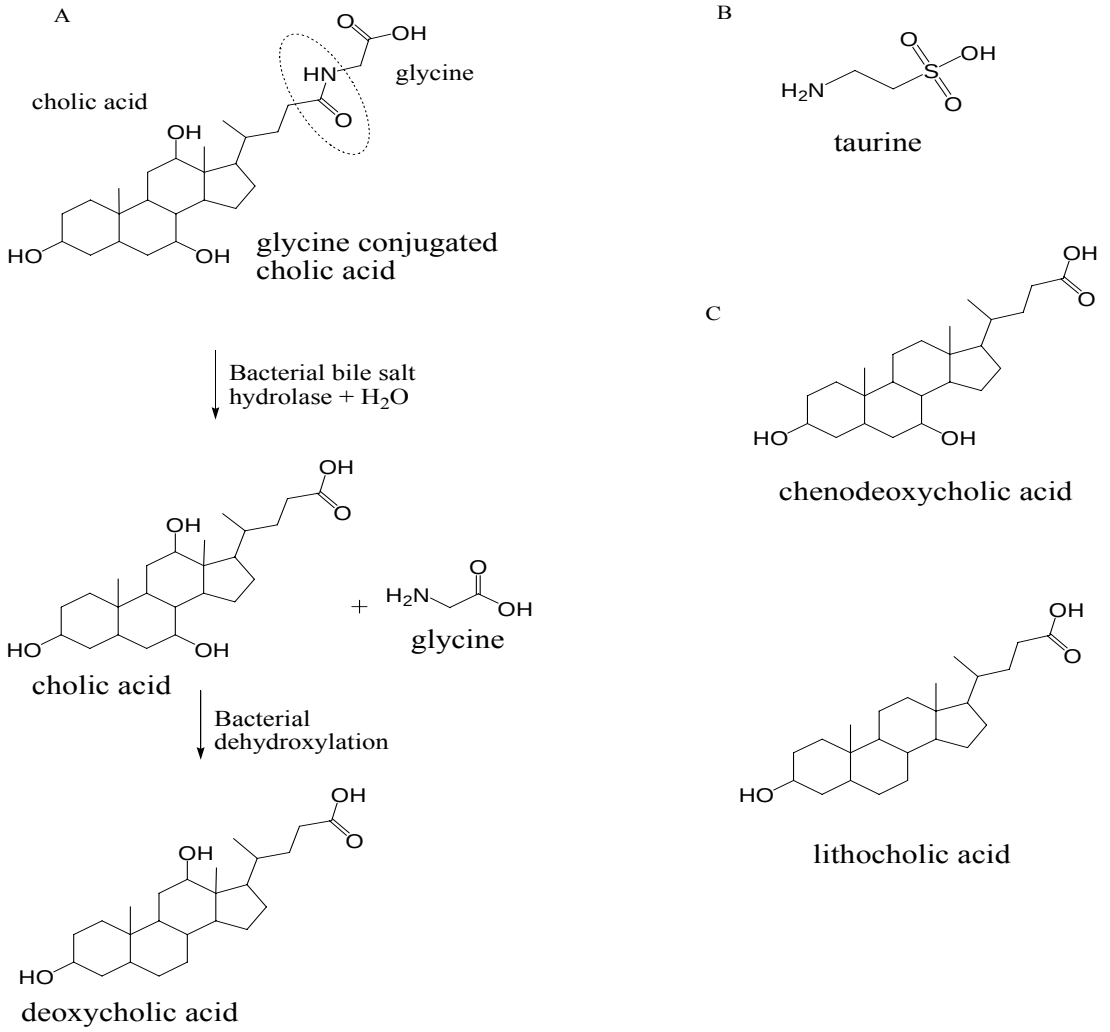


Figure 1.1 A) Modifications of bile acids by bacteria. Bile acids are conjugated with different amino acids by peptide bonds. Glycin conjugated cholic acid is cleaved to cholic acid and glycine by bile salt hydrolases. Cholic acid can again be modified by 7α-dehydroxylation to form deoxycholic acid. B) Taurine; bile acids can also be conjugated with taurine. C) Two other common bile acids; chenodeoxycholic acid and its dehydroxylated derivate; lithocholic acid.

The major function of bile is the solubilisation and emulsification fats. This also makes bile a potent antimicrobial agent and it has an important role in the innate

immune-system. The detergent properties of bile are mostly due to bile acids (salts) which make up approximately 67 % (v/v) of bile from healthy humans (Carey & Duane, 1994). Although it is the detergent property that has most destructive effect on bacterial membranes, bile acids can also harm bacteria in other ways: Bile salts may induce secondary structure in RNA and induce DNA damage (Kandell & Bernstein, 1991; Payne *et al.*, 1998; Powell *et al.*, 2001; Zheng & Bernstein, 1992). The detergent actions of bile salts *may* also lead to misfolding of proteins and the induction of chaperone genes as a response to bile acids, observed in many bacteria, is strong indicator to that denaturation may occur (Flahaut *et al.*, 1996; Leverrier *et al.*, 2003; Schmidt & Zink, 2000). Bile may cause oxidative stress through the generation of free radicals (Lechner *et al.*, 2002; Sokol *et al.*, 1995) and in addition bile acid may chelate calsium and iron (Rajagopalan & Lindenbaum, 1982; Sanyal *et al.*, 1991)

1.2.2 Bacteria in the gastrointestinal tract

Bile tolerance

Some bacteria can tolerate very high levels of bile, e.g *Salmonella typhimurium* and *E. coli* have the ability to colonize the gallbladder (Prouty *et al.*, 2002a), and *E. coli*, C*amplyobacter* spp., *Streptococcus* spp. and *Clostridium* spp. has been isolated directly from bile (Brook, 1989; Flores *et al.*, 2003). *Salmonella typhimurium* has the ability to survive prolonged incubation in 60 % bile (oxbile) *in vitro* (Van Velkinburgh & Gunn, 1999). Other entero-pathogeneic bacteria also have a rather high tolerance to bile; e.g. *Salmonella typhi* tolerate 12 % oxbile in the growth media (Van Velkinburgh & Gunn, 1999) and *Listeria monocytogenes* survives in broth supplemented with 15 % human bile, and 30 % oxbile (Begley *et al.*, 2002). Interestingly many commensial bacteria have much lower bile tolerance. Hyronimus *et al.* (2000) et al. investigated bile tolerance in spore forming lactic acid bacteria, and found that only 5 of 13 tested strain tolerated 0.3 % oxbile in the growth media. For different *Lactobacillus* strains tested by Jacobsen *et al.* (1999) 31 of 47 strains completely stopped replication, and 5 strains had delayed growth.

Although some genera such as *Listeria* are considered bile tolerant, it seems likely that for most bacteria bile tolerance is a strain specific property. For example is *Enterococcus faecalis* ATCC 19433 rapidly killed by 0.3 % bile salts (Flahaut *et al.*, 1996), while other *Enterococcus faecalis* strains have been isolated directly from bile

(Flores *et al.*, 2003). Of the 47 *Lactobacillus rhamnosus* strains investigated by Chateau *et al.* (1994) 3 strains were classified as resistant, 5 as tolerant, 3 showed low tolerance, and 11 were classified as sensitive, when subjected to 0.3 % bile salts. Similar results are also seen for other bacteria such as *Bifidobacterium*, *Propionibacterium* and other *Lactobacillus* strains. For details see: (Buck & Gilliland, 1994; Gupta *et al.*, 1996; Ibrahim & Bezkorovainy, 1993; Jacobsen *et al.*, 1999; Zarate *et al.*, 2000). To my knowledge only one study is published about bile tolerance in *B. cereus;* Spinosa *et al.* (2000), showed that *B. cereus* AF172711 had a minimum inhibitor concentration (MIC) of bile salts below 0.01 %.

It should be noted that the bile tolerance observed for many bacteria *in vitro* may not truly reflect the bile composition and other factors encountered *in vivo*. In periods of starvation the bile concentration in the intestine can be rather low, and exposure to such low concentrations (and other type of stresses) may elevate many bacteria's tolerance to bile. Flahaut *et al* (1996) discovered that when *E. faecalis* was challenged with 0.3 % bile salts only 0.05 % of the bacteria survived. When these bacteria were pre-exposed to 0.08 % bile salts for only 15 seconds 85 % of the bacteria survived. Experiments done with *Bifidobacterium adolescentis* revealed that pre-exposure to 0.1 % bile resulted in 300-fold protection against 0.3 % bile (Schmidt & Zink, 2000). Pre-conditioning to other type of stresses may also elevate bile tolerance. In *Propionibacterium freudenreichii* pre-treatment with SDS, heat (42 ºC) and elevated osmolarity induced good protection against otherwise lethal levels of bile salts (Leverrier *et al.*, 2003).

Genes involved in bile tolerance

The genetics of bile tolerance are poorly understood, but this is an area of active investigation and some studies have been published. Upon bile challenge bacteria initiate stress responses that in many ways are similar to the responses seen when challenged with other stresses such as heat, osmolarity, pH etc. Many general stress proteins are induced, e. g. GroESL, DnaK, alternative sigma factors, DNA repair genes among others (Bernstein *et al.*, 1999; Flahaut *et al.*, 1996; Gahan *et al.*, 2001; Leverrier *et al.*, 2003; Leverrier *et al.*, 2004; Rince *et al.*, 2000). Although general stress response seem crucial for bile tolerance, more specific responses are also essential. The most important factors seem to be active transport of bile acids out of

the cell and the action of bile salt hydrolases. In several bacteria a number of transporters are up-regulated by bile challenge, and deletion mutants for this type of transporter are bile sensitive. Many of these transporters are multidrug transporters and also important for resistence other toxic compounds such as antibiotics and heavy metals, for details see: (Elkins *et al.*, 2001; Lin *et al.*, 2002; Lin *et al.*, 2005; Thanassi *et al.*, 1997; Yokota *et al.*, 2000) Bile salt hydrolases cleave the peptide bond in conjugated bile acids, and seem to be an important factor in bile tolerance (Dussurget *et al.*, 2002; Elkins *et al.*, 2001; Grill *et al.*, 2000). It has also been proposed that bacteria may incorporate unconjugated bile acids and cholesterol in the cell membrane, and that this somehow will promote tolerance (Dambekodi & Gilliland, 1998; Taranto *et al.*, 2003). Not unexpectedly *Lactobacillus reuteri* (and probably many other bacteria) alter the membrane upon bile challenge (Taranto *et al.*, 2003). The 7α-dehydroxylation may also be involved in bile-tolerance, but the mechanism behind this is not understood. In gram negative bacteria the lipopolysaccaride (LPS) has an important role in response to bile challenge (Lacroix *et al.*, 1995; Nesper *et al.*, 2001; Nesper *et al.*, 2002; Prouty *et al.*, 2002b) Although many of the above mentioned genes are involved in protection against bile, we still know very little about the molecular mechanisms behind these protective genes.

Pathogenisis in the Gastroontestinal tract

Strict regulation of virulence (and other) genes in response to the microenvironment is highly important to ensure that cellular energy is not wasted on the expression of un-necessary genes. Enteric pathogens meet a number of challenges as they enter the GI-tract. Although these challenges (pH, temperature, iron starvation, bile etc.) are potentially harmful to bacteria, many bacteria uses such conditions as environmental signals for the induction of a virulent response. (Reviewed by Mekalanos, 1992). It seems that many enteric pathogens have co-opted bile as an environmental cue to determine the organism's intestinal location so that a temporal pattern of virulence factor expression is established (discussed below).

Salmonella typhimurium represses both invasion and flagella genes and thus this bacteria represses its invasive capacity in the intestinal lumen where bile concentration are high, and invasion then can be initiated where bile concentration are lower, e.g. in the mucus layer distal ileum (Prouty & Gunn, 2000; Prouty *et al.*, 2004;

Wells *et al.*, 1995). In *Vibro cholera* bile acids directly induce expression of cholera-toxin (Hung & Mekalanos, 2005) and motility genes, while other virulence genes are down-regulated (Krukonis & DiRita, 2003; Schuhmacher & Klose, 1999), suggesting an attempt to move towards lower bile concentration. Both *Shigella* spp (Pope *et al.*, 1995) and *E. coli* (de Jesus *et al.*, 2005) have an elevated ability to adhere to epithelial cells which promotes invasion after stimulated with bile. When *Enterococcus faecalis* is incubated with bile it alters the physicochemical properties of its surface, which elevates its ability to invade biliary drain materials (Waar *et al.*, 2002). There is not much data published on the correlation between bile and pathogenicity and *Listeria monocytogenes*. However, a bile salt hydrolase, under the control of the principal regulator of *Listeria* virulence genes (PrfA), is important for survival in the presence of bile and implies a connection between bile and virulence (Dussurget *et al.*, 2002).

When enteropathogenic bacteria have survived the bile challenge, and some of them have also initiated the preparation for colonization/invasion by induction of virulence genes, they face a new challenge before pathogenicity can be induced; the mucus layer. Bacteria have to attach to the mucin layer and to some degree (dependent on colonization or invation) also penetrate if they will avoid transportion with the foods. The ability to break down mucin is important as bacteria have to penetrate a mucus-layer that is 25 to 200 times their own length. Little information about bacteria's interaction with the mucus layer is available, but this has been investigated to some degree in a few species: V*ibrio cholera* induces expression of a protease (Hap) when supplemented with mucin in the growth media. This protease hydrolyses mucin and is necessary for penetration of a mucin containing gel. In addition it has a role in the activation of the cholera toxin (Silva *et al.*, 2003). Alvarez *et al.* (2003) discovered that glyceraldehyde-3-phosphate dehydrogenase is involved in adhesin to the mucus layer. In addition the ability to break down carbohydrate monomers may be an important factor in degradation and/or adhesion to mucus (Reviewed by Lingwood, 1998). Some strains of *B. cereus* do have such an enzyme; 1,2-α-L-fucosidase, which cleaves, by hydrolysis, fucose residues in oligosaccharides (Miura *et al.*, 2005).

1.3 Aims of this study

Problem

Are pathogenicity and colonization factors induced in *Bacillus cereus* ATCC 14579 when factors such as, bile, bile salts or mucin, are encountered *in vitro*?

The aims of this study are:

(1) Devise a method for the simulation of some of the conditions found in the human Gastrointestinal tract, using bile, bile salts and mucin in the culture media.

(2) Determine the physiological response / tolerance of *B. cereus* ATCC 14579 to bile, bile salts and mucin.

(3) Determine the *B. cereus* ATCC 14579 genetic response to such conditions, with focus on surface proteins, using

   i. Real-time-RT PCR
   ii. Microarray (whole genome microarrays)
   iii. 2-D protein gel-electrophoresis

Part of the following work was carried out in collaboration with Solveig Sirnes, (real-time RT-PCR analysis), Ine Robertsen (Bacterial growth and RNA isolation) and Dr. Solveig Ravnum (microarray analysis).

2 MATERIALS AND METHODS

2.1 Introduction to the methods section

2.1.1 Materials, chemicals and buffers

All materials/kits/solution/buffers, used in this work, and their suppliers, are specified in the text where first mentioned. Salts, simple organic compounds and other routinely used chemicals were of *pro analysi*s quality and suppliers are not listed.

All buffers were adjusted to the correct pH with 5 M NaOH or 5 M HCl with the exception of the phosphate buffer, where 0.2 M Na_2HPO_2 or 0.2 M NaH_2PO_2 was used.

2.1.2 Collaboration

Part of this work was carried out in collaboration with co-students and other researchers. Collaboration is specified in the methods section and in the introduction.

2.2 Growth of Bacteria and shift experiments.

2.2.1 Bacteria strain, culture conditions

Bacillus cereus ATCC 14579, the type strain of *Bacillus cereus*, was obtained from Professor Anne Britt Kolstø, School of Pharmacy, Oslo University. This strain was used in all the experiments. Bacteria were grown in IR-media (Ine Robertsen media, table 2.1), which is composed of 50 % LB, 50 % mineral media and glucose. This medium gives less variation in the pH than the more "traditional" media such as LB or TSB (Robertsen, 2005). The growth medium was sterilized by filtration through a 500 ml Stericup® 0.22 μm filter (Millipore, Billerica, Massachusetts, USA). Growth

on agar plates was carried out using LB-agar (table 2.1). The LB-agar was sterilized by autoclaving and plates were prepared either using a PourMatic® MP-1000 (New Brunswick Scientific, N.J. USA) or poured by hand. LB-agar plates with bile salts were prepared by autoclaving LB-agar and adding sterile 20 % (w/v) bile salts, Na-cholate and Na-deoxycholate in 1:1 relation (Fluka, Buchs, Switzerland) to correct concentration before pouring the plates.

Table 2.1 Bacteria growth media; IR-media (50 % LB and 50 % glucose mineral media) and LB-plates. (1) From Becton, Dickinson and Company, Le Pont de Claix, France.

IR-medium		**LB-agar**	
K_2HPO_4	0.8 g	Tryptone (1)	10 g
KH_2PO_4	0.2 g	Yeast extract (1)	5 g
$CaSO_4 \cdot 2H_2O$	0.05 g	NaCl	10 g
$MgSO_4 \cdot 7H_2O$	0.25 g	Agar (1)	20 g
Glucose	5 g	dH_2O	to 1 L
Tryptone (1)	5 g	Dissolve in dH_2O and adjust volume to 1 L. Autoclave and cool to ~50 °C before plating out.	
Yeast extract (1)	2.5 g		
NaCl	5 g		
dH_2O	to 1 L		
Dissolve $CaSO_4 \cdot 2H_2O$ in ~500 ml dH_2O, then add the remaining ingredients and adjust volume to 1 L. Sterile filtration.			

The mucin containing medium was very viscous, and impossible to sterilize by filtration. Autoclaving could not be used because it would lead to denaturation of the mucin proteins, and the sugar molecules would react with peptides or proteins in the media. Therefore, the medium containing mucin was prepared by dissolving 5 g mucin (from porcine stomach, type III, Sigma, Steinheim, Germany) in 85.6 ml 1 M NaOH and incubated over night (ON) at room temperature (RT). This was then neutralized by adding 1 M HCl (~85.6 ml). The NaOH and HCl correspond to 5 g NaCl, and the IR-medium was prepared without the addition of NaCl. The dissolved

mucin was added to sterile IR-medium (without NaCl) prepared in a total of 829 ml dH_2O to make IR-medium containing 0.5 % (w/v) mucin. The mucin media were streaked out on LB-plates to check if previously detected bacteria were present.

Unless otherwise noted, bacteria were grown at 37 °C with shaking at 175 rpm in 1 L Erlenmeyer flasks containing 100 ml IR-medium. The growth medium was preheated to 37 °C ON and checked for contamination prior to use. One vial of ON culture (10 µl suspension with 3 µl 87 % glycerol), section 2.2.2, was used as the inoculum per 100 ml growth medium. Standard sterile technique was employed in all experiments and the bacterial suspensions were checked for contamination by microscopic examination in a Biomed phase contrast microscope (Leitz, Portugal).

2.2.2 Generation of over night cultures

To eliminate possible changes in the bacterial strain during the period of this work a large stock of ON cultures was prepared. Approximately 10 µl of a *Bacillus cereus* ATCC 14579 suspension was plated out on LB plates and grown ON at 30 °C. Twenty ml IR-media in a 100 ml Erlenmeyer flask was inoculated with one well isolated colony and grown ON (~16 h.) at 37 °C with shaking at 175 rpm. The cell density of the suspension was determined by flow cytometry. Approximately 50 x 10 µl suspensions were mixed with 50 X 3 µl 87 % glycerol and stored at -70 °C until use.

2.2.3 Growth curves and measurement of pH, glucose and the cell density

To establish the growth curve one batch of ON cultures was inoculated into 100 ml IR-media and grown until the culture was well into the stationary phase. Samples for the measurement of pH, glucose and the cell density were taken every hour.

pH was measured by applying 20 µl suspension to Neutralit® or Acilit® pH strips provided by Merck KgaA (Darmstadt, Germany).

The concentration of glucose in the media was measured using Glucose (GO) assay from Sigma. One ml of bacteria solution was removed every hour for a total of 10 hours after inoculation. The bacteria solution was filtrated through a 0.22 µm pore filter and stored at 4 ºC over night. One glucose-oxidase/peroxidase-reagent capsule was dissolved in 39.2 ml dH_2O, before 0.8 ml o-Dianisidine Reagent was added. All the samples were diluted in dH_2O to give a glucose concentration below 50 µg/ml. IR-medium was used to make a standard with concentrations between 0 and 60 µg/ml glucose. Two ml assay reagent was added to each sample in a 5 ml polystyrene tube. The tubes were incubated in the dark for 30 min at 37 ºC. The reaction was terminated by adding 2 ml 6 M H_2SO_4. Absorbance was measured at 540 nm on a PU 8750 UV/VIS Scanning spectrophotometer from Philips (UK).

The bacteria cell density was measured using the Bacteria Counting Kit from Molecular Probes (Eugene, Orgeon, USA) and counted on a Becton Dickinson FACSCalibur Flow Cytometer, with the software: BD CellQuest Pro (both: Becton and Dickinson, San Jose, California, USA). The bacteria suspension, 500 µl, was diluted 5X, 10X, 100X or 1000X to give a measurement between 200 and 2000 fluorescent units, and a number of reference sphere fluorescent units between 100 and 1500. One µl of florescent dye and 10 µl microspheres were added to 1 ml of the diluted bacterial suspension. The forward scatter emission was measured at 500 nm for both the bacteria and the spheres and the bacterial density was calculated using formula (1) shown below. Three measurements were made for each time point sample and the average cell number was used. The standard deviation between each measurement was below 10 %.

$$\text{Cell density (cell\#/ml)} = \text{fs(bacteria)/fs(spheres)} \times 10^6 \times \text{dilution factor}, \quad (1)$$

where fs is the number of florescent units from either the bacteria or the spheres.

2.2.4 Calculations

Generation time

The generation time in exponential phase was estimated using the following formula (2)

$$n = 3{,}3 \times (\log N - \log N_0)$$
$$g = t/n, \qquad (2)$$

where n is the number of generations, N is the final cell number and N_0 is the start cell number. g is the generation time and t is the time in minutes.

Standard deviation

The standard deviation between parallel measurements (cell density, log2 ratios, etc) in this work was calculated with the non-biased method, formula (3).

$$\text{Standard deviation} = \sqrt{\frac{\sum(x_i - x)^2}{n}} \qquad (3),$$

where n is the number of observations, x_i is the value of each observation and x is the average of all observations

2.2.5 Growth of *Bacillus cereus* in the presence of bile and bile salts.

A concentration screening was performed to investigate *Bacillus cereus* ATCC 14579 tolerance towards bile and bile salts. Two vials with frozen ON-cultures were cultured in 205 ml IR-medium in a 1 L Erlenmeyer flask at 37 °C and 175 rpm. After 2.5 hours (for the bile culture) and 3 hours (bile salts culture) the culture was portioned into (volume?) four 1 L flasks and bile (Ox-bile, partially purified, Fluka) or bile salts were added. The final concentrations were, bile; 0 %, 0.0125 %, 0.0375 % and 0.375 % (all w/v) and bile salts; 0 %, 0.001 %, 0.005 % and 0.01% (all w/v) respectively.

2.2.6 Bile salts adaptation

The following experiment was performed to test if *B. cereus* ATCC 14579 would tolerate higher concentration of bile salts after a prior exposure to a low concentration..

B. cereus ATCC 14579 was grown for 3 hours in 205 ml IR-media. The culture was divided in two and shifted to new media; 100 ml was shifted to 100 ml IR-medium

with no additives. 100 ml was shifted to 100 ml IR media containing 0.01 % (w/v) bile salts, to give a final concentration of 0.005 %. Samples were removed after 5 (bile salt culture) and 30 minutes (bile salt and control). The suspensions were diluted 2000X (bile salts, 5 min), 3333X (bile salts, 30 min), and 3846X (control, 30 min) and 100 µl were plated out on LB-agar plates with concentrations of bile salts ranging from (all w/v): 0.001 %, 0.005 %, 0.01 %, 0.02 %, 0.05 %, 0.10 %, 0.20 %, 0.50 %, and 1 %. The diluted suspensions were plated out in triplicate for each concentration of bile salts. To determine the number of bacteria plated out from each dilution, each dilution was diluted further; 10X and 100X and plated on LB-agar plates. Plates were incubated ON at 37 °C. The experiment was repeated once.

2.2.7 Shift experiments

A culture of *B. cereus* ATCC 14579 in exponential growth was shifted to new media containing different additives (bile, bile salts and mucin). A start culture with 410 ml IR media in a 3 L Erlenmeyer flasks was inoculated with 4 vials of ON culture. The start-culture was grown for 3 hours and 4 x 100 ml aliquotes were removed and added to 4 new flasks containing: 1) 100 ml IR medium (control); 2) 100 ml IR-media containing 0.06 % (w/v) bile which gives a final concentration of 0.03 % (w/v) bile; 3) 100 ml IR-media containing 0.01 % (w/v) bile salts which gives a final concentration of 0.005 % (w/v) bile salts; 4) 100 ml IR-media containing 0.5 % (w/v) mucin which gives a final concentration of 0.25 % (w/v) mucin. All pre-equilibrated to 37 °C. A schematic presentation is shown in figure 2.1.

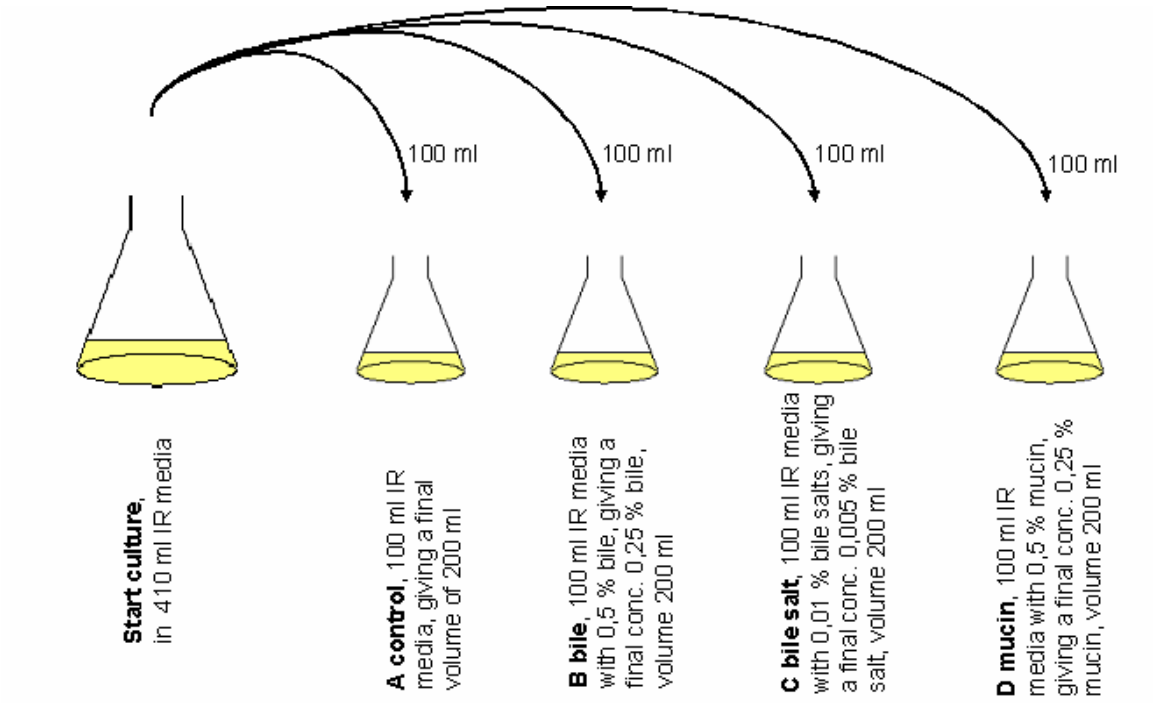


Figure 2.1 Shift experiment. After three hours of growth the start culture was shifted to four new flasks containing 100 ml IR to give four new 200 ml cultures containing: A – no additives, B 0.03 % (w/v) bile, C 0.005 % (w/v) bile salts and 0.25 % (w/v) mucin.

Samples for RNA isolation and cell counting for RealTime RT PCR were removed after 0, 5, 10, 20, and 30 minutes (in collaboration with Solveig Sirnes).

Samples for RNA isolation and cell counting for microarray analysis were removed after 0, 15, 30, and 60 minutes. The mucin shift culture was not used. (In collaboration with Ine Robertson.)

Samples for protein isolation and cell counting were removed at 0 and 30 minutes from the control (A) and after 30 minutes for bile (B) and bile salts (C) cultures. The mucin shift culture was not used in the 2D-protein electrophoresis experiment.

2.3 *In silico* investigation of potential virulence genes and PCR-primer selection

2.3.1 Criteria for the selection of potentially interesting genes.

Potentially interesting genes for use in real-time RT-PCR were selected using the following criteria; they should in some way be involved in virulence, they should be associated with the cell wall or membrane and finally, they should possibly be up-regulated when the bacteria encounter the types of conditions which are found in the human intestine. Few cell wall/membrane virulence factors are documented in *Bacillus cereus* therefore the presence, in *Bacillus cereus*, of homologues of known virulence factors in other gram positive bacteria was investigated. In addition, the presence of a plcR–box in the promoter region of the said genes was also investigated. On the basis of these criteria several genes were identified (see section 3.2.1) and three of them were selected for further analysis using Real-time RT PCR. The selected genes were two internalin A genes and a fibronectin binding protein gene (primers named; intA, intB, and fbp, respectively) The gene for glyceraldehyde-3-phospate dehydrogenase (primer named gap) was chosen as a reference.

2.3.2 Analysis of genes coding for potential virulence factors using bioinformatics

The amino acid sequence similarity between *Bacillus cereus* ATCC 14579 genes and known virulence factors from *Listeria monocytogenes* and *Streptococcus spp.* (sequences obtained form NCBI, www.ncbi.nlm.nih.gov/) was detected using the basic local alignment program, “blastp” or “tblastn” provided in the EMBOSS package (http://emboss.sourceforge.net/). The similarity between potentially homologous genes was further investigated by generating a Needleman-Wunch global alignment, with the BLOSUM62 matrix (or the BLOSUM20 matrix for distantly related sequences), between the sequences of interest, using the “needle” algorithm in the EMBOSS package. Multiple alignments were generated with the clustalW program (ver. 1.83), with the BLOSUM62 matrix in the EMBOSS package. The genes were investigated for known domains or motifs by using the integrated motif/domain database; Interpro (www.ebi.ac.uk/InterProScan). Interpro scans several domain/motif databases: BlastProDom, HMMTigr, TMHMM, FPrintScan, ProfileScan, HMMPanther, HMMPIR, ScanRegExp, HMMPfam, SuperFamily,

HMMSmart and SignalPHMM. In the results section reference is made to the actual database in which the domain/motif was found, even though the search was performed at Interpro. To scan for plcR consensus binding sequences an embl formatted sequence of the *Bacillus cereus* ATCC 14579 genome (downloaded from http://www.ebi.ac.uk/cgi-bin/sva/sva.pl?) was uploaded to Artemis, and the sequence was queried with the consensus sequence (plcR: tatgnannnntncata) with variables. The gene was interpreted as a putative plcR regulated gene if the consensus sequence was found 20 to 200 nt upstream of the translation start of the gene in question (Slamti & Lereclus, 2002).

2.3.3 Oligonucleotide primer selection

The DNA sequences for BC0552 (IntA), BC1331 (IntB), BC3873 (fbp) and BC5141 (gap) were obtained from the KEGG (Kyoto Encyclopaedia of Genes and Genomes, www.kegg.com) database. Primers were selected using eprimer3 in the EMBOSS package. The optimum melting temperature was set to 60 °C, the primer size to ~20 nt and the product size to ~200 nt. Primers containing 4 or more repeats of the same base were excluded. The primers were then checked for similarities in the *Bacillus cereus* genome using "blastn" in the EMBOSS package. The primers were synthesized by MWG-BIOTECH AG (Ebersbergand, Germany) and are listed in table 2.2.

Table 2.2 Oligonucleotide primers: F – forward primer, R – reverse primer. The full gene names are given in table 3.2. The melting temperature (T_m) is the one calculated by MWG, not with the design program eprimer3.

Primer name	Oligonucleotid sequence	T_m	Product size
fbpF	TGGCTCTCACGTTGTCATTC	57.3 °C	193
fbpR	TACGGTTTGCTGATTGTCGT	55.3 °C	
intAF	ACGAGCCAACAACAGGAGTT	57.3 °C	200
intAR	TTGTCCACCTGTTGCTCCTA	57.3 °C	
intBF	TGAAACGGCTATTGATGCAG	55.3 °C	196
intBR	CACATATCCTTTCGCCCAAT	55.3 °C	
gapF	ACGCGGAATGATGACAACAA	55.3 °C	183
gapR	ACAGCGCCACCGTTTAATTT	55.3 °C	

2.4 Real-time RT-PCR (in collaboration with Solveig Sirnes)

Expression pattern of internalin and fibronectin binding protein genes under bile, bile salts and mucin stimuli was investigated by Real-time RT-PCR. RNA was isolated from all cultures (A, B, C, and D, see section 2.2.7) at different time intervals (0-5-10-20-30 min.) after the shift.

2.4.1 RNA isolation

RNase free filter tips and DEPC (DiEthylPyroCarbonate)-treated water (0.1 % (v/v) DEPC in dH_2O, stirred for 24 h and autoclaved) was used in all the experiments in this section.

Samples (10 to 20 ml bacteria suspension corresponding to ~$1x10^9$ cells) were removed from the shifted cultures and added to an equal volume of ice-cold methanol and incubated for 5 minutes at RT to kill the bacteria. The suspensions were then stored on ice until they were pelleted by centrifugation at 4 °C and 4000 x g (rcf) in an IEC centra cl3 centrifuge (Thermo Electron Corporation). The supernatant was removed and the bacteria pellets were stored at – 70 °C until the day of usage (maximum 2 days).

Total RNA was isolated using the FastRNA® Pro Blue Kit and the FastPrep® FP 120 Instrument from Qbiogene (Illkirch Cedex, France) according to the manufactures protocol (appendix I). The pellet from the shift experiments was used. The samples from the medium containing mucin were re-extracted using chloroform:isoamyl alcohol (24:1 v/v) (point 12 in the protocol, see appendix I). The isolated RNA was dissolved in 100 µl DEPC-treated H_2O and stored at -70 °C.

RNA concentrations were determined by diluting RNA in DEPC-treated water and measuring the absorbance 260 nm on a PU 8750 UV/VIS Scanning spectrophotometer. The RNA purity was estimated by measuring the ratio of the absorbance at 260 nm and 280 nm. The RNA was diluted to give absorption (A_{260nm}) between 0.1 and 0.9 before measuring, and the concentrations were calculated using following formula (4).

$$\mu l\ RNA/ml = Dilution\ factor \times 40 \times A_{260nm} \quad (4)$$

RNA purity was estimated by dividing the A_{260nm} on A_{280nm}.

Denaturing formaldehyde gel electrophoresis was used to estimate the RNA integrity and possible DNA contamination. To prepare the gels, 1.2 % (w/v, final concentration) agarose was melted in DEPC-treated water by heating in microwave oven. After cooling 10X MOPS-buffer and formaldehyde (12.3 M) was added to final concentrations of 1X and 2.2 M, respectively. MOPS-buffer (10X) contains: 2 M 3-N-morpoline propane sulfonic acid (MOPS), 800 mM Sodium acetate and 100 mM EDTA (pH 8).

The following: 5.0 µl RNA or RNA ladder; 2.0 µl 10xMOPS buffer; 1.0 µl DEPC-water; 2.0 µl formaldehyde and 10.0 µl formamide were mixed in a PCR tube and samples were denatured by heating at 70 °C for 15 minutes. Eight µl of the samples were then mixed with 2 µl GEL loading buffer (New England Biolabs, Ipswich, MA, USA) and loaded on the gel. RNA ladder (10 µl) was also loaded (New England Biolabs). The gel was run in 1X MOPS buffer for ~70 minutes at 3.5 V/cm (70 V for 20 cm gels) in a fume hood. The gel was stained using 0.5 µg/ml ethidium bromide in 0.1 M ammonium-acetate (in DEPC-treated H_2O) for 45 minutes then destained in DEPC-treated H_2O ON.

2.4.2 Testing of the PCR-Primers

The specificity of the primers were tested by with the amplification of genomic DNA extracted from *Bacillus cereus* ATCC 14579 followed by agarose gel electrophoresis of the PCR product.

The following was mixed in a PCR tube (A master-mix was used for buffer, dNTPs, water, and DNA template):

2.5 µl 10x PCR reaction buffer (Finnzymes, Espoo, Finland)
1.25 µl dNTP (200 µM)
16.25 µl dH_2O
2.0 µl DNA template
2.5 µl Forward and reverse primers (0.2 µM of each)
0.5 µl DyNAzyme™ II DNA polymerase (Finnzymes)

PCR was performed with following protocol:

Initial denaturation	2 min	94 ˚C
Denaturation	30 sec	94 ˚C ┐
Annealing	30 sec	58 ˚C 30x
Elongation	30 sec	72 ˚C ┘
Final elongation	5 min	72 ˚C

A 1.5 % (w/v) agarose gel was prepared by mixing 0.75 g agarose (Sigma) in 50 ml 1X TAE and heating in microwave oven until all the agarose was dissolved. Five µl PCR product or 5 µl 100 bp ladder (New England Biolabs) were mixed with 5 µl 1 X Gel loading buffer (New England Biolabs) and loaded on the gel. Electrophoresis was preformed at 80 V for ~50 min. The primers tested are listed in table 2.2

TAE (1X) contains 4.84 g Tris (2-Amino-2-(hydroxymethyl)-1,3-propanediol), 1.14 ml glacial acetic acid, 2.00 ml 0.5 M EDTA (ethylenediaminetetraacetic acid) (pH 8.0), to 1 L with dH_2O.

2.4.3 Real-time PCR

The mRNA levels of fbp, intA and intB in the shift experiments were determined by real-time RT-PCR.

DNase treatment

Samples were treated with DNase to remove any traces of genomic DNA prior to reverse transcription. The Deoxyribonuclease I, Amplification Kit from Invitrogen

Ltd. (Paisley, UK) was used. For each target sequence (to be amplified by reverse transcriptase) one µg RNA was used. The following was mixed in a tube:

1 µg RNA

1 µl 10 X buffer

1 µ1 DNase was added. The reaction volume was adjusted to

to 10 µl with DEPC-treated H_2O.

The reaction was incubated at RT for 15 minutes, stopped by adding 1µl 25 mM EDTA for each 10 µl reaction volume and incubated at 65 °C for 10 minutes.

Reverse transcriptase

Synthesis of cDNA was performed by reverse transcription of mRNA using the Super Script™ II Reverse Transcriptase Kit (Invitrogen). The following was mixed in a tube:

11 µl DNase treated RNA (1 µg)

1 µl gene specific reverse primer (2 pmol/µl)

1 µl dNTP mix (10 mM each)

The mix was incubated at 65 °C for 5 minutes, chilled on ice and 4 µl 5 X First strand buffer and 2 µl 0.1 M DTT was added. The mix was pre-heated for 2 minutes at 42 °C before 1µl Superscript™ II reverse transcriptase (200 U/µl) was added. The reaction was incubated at 42 °C for 50 min and terminated by heating at 70 °C for 15 minutes.

Real-time PCR, principle

Real-time PCR can be used in the relative quantification of cDNA (mRNA). A fluorescent dye (SYBR® Green), which binds to dsDNA, can be used to estimate the amount of PCR product formed after each PCR cycle. This data can in turn be used to determine the relative abundance of one population of starting molecules with another. The point where each reaction reaches the logarithmic growth, called the Ct-value, is proportional to the amount of starting material. A reference gene comparison is necessary in order to eliminate differences between samples caused by differences in RNA (cDNA/DNA) concentration in the starting population. The expression of each gene is presented relative to the reference gene (when using the Pfaffl-method).

Procedure

Real-Time PCR was performed on a LightCycler® instrument provided by Roche (F. Hoffmann-La Roche Ltd, Diagnostics Division, Basel, Switzerland). The reaction was carried out using the DyNAmo™ Capillary SYBR® Green qPCR kit from Finnzymes in LightCycler® Capillaries (Roche). The cDNA samples were diluted in series to 10^{-1}, $10^{-1.5}$, 10^{-2}, $10^{-2.5}$ and 10^{-3} dilution (see figure 2.2).

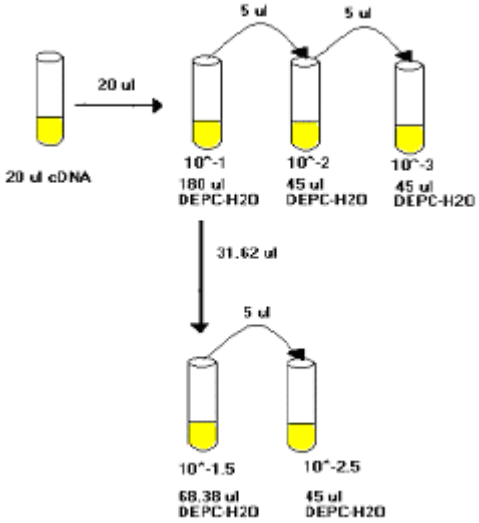


Figure 2.2 Dilution series describing how to prepare 10^{-1}, $10^{-1.5}$, 10^{-2}, $10^{-2.5}$, 10^{-3} dilutions of reverse transcription products prior Real-time PCR.

Diluted cDNA (5 µl), primer (5 µl, forward and reverse, 2 µM each), and 2X Master mix (10 µl, containing SYBR® Green, enzyme and reaction buffer) were gently mixed and loaded in the capillaries, spun down and placed in the LightCycler® rotor. DNase treated RNA was used as a negative control. This was diluted $10^{-1.26}$ to obtain the same dilution as the 10^{-1} diluted cDNA (The DNase treated RNA is further diluted through the reverse transcription, hence the extra dilution.) The PCR was run using the program listed in table 2.3. The results were analyzed using Roche Molecular Biochemicals LightCycler Software version 3.5

Table 2.3 Cycle program for Real-time PCR for analysis of expression and melting curves.

Table: Light cycle program					
Step	**Temperature (°C)**	**Time (min:sec)**	**Temperature transistion rate (°C/sec)**	**Aquisition mode**	**Analysis mode**
Initial Denaturation	95C	10:00	20,00	None	None
Amplification x 40					
Denaturation	95C	10	20,00	None	Quantitiativ
Annealing	60C	20	20,00	None	Quantitiativ
Extension	72C	10	20,00	Single	Quantitiativ
Melting curve					
Denaturation	95C	0	20,00	None	Melting curve
Re-annealing	57C	15	20,00	None	Melting curve
Denaturation	98C	0	0,10	Cont	Melting curve
Cooling	40C	10	20,00	None	None

Melting curve analysis was performed on the PCR products in order to check for product contamination from primer dimers, chimer formation and mispriming. SYBR® Green binds to dsDNA and the fluorescent signal will be reduced as the dsDNA melts. By plotting the time-derivate of the relative fluorescent units (RFU) on the Y-axis (-d(RFU)/dT) and degrees on the X-axis a peak for each dsDNA product will be generated. More than one peak indicates more than one PCR product.

The following genes were investigated; intA, intB and fbp at time 0 and 30 minutes after the shifts in all cultures (A – D).

The real-time PCR products were subjected to 1.5 % agarose gel electrophoresis in order to verify that the correct product was amplified. Five µl real-time PCR product were mixed with gel loading buffer and run for ~50 minutes at 80 V. (For details about agarose gels; see section 2.4.2) Each sequence was loaded (intA, intB, fbp and gap) and in addition two products that showed a large difference in expression; intB and gap with Ct values of 34.33 and 12.33, respectively.

2.4.4 Mathematical and statistical Analysis of Real-time RT-PCR results

2.4.4.1 Amplification efficiencies

The amplification efficiency is a measurement of the efficiency of the PCR-amplification. Generally, each primer pair and its target sequence has its own amplification efficiency that is unique for this sequence. It describes how efficient the PCR reaction is under the specified conditions. To determine the amplification

efficiency for a sequence, a dilution series for the sequence is generated, a regression curve is plotted, and a regression curve analysis is preformed. (Lunde *et al.*, 2003) The following formula (5) describes a PCR reaction in the exponential phase:

$$X_n = X_0 * (1 + E_X)^n \qquad (5)$$

Where X_n is the number of target molecules in cycle n, X_0 is the number of target molecules before amplification, E_x is the amplification efficiency of the target sequence and n is the number of cycles. The formula can be reorganized to give amplification efficiency (6):

$$E_x = (X_n / X_0)^{1/n} - 1 \qquad (6)$$

A regression line was generated for each different sequence by plotting the Ct-value (Y-axis) against the $\log_{10}$ of dilution factors (X-axis). The regression line is found by the least square method. A minimum of 4 points was used to generate the regression line and 0,990 was the cut-off for the correlation efficient (R_2) (The correlation efficient shows how well each point is fitted to the straight line), hence all lines generated with less then 4 points or a correlation efficient < 0,990 were discarded. An example of a regression curve is shown in figure 2.3.

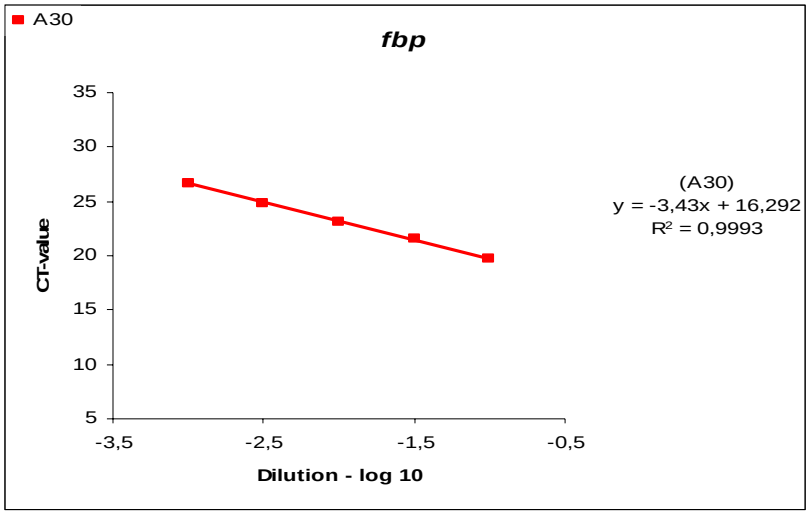


Figure 2.3 Example of a regression curve for the calculation of amplification efficiencies. Log_{10} of the dilution factors were plotted against the respective Ct-values. The absolute value of the slope (here: 3.43) is the number of cycles needed to get a ten-fold doubling of the initial cDNA. This example is from one of the regression curves for *fbp*.

Each unit at the X-axis corresponds to the ten-fold increase in the amount of product, and thus X_n/X_0 can be set to 10. The absolute value to the slope of the regression line then describes how many cycles are necessary to ten-double the amount of the product sequence. The amplification efficiency of a sequence can thus be given by the formula (7):

$$E_x = 10^{1/n} - 1, \qquad (7)$$

where n is the absolute value to the slope

2.4.4.2 Reference gene

The reference gene is used to normalize the PCR for the amount of RNA added to the reverse transcription reactions. Usually, housekeeping genes are selected, since they are thought to be equally expressed throughout the cell cycle. The expression of glyceraldehyde-3-phosphate dehydrogenase (gap, BC5141) was measured throughout the 30 minutes time-window after the shift in which the samples were taken. The expression of a reference gene is presented as $2^{-\Delta CT}$, where ΔCT ($CT_{time\ x} - CT_{time\ 0}$), and time 0 represents the 1-fold expression of each gene (Schmittgen & Zakrajsek, 2000).

2.4.4.3 The Pfaffl method, relative quantification

Pfaffl presented in 2001 a new method to quantify gene expression using RealTime RT-PCR (Pfaffl, 2001). This method will normalize the expression of a target gene to the expression of the reference gene, thus removing sources of error such as unequal RNA concentrations in different samples and pipetting inaccuracies and is given by the following formula (8). In addition, this method will account for different amplification efficiencies between different sequences.

$$\text{Ratio} = \frac{(E_{target})^{\Delta CTtarget\ (CTcontrol - CTsample)}}{(E_{ref})^{\Delta CTref\ (CTcontrol - CTsample)}} \qquad (8)$$

Where “target” is the sequence under investigation and “ref” is the reference gene; E_{target} and E_{ref} describe the amplification efficiency + 1 of the target sequence and the reference sequence, respectively. ΔCT is the deviation in CT value between the control (in this work A0) and the sample we are investigating the expression of, for both the target gene and the reference gene. The ratio thus describes the fold increase of expression in the sample compared to the control (relative to the expression of the reference.) By using this method, both the difference in amplification efficiencies for different sequences and sample to sample differences, will be accounted for.

2.5 Microarray (in collaboration with Ine Robertson and Solveig Ravnum)

2.5.1 Shift experiments and RNA isolation for microarray

Shift experiments were performed as described in 2.2.7, but without the mucin culture. Samples for RNA isolation was removed at 0, 15, 30, and 60 minutes after the shift.

The RNA isolation was performed by Ine Robertsen as described in 2.4.1. Concentrations and A-260nm/A-280nm-ratios were measured and RNA integrity was examined on denaturing agarose formaldehyde gels as described (2.4.1).

2.5.2 cDNA labeling and microarray hybridization

The microarray hybridization was performed by Solveig Ravnum. cDNA synthesis and labeling was done with the FairPlay™ microarray labeling kit (Stratagene, CA, USA). Except for the use of random hexamers (500 ng, Applied biosystems (CA, USA)) as primers, the protocol (appendix II) was followed in all steps. In brief; RNA (10 µg) and spike mixture (Stratagene) were reverse transcribed, purified and labeled with amino-allyl coupling of Cy3 and Cy5 dyes (Amersham, Uppsala, Sweden). The labeled cDNA was denatured (95 °C, 2 min.) and left at 42 °C for 20 minutes before hybridization.

Hybridization was done according to Cornings (MA, USA) manual (UltraGAPS™ Coated Slides instruction manual (appendix II). The slides was pre-hybridized, hybridized over night at 42 °C and washed, see appendix II for details.

The cDNA was hybridized on arrays of 70-mer oligos of the whole genomic *B. cereus* ATCC 14579 ORFs. The 70-mers were designed and synthesized by Qiagen-Operon (CA, USA) and printed on UltraGAPS™ gamma amino silane coated slides from Corning, with the Midigrid Biorobotics arrayer at the Norwegian Radiumhopspital (Oslo, Norway). In addition the array has both positive/negative controls and 70-mer oligos corresponding to *Arabidopsis thaliana* RNA spikes. Each 70-mere is replicated in pairs, and each array is replicated once on the slide, thus each 70-mere is in 4 replica on each slide.

Since the two dyes Cy3 and Cy5 have somewhat different affinity towards the same sequence a dye-swap was performed: Two replicates were performed per hybridization where the hybridization-pair was labeled opposite in the replicate.

RNA from cultures A30, B30, and C30 was hybridized against A0. This was done in two biological replicates, where each of these was replicated by dye-swap. Ten µg RNA from each sample was used.

2.5.3 Microarray analysis

The slides were scanned with an Axon 4000B (Molecular Devices Corporation, CA, USA) Gridding and spot annotation were done using the GenePix Pro 6.0 software (Molecular Devices Corporation), and results for each spot were calculated with the same software.

The microarray data was analyzed with an R-script developed by Karoline Fægri, Institute of pharmacy, Oslo University, in R 2.0.1 (R-Development-Core-Team, 2004) with the package Limma (Smyth, 2005). The gene-pix files were imported to R and the package Limma was used for filtering, normalization and further analysis. The dye-swap was inverted. The mean red and green foreground values and median red and green background values were used. The data were first filtered so that all control spots were weighted to zero, while spots that were flagged bad in GenePix, saturated in both channels or weak in both channels were weighted to no answer. The saturation limit was set to 60 000. Lower threshold value was set to intensity lower than 2 x

background. Spots that were weak in one channel and saturated in the other were not removed, because the effect is significant even if the ratio is not absolutely correct.

Background correction was done using the Edwards method (Edwards, 2003). Normalization within each slide was done with the loess method (Cleveland *et al.*, 1993). Spots that were not present in at least two replicas per slide were filtered out. The average of each spot in each slide was fitted to the corresponding spot in the biological replicate. The model was evaluated using Bayesian statistics and moderated t-statistics were computed. On basis of this, P-values were computed.

The average log2 ratio (A30, B30 or C30 divided on A0) for each spot is given along with the P-value. All spots with P-value equal to or above 5 % were filtered out. For B30/A0 and C30/A0 only spots with absolute log2 ratio above 0.59 were selected and listed. All spots in A30/A0 with P-value under 5 % were included in this list. Lists of filtered spots were generated.

The lists of significant spots were further analyzed in excel, and grouped according to function by using information about each gene available from KEGG (www.kegg.com).

2.6 Protein extraction and 2-D electrophoresis

2.6.1.1 Determination of protein concentration

The concentration of protein was determined using the Bio-Rad Protein Assay (Bio-Rad Laboratories, CA, USA). Protein extract (0.5 µl to 5 µl) was diluted in 20 mM Tris (pH 7.5) to a total volume of 160 µl and dispensed into a microtitrer plate. Forty µl undiluted dye reagent was added, mixed thoroughly and incubated at RT for 8 minutes. The absorbance was measured at 595_{nm}. The A_{595nm} was the compared to a standard curve (see below), and the protein concentration was determined.

Two standard curves were prepared using BSA (Bovine Serum Albumin): 1) from 1 µg to 5 µg (A_{595nm} between 0.420 and 0.600) and 2) from 5 µg to 20 µg (A_{595nm} over 0.600). Samples with A_{595nm} under 0.420 or over 0.900 were concentrated or diluted

to give an A_{595nm} in the correct range. The same standard curves were used for all experiments.

2.6.1.2 Extraction of total cell proteins

Principle

Two procedures were used in the extraction of total cell proteins; one in which the cell pellet was suspended in 20 mM tris (pH 7.5) containing protease inhibitor for use in the aminopeptidase C assay (section 2.6.2.2) and one in which the cell pellet was suspended in lysis buffer containing strong denaturants. The lysis buffer is more hydrophobic and better recovery of hydrophobic proteins (such as membrane proteins) is expected. The FastPrep® method was used to disrupt the cells. The angular motion of the FastPrep® instrument causes the lysing matrix particles to impact the cells from all directions simultaneously, and thus disrupting them. Both buffer extracts were analyzed by 2-D protein gel electrophoresis.

Procedure

The protease inhibitor used in the following experiments was Complete Protease Inhibitor Cocktail Tablets (Mini, EDTA-free) from Roche. One tablet was dissolved in 500 µl dH_2O to make 21X concentration. This was added to the buffers to a final concentration of 1X.

IR-media (200 ml) was inoculated with 2 vials of ON-culture and grown for 3 h. at 37 °C, at 175 rpm. in a 1 L Erlenmeyer flask. The culture was shifted to 2 x 100 ml fresh IR-media (with no additives) and harvested immediately by centrifugation, 4000 x g (rcf), 4 °C for 15 minutes Sorvall® RC-5B Refrigerated Superspeed Centrifuge (DuPont instruments, France). The cell pellets were washed for 1 minute with shaking (175 rpm, 4 °C) in 20 mM Tris (pH 7.5) containing protease inhibitor and centrifuged at 4000 x g at 4 °C for 10 minutes. One cell pellet was suspended in 1 ml 20 mM Tris (pH 7.5) containing protease inhibitor and the other cell pellet was suspended in 1 ml lysis buffer ((7 M Urea, 2M Thiourea and 4% (w/v) CHAPS (3-[(3-Cholamidopropyl)-dimethylammonio]-propanesulfonic acid)). This is the same as the rehydration buffer described in 2.6.3.2, but without bromophenol blue. Both samples

were transferred to lysing matrix A tubes and homogenized in the FastPrep® instrument at setting 6 for 40 seconds. The samples were centrifuged at 13 000 rpm for 2 minutes in a microsentrifuge, transferred to new tubes and centrifuged again for 5 min (13 000 rpm) in order to remove any protein aggregates or cell debris. The supernatants were transferred to new tubes. Protein concentrations were measured, and samples for the determination of aminopeptidase C activity were collected.

The samples were concentrated as described below in section 2.6.2.1 prior to 2D protein electrophoresis. There were some exceptions for the proteins extracted with the lysis buffer; lysis buffer was used as the dialysis buffer and because of the high viscosity of the lysis buffer the centrifugation time was increased to 3 hours and performed only twice.

2.6.2.1 Testing of different extraction buffers

A number of different buffers were tested in the isolation of proteins "loosely" associated with the bacterial cell. The principle of this method is that relatively mild buffers will dissociate loosely attached surface proteins without disrupting the integrity of the cells. Covalently, or strongly attached surface proteins, however, will not be dissociated.

IR-media (400 ml) was inoculated with 40 µl ON-culture and grown for 3 h. at 37 °C, 175 rpm. The culture was shifted to 4 x 100 ml fresh IR-media (with no additives) and harvested immediately by centrifugation, 4000 x g (rcf), 4 °C, for 15 minutes. The cell pellets were washed in 1 ml 20 mM Tris (pH 7.5) containing protease inhibitor. The supernatant from the washing step was collected. The cell pellets were extracted with different buffers (see table 2.4 below) for 10 minutes with gentle shaking (175 rpm) at 4 °C and pelleted by centrifugation. The supernatants were collected and designated "buffer extracts". For comparison the remaining cell pellets were supended in 20 mM Tris (pH 7.5) and lysed using FastPrep® method (as described in 2.6.1.2). Both the washing extraction and the buffer extractions were additionally centrifuged for 10 min at 4000 x g (rcf), 4 °C and transferred to new tubes. All extractions and the washes were analyzed for aminopeptidase C activity.

Table 2.4 Extraction buffers for protein isolation.

Buffer[#]
20 mM Tris (pH 7.4) [(1)]
1X PBS[(2)] (pH 7.0)
1 M Tris (pH 7.4)
1 M KSCN[(3)] in 20 mM Tris (pH 7.5)
0.01 % NP-40[(4)] in 20 mM Tris (pH 7.5)
0.1 % (w/v) CTAB[(5)] in 20 mM Tris (pH 7.5)
0.01 % (v/v) Triton X-100 in 20 mM Tris (pH 7.5)
1.5 M LiCl in 20 mM Tris (pH 7.5)

[#]All buffers contain 1X protease inhibitor.

[(1)]Wash step – 1 min only

[(2)]Phosphate buffer saline (0.26 g NaH_2PO_4 x H_2O, 1.49 g Na_2HPO_4 x $2H_2O$, 8.77 g NaCl to 1 L with dH_2O)

[(3)]Potassium thiocyanate

[(4)]Nonidet® P 40 (Shell chemicals)

[(5)]Hexadecyltrimethylammonium bromide

Concentration of protein samples prior to SDS-PAGE

Samples were concentrated by ultrafiltration; the samples were transferred centricon 3 ultrafiltration devices (3 kDa cut-off, from Millipore) and centrifuged at 6500 x g (rcf) at 4 °C for 2 hours. To collect the concentrated samples the tubes were inverted and centrifuged (600 x g (rcf), 4 °C, 1 min.). To remove traces of proteins retained in the filter 5 µl 5 mM Tris (pH 7.5) containing protease inhibitor was added and the centrifugation was repeated (600 x g (rcf), 4 °C, 2 min.).

Concentration and dialysis of protein samples prior to 2-D electrophoresis

In 2-D electrophoresis the salt concentration is crucial, and should not exceed 20 mM. In order to achieve this 1 ml 5 mM Tris (pH 7.5) containing protease inhibitor was added to the samples and transferred to the ultrafiltration tubes. The samples were centrifuged for 2 hours (6500 x g, 4 °C). Another 2 ml 5 mM Tris (pH 7.5) containing protease inhibitor was added and the centrifugation was repeated. This last step was repeated once more, and the now dialyzed and concentrated samples were collected as described above.

2.6.2.2 Aminopeptidase C assay

Principle

The aminopeptidase C assay was used to estimate the level of cytosolic contamination in the buffer extracts. Aminopeptidase C is an enzyme that hydrolyses both peptides and aminoacyl–*p*-nitroanilides (Mistou & Gripon, 1998) that is only found in the cytosol. By incubating protein extracts with L-arginine-*p*-nitroanilide any aminopeptidase Cs present will hydrolyze the compound and give two products; L-arginine and *p*-nitroaniline. *p*-nitroaniline is a yellow compound with an absorption maximum at 410 nm (Mistou & Gripon, 1998). By measuring the reaction spectrophotometrically, any absorbance at 410 nm (that is above the present background absorbance) will indicate aminopeptidase C activity, and thereby be an indication of cytosolic contamination present in the protein extract. The use of this assay to document cytosolic contamination was first described by Schaumburg *et al.* (2004).

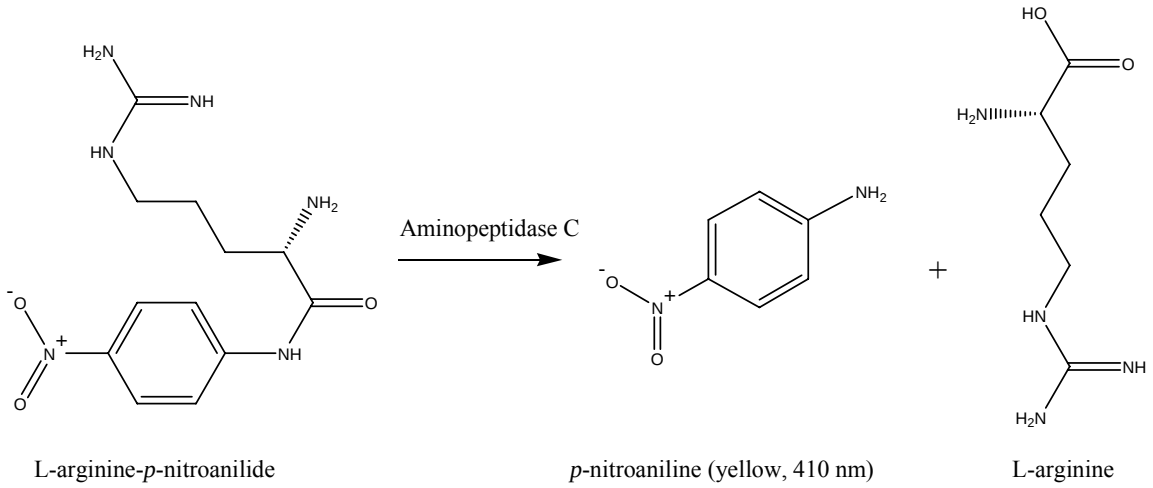


Figure 2.5 The cytosolic protein aminopeptidase C hydrolyses L-arginine-*p*-nitroanilide into L-arginine and *p*-nitroaniline. *p*-nitroaniline is an yellow compound with absorption maximum at 410 nm.

Procedure

Fresh protein extracts (10 µl) were added to 188 µl 20 mM Tris (pH 7.5) in a microtitrer plate. The plate was incubated at RT for 10 minutes before 2 µl L-arginine-p-nitroanilide (200 mM in 20 mM Tris (pH 7.5)) was added to each well. The absorbance at 405 nm was measured, using a Wallac 1420 Multilabel counter (Wallac, Turku, Finland) reader, every fifth minute over a period of 20 minutes. A blank containing no protein extract was also analyzed in each run.

All the buffer extracts and their respective washes, cytosolic fractions and the supernatant containing the extracellular proteins, were analyzed. Whole cell extracts were also analyzed. Analysis was performed immediately after harvesting, before any concentration or precipitations.

The effect of the different buffers on the assay was investigated. One µl of fresh whole cell extract was incubated for 15 minutes with 10 µl of each buffer, as specified in table 2.4 and assayed as described above.

2.6.2.3 1-D SDS Polyacrylamide-gel electrophoresis of protein extracts

In 1-D gel electrophoresis the proteins are separating according to their molecular weight. SDS (Sodium dodecyl sulfate) will bind the polypeptide backbone and make the proteins negatively charged, thus all proteins will migrate towards the anode with speed proportional to each proteins MW. The proteins are denatured by heating and disulfide bridges are reduced with 2-mercaptoethanol.

The buffer extracts (listed in table 2.4) and their corresponding total protein fractions extracted from the remaining cell pellet after buffer extraction were analyzed by 1-D SDS polyacrylamide gel electrophoresis. In addition the wash extract (the supernatant after the washing step, see 2.6.2.1) was investigated by SDS PAGE for comparison of protein patterns.

Protein samples and Prestained Protein Marker (from New England Biolabs) were reduced and denatured by heating at 100 °C for four minutes in 5X sample buffer in a 4:1 relationship. The 5X sample buffer contains; 0.6 ml 1 M Tris-HCl (pH 6.8), 5 ml 50 % (v/v) glycerol 2 ml 10 % (w/v) SDS, 0.5 ml 2-mercaptoethanol, 1 ml 1 % (w/v) bromophenol blue, and 0.9 ml dH_2O.

SDS PAGE (Sodium dodecyl sulfate polyacrylamide gel electrophoresis) was performed on the Bio-Rad Mini Protean II system. A 10 % separating gel was prepared by mixing:

5 ml 40 % (w/v) acrylamide
5 ml separating buffer (75 ml 2 M Tris (pH 8.8), 4 ml 10 % SDS, and 21 ml dH_2O)
10 ml dH_2O
100 µl 10 % (w/v) ammonium persulfate
10 µl TEMED (N,N,N',N'-tetramethylethylenediamine)

The solution was gently mixed and poured in the gel casting chamber. ~2 mm dH_2O was gently layered on top to make an even surface. The gel was polymerized for ~45 min at RT.

A stacking gel was prepared by mixing:
0.5 ml 40 % (w/v) acrylamid
1.0 ml stacking gel buffer (50 ml 1 M Tris (pH 6.8), 4 ml 10 % SDS, and 46 ml dH_2O)
2.5 ml dH_2O
100 µl 10 % (w/v) ammonium persulfate
10 µl TEMED

Excess water on top of the gel was removed and the stacking gel was poured on top. A comb was inserted and any air bubbles were removed. The gel was polymerized for ~30 min at RT.

The gel was placed in the gel chamber and the chamber was filled with PAGE running buffer (3 g Tris, 14.4 g glycin, 1 g SDS, dH_2O to a total volume of 1 liter). Protein samples and marker were loaded and electrophoresis was carried out at 110 V for ~100 min.

The gels were stained with Coomassie Brillant Blau R-250 (AplliChem, Darmstadt, Germany). CBB R-250 (1.0 g) was mixed in 450 ml dH_2O, 450 ml methanol and 100ml glacial acetic acid to make the gel staining solution. Each gel was stained in this solution for ~10 minutes under agitation. The gels were destained ON with destaining solution (100 ml methanol, 100 ml glacial acetic acid and 800 ml dH_2O).

2.6.3 Investigation of protein expression patterns

2.6.3.1 Shift experiment and protein isolation

The LiCl extraction buffer (1.5 M LiCl in 20 mM Tris (pH 7.5) containing protease inhibitor) was selected for the protein extractions in the following experiments. Shift experiments were performed as described in section 2.2.7 with some exceptions; 1) the mucin culture was not used. 2) Samples were taken at 30 minutes after the shift for control, bile and bile salts cultures and immediately after the shift for the control only. 3) The entire cultures (200 ml) were harvested.

The protein extraction was performed as described for the LiCl extraction described in section 2.6.2.1. In brief; the culture was harvested by centrifugation, washed and suspended in 1.5 M LiCl in 20 mM Tris (pH 7.5) containing protease inhibitor, incubated for 15 min on ice and the proteins in the supernatant were harvested. The experiments were done in three parallels. The protein yield of buffer extracts for the control culture at 0 minutes (A0) and the bile culture at 30 minutes (B30) were low. The proteins from two parallel extractions were therefore combined in order to increase protein yield. The LiCl extracts were analyzed by the aminopeptidase C assay (2.6.2.2), concentrated and dialyzed (see section 2.6.2.1). The protein concentrations were measured and 5 µg of each extract was subjected to analysis by 2-D electrophoresis

2.6.3.2 Two-dimensional electrophoresis

Principle

2-DE is a method for separation of proteins in two dimensions on a gel. In the first dimension the proteins is separated according to each proteins isoelectric point (pI) by isoelectric focusing. In the second dimension the proteins are separated according to their mass.

Procedure, isoelectric focusing

IsoElectric Focusing (IEF) was performed on 7 cm pH 3- 10 NonLinear IPG strips (Invitrogen (ZOOM® strip pH 3 – 10 NL)) and run in the ZOOM® IPGRunner™ MiniCell (Invitrogen).

For each sample, 5 µg protein in a total of 34 µl ddH_2O was mixed with 116 µl 1.33X rehydration solution (4.2 g urea, 1.52 g thiourea and 0.4 g Chaps and a trace of bromophenol blue in 7.7 ml ddH_2O) to give 5 µg proteins in a total of 150 µl 1X rehydration solution (7 M urea, 2 M Thiourea and 4 % (w/v) Chaps). The entire mix (150 µl) was loaded into the ZOOM® IPGRunner™ cassette and IPG strips were inserted. The cassette was sealed and the strips were rehydrated for 2 hours. The sealing tape was removed, dH_2O was added to the electrode wicks and IEF was performed according to the program shown below. Four to six samples were focused in each run.

Step 1:	100 V, 20 min
Step 2:	200 V, 15 min
Step 3:	450 V, 15 min
Step 4:	750 V, 30 min
Step 5:	1500 V, 30 min
Step 6:	3000 V, 120 min

The strips were either stored at –70 °C ON or separated in the second dimension immediately.

Second dimension

The focused IPG strips (7 cm, pH 3- 10NL) were equilibrated in 10 ml equilibration solution (6 M urea, 30 % (v/v) glycerol, 2 % (w/v) SDS 75 mM Tris (pH 8.8) and trace of bromophenol blue) and 100 mg DTT at RT under gentle shaking for 15 minutes in order to reduce any oxidized proteins. The equilibration solution was removed the strips were incubated at RT with gentle shaking for 15 minutes in 10 ml equilibration solution containing 250 µg iodoacetamide in order to carbamidomethylate free sulfur groups (and remaining DTT) and thus prevent formation of new disulfide bonds.

The IPG strips were then loaded onto 4 -12 % Bis-Tris gels (NuPAGE® Novex 4-12% Bis-Tris ZOOM® Gels, 1.0 mm. Invitrogen) and ~400 µl hot 0.5 % (w/v) agarose, with a trace of bromophenol blue, was added to the well on top of the strip. Electrophoresis was performed in 1X MOPS buffer (Invitrogen) at 200 V for ~75

minutes (until the bromophenol blue left the gels). The gels were immediately placed in fixing solution (see silver staining below).

Silver staining

Silver staining of 2-D gels was carried out with a method based on the Amersham Biosciences PlusOne Silver Stain Kit. This method is compatible with MS analysis.

All water used for washing and the staining solutions was Milli-Q water (18.2 MΩ dH_2O, Millipore, here; abbreviated ddH_2O) and all solutions were freshly prepared. The gels were under gentle agitation throughout the procedure and all solutions were removed by aspiration. The protocol described below was followed:

1) The gels were fixed for 30 min or ON in fixation solution (100 ml ethanol, 125 ml ddH_2O, and 25 ml glacial acetic acid.)
2) Sensitizing was performed for 30 min in sensitizing solution (75 ml ethanol, 10 ml 5 % (w/v) sodium thiosulfate, and 17 g sodium acetate in total 250 ml ddH_2O.)
3) The gels were washed gels 3 x 5 minutes in ddH_2O
4) Staining was performed in 0.25 % (w/v) silver nitrate for 20 minutes
5) The gels were additionally washed for 2 x 1 minute
6) Developing was performed by adding developing solution until the solution becomes brownish (10 – 30 sec.) The developing solution was removed and fresh developing solution was added. The gels were allowed to develop for 2-5 minutes, until a known pattern became visible. To make developing solution 10.6 g potassium carbonate and 50 µl formaldehyde was mixed in 250 ml ddH_2O.
7) The reaction was stopped by adding stop solution (3.65 g $Na_2EDTA{\cdot}2H_2O$ in 250 ml ddH_2O) and agitation for ~10 minutes.

Stained gels were kept in ddH_2O.

2.6.3.3 Analysis of expression pattern on 2-DE gels

Expression patterns detected by 2-D electrophoresis were analyzed using ImageMaster™ 2DE Platinum 6.0 software from GE healthcare. Pictures of the gels were taken to 16 bits images. The gels were normalized, grey levels/contrast was adjusted and spots were detected. The spots were quantified using the relative volume (vol %), meaning that each spot is presented as the volume % of the total spot volume in the entire gel. For each treatment and time-point (A0, A30, B30 and C30) three gels (each from a different biological replicate) were matched together (in match-sets; A0, A30, B30 and C30). For ease of comparison synthetic gels were made for each match-set based on the average volume % of the matched spots.

The spots from all the match-set were then matched to each other. Spots that displayed different regulation in B30 and/or C30 when compared to A0 were selected. These spots were then filtered by; (1) removing spots that had an internal standard deviation (between the replicates in the match-set) that overlaid with the standard deviation of the matching spot in A0. (2) Removing spots that showed a similar regulation in A30 and (3) filtering out compared spots that had a log2 ratio between 0.59 and -0.59. All spot regulations were in addition confirmed by visual comparison.

2.6.4 Identification of proteins; Protein mass fingerprinting

Principle

Protein Mass Fingerprinting (PMF) is a method for the identification of proteins. Excised protein spots are digested to give peptides for which the masses are determined by MS. A comparison is made to the peptide masses with all possible *in silico* digested peptide masses in a database: The mascot program used for PMF will take all theoretical proteins in the specified database (here: NCBInr) and generate all potential peptide masses if these proteins were digested with a specified protease (here: trypsin). The theoretical masses of these peptides are then compared to the masses obtained for trypsin digested proteins of interest.

2.6.4.1 Trypsin digestion

Protein spots of interest were cut out of the gels using pipette tips and kept at -20 °C until the day of analysis. Milli-Q water (ddH_2O) was used in all steps. Eight spots, four from B30 and four from C30 were excised and analyzed by Protein Mass Fingerprinting (PMF). This was done in two parallels, with each parallel spot located on parallel gels

The gel spots were destained in farmers reducing agent (5 % (w/v) sodium thiosulfate/ 0.25 % (w/v) freshly prepared potassium ferracyanide,) until the samples were color less (~10 minutes). Samples were then washed 2 x 20 minutes in 20 mM ammonium bicarbonate/ 50 % (v/v) acetonitrile and washed once for 5 minutes in 100 % acetonitrile. The gel pieces were dried in a SpeedVac for 30 minutes. Fifteen µl 0.02 µg/µl trypsin (Sequencing Grade Modified Trypsin from Promega (Madison, WI, USA)) in 20 mM ammonium bicarbonate was added and the gel pieces were allowed to swell for 15 minutes. Excess liquid was removed and 20 mM ammonium bicarbonate was added until it covered the gel pieces (10 – 20 µl). Digestion was performed ON (~18 h.) at 37 °C. The liquid was removed and kept on ice until MS analysis. The gel pieces were washed twice with shaking in 20 µl 0.2 % (v/v) TFA (Trifluoroacetic Acid) for 45 min on 37 °C. The washes were harvested and combined.

2.6.4.2 MALDI-TOF analysis of trypsin digests

MALDI-TOF MS (Matrix Assisted Laser Desorption Ionization – Time of Flight Mass spectrometry) was used to identify the trypsin digests and was performed on a Voyager – DE™ RP BioSpectrometry™ Workstation Mass spectrometer from PerSeptive Biosystems inc. (Houston, Texas, USA).

An α-cyano-4-hydroxycinnamic acid matrix was used. This was prepared by mixing 10 µg α-cyano-4-hydroxycinnamic acid in 1 ml 50 % acetonitrile/0.1 % (v/v) TFA. The solution was vortexed for 1 minute and centrifuged at 13000 rpm for 30 seconds. 500 µl of the supernatant was collected, combined with 500 µl 50 % acetonitrile/0.1 % (v/v) TFA and 24 µl 5 mg/ml ammonium citrate was added.

The trypsin digested protein samples, (1 µl) of each sample, were spotted on the MALDI-plate and 0.6 µl matrix was added to each spot. The peptide/matrix mix was air-dried and the MALDI-plate was inserted into the MS. The mass-spectra were recorded in Voyager Instrument Control Panel (version 5.10) software from Applied biosystems. Three spectra of each 80 shots were combined for each sample. Spectra were recorded for masses ranging from 1 kDa to 4 kDa only because of a very high noise level in the lower part (700 – 1000 Da) of the spectra. The spectra were baseline correlated and the noise was removed with the Data Explorer version 4.0.0.0. software. The mass list was exported to the Mascot program.

2.6.4.3 Identification of digested proteins

Peptide masses were detected with the Mascot program, available at http://www.matrixscience.com. The peptide masses were queried against the NCBInr (non-redundant) database, with trypsin as the digestion enzyme, carbamidomethyl as fixed peptide modification, and oxidation of methionine as a variable peptide modification. Monoisotopic masses were used and the peptide tolerance was set to ±1.0 Da. Only peptides with a MOWSE-score above the cut-off were used and each identified peptide was also confirmed by matching its theoretical mass and pI with its localization on the gel.

2.7 Practial Proteomics in York

In April 2005 I attended to the Practical Proteomics course I at University of York. In this course my own protein extracts and excised gel bands were analyzed. In addition the 2-DE separation of an *E. coli* protein extract, on the Invitrogen Mini gel system, was demonstrated.

The following work was carried out: The protein sample was a combination of two CTAB extracts (as described in 2.6.2.1). This was concentrated and ~60 µg was diluted in 350 µl rehydration solution, applied to 18 cm 3-10 linear IPG strips (Immobiline DryStrip, Amersham) and rehydrated ON. IEF was performed with the following program; 300 V for 6 min, gradient V-increase to 3500 V in 3 hours, 3500 V for 3 hours, 8000 V for 64000 Vh. The strip was equilibrated, reduced,

carbamidomethylated and applied to a 9 – 16 % gradient gel. Electrophoresis was carried out at 200 volt for ~6 hours. The gel was silver stained as described in 2.6.4.3.

The excised 1-D bands are indicated in figure 3.19 (bands named A, B, and C). These were destained, equilibrated as the IPG strips (section 2.6.4.2), and digested as described in 2.6.5.1. The samples were analyzed both by MALDI-TOF MS and MALDI-TOF-TOF MS/MS.

3 RESULTS

3.1 Bacterial growth and shift experiments

3.1.1 Growth, pH and glucose concentration curves

Bacillus cereus ATCC 14579 growth, pH and glucose concentration curves are shown in figure 3.1. 100 ml IR-medium was inoculated with one batch of ON-culture in a 1 L Erlenmeyer flask and incubated at 37 °C and 175 rpm. Samples were taken every hour. The cell density was measured by flow cytometry, pH was measured with pH strips from Merck and glucose with (GO) glucose kit from Sigma.

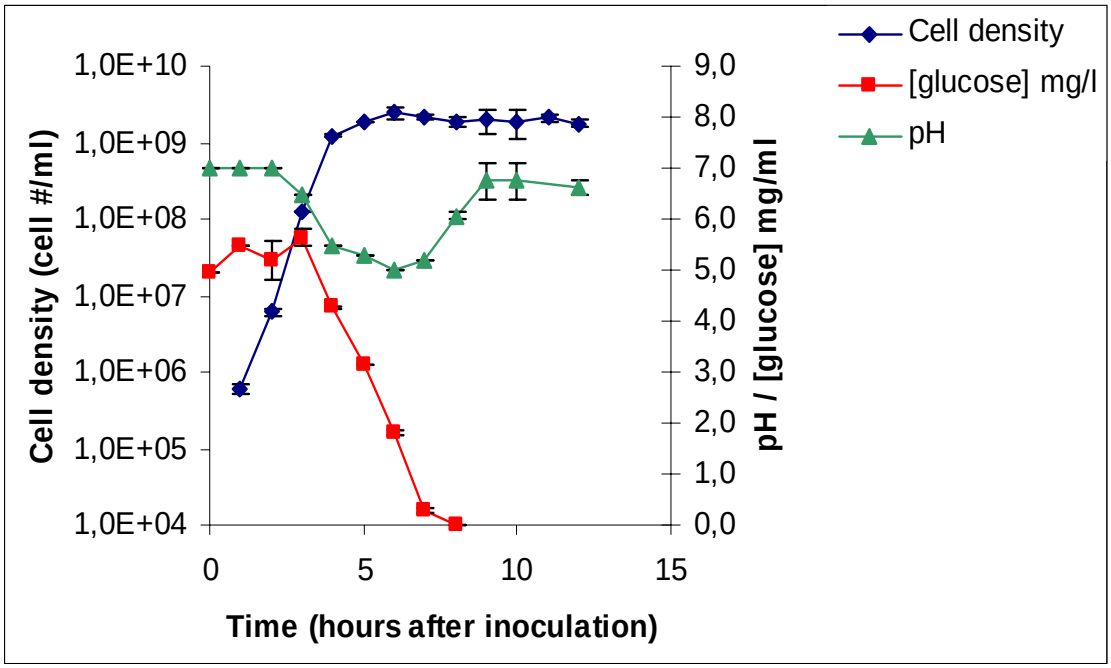


Figure 3.1 Bacteria cell density, pH and [glucose concentration] during growth of *Bacillus cereus* ATCC 14579 in IR-medium. The data represents the mean of at least two experiments, and the error bars indicate standard deviation.

3.1.2 Survival of *B. cereus* ATCC 14579 in bile and bile salts

B. cereus was grown in the presence of different concentrations of bile and bile salts in order to select appropriate concentrations to be used in shift experiments. The results are shown in figures 3.2 and 3.3. On the basis in these results; concentrations of 0.03 % (w/v) and 0.005 % (w/v) of bile and bile salts respectively, were selected for use in the shift experiments.

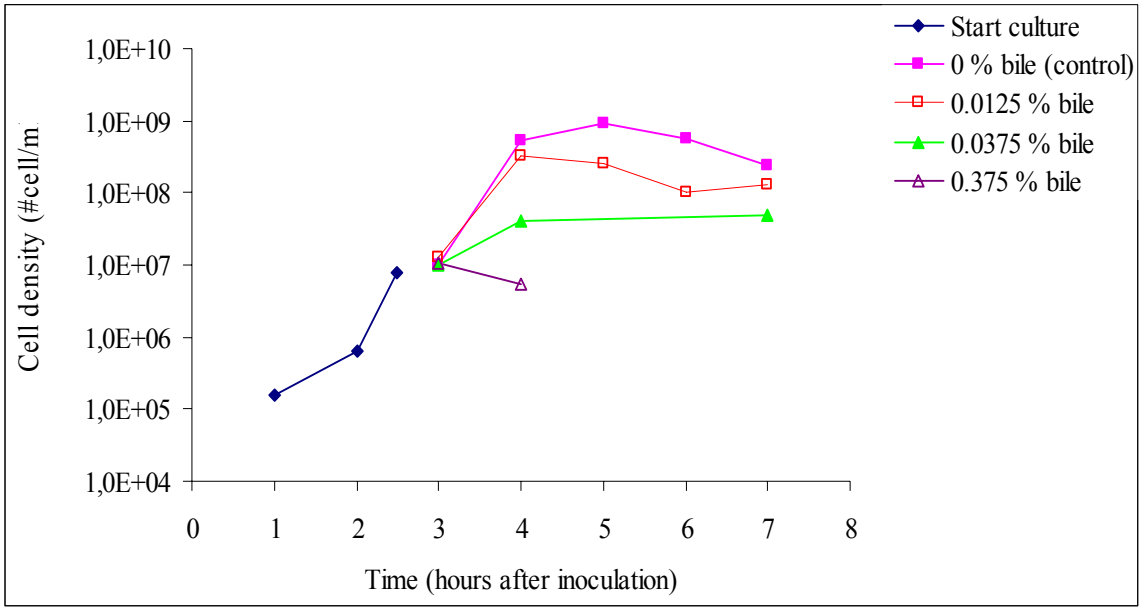


Figure 3.2 Growth of *Bacillus cereus* ATCC 14579 in medium containing different concentrations of bile. The start culture was divided into four portions after 3 hours of growth and bile was added to the concentrations indicated in the figure. The cell density was measured by flow cytometry.

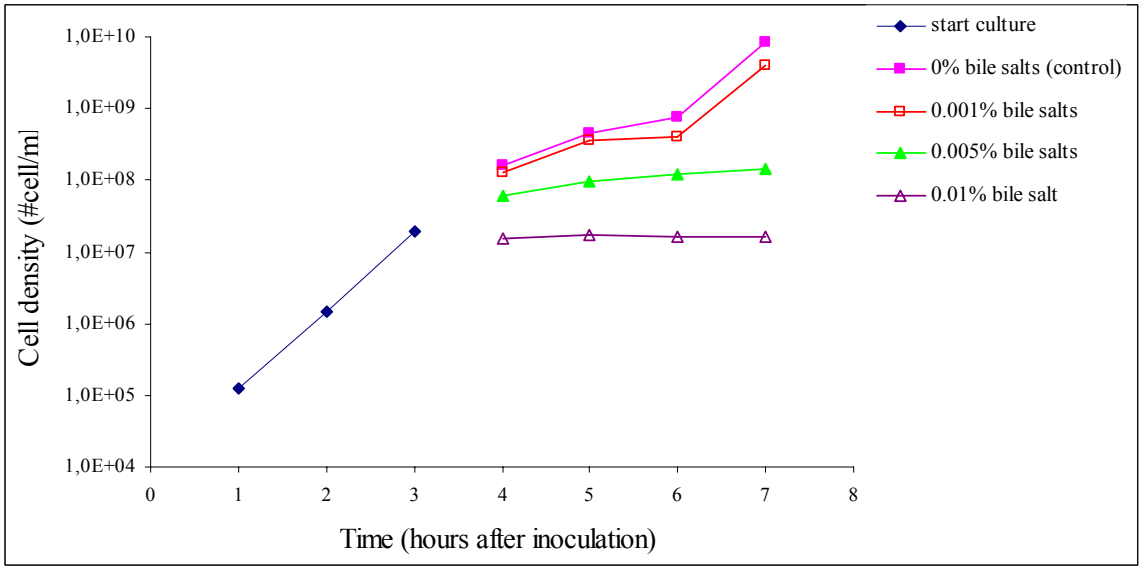


Figure 3.3 Growth of *Bacillus cereus* ATCC 14579 in medium containing different concentrations of bile salts. The start culture was divided into four portions after 3 hours of growth and bile salts were added to give the concentrations indicated in the figure. The cell density was measured by flow cytometry.

3.1.3 Bile salts adaptation test

Exponentially growing *B. cereus* ATCC 14579 was shifted to new media containing bile salts, (0.005 % final concentration, w/v) and incubated for 5 minutes before samples were plated out on LB-agar plates containing different concentration of bile salt. No colonies were observed on plates containing more than 0.01 % bile salts after ON incubation. Incubation for a further three days did not give any new colonies. The same results were obtained after preincubation of *B. cereus* with 0.005 % bile salts for 30 min. The results are shown in table 3.1 which includes, cell densities at point of harvest, number of bacteria plated out, and number of bacteria colonies observed in the different concentration of bile salt and the percent survival.

Table 3.1 Results of test for adaptation of *B. cereus* ATCC 14579 to bile salts. Exponentially growing *B. cereus* cultures were shifted to media containing 0.005 % bile salt, and incubated for 5 or 30 minutes before plated out on LB-agar plates containing different concentration of bile salt. A control culture which shifted to media without bile salts were also plated out. The average cfu and st.dev results are from three plates of the same culture. The cfu number and st.dev. in the control plates results from a 10X dilution - the numbers counted was $1/10^{th}$.

	Control – 0 % bile salts			**Survival in bile salts**				
		# bacteria plated out		**0.001-0.005 % bile salts**	**0.01 % bile salt**			**0.02 - 1.0 %;**
Pre-incubation conditions	**Cell density (cfu #/ml)**	**average # cfu**	**st.dev**	**# cfu**	**average # cfu**	**st.dev**	**Percent survival**	**# cfu**
Bile salts 0.005 % - 5 min #1	~6,0 x 10^6	301	39	confluent growth	195	10	65 %	No colonies observed
Bile salts 0.005 % - 5 min #2	~6,5 x 10^6	326	25	confluent growth	259	25	79 %	No colonies observed
Bile salts 0.005 % - 30 min #1	~2,0 x 10^7	590	74	confluent growth	331	34	56 %	No colonies observed
Bile salts 0.005 % - 30 min #2	~1,5 x 10^7	435	22	confluent growth	252	36	58 %	No colonies observed
Control (no bile salt. 30 min) #1	~1,1 x 10^7	288	31	confluent growth	177	18	61 %	No colonies observed
Control (no bile salt. 30 min) #2	~1,4 x 10^7	371	41	confluent growth	241	17	65 %	No colonies observed

3.1.4 Growth of *Bacillus cereus* ATCC 14579 in shift experiments

Growth and pH curves for *Bacillus cereus* ATCC 14579 grown in IR-medium and shifted to IR-medium containing; no additives (control), 0.03 % (w/v) bile, 0.005 % (w/v) bile salts and 0.25 % (w/v) mucin are given in figure 3.4 and 3.5. The cell density, measured by flow cytometry, and the pH were determined at the times indicated in figures (3.4 and 3.5). Figure 3.5 shows the growth immediately after the shift.

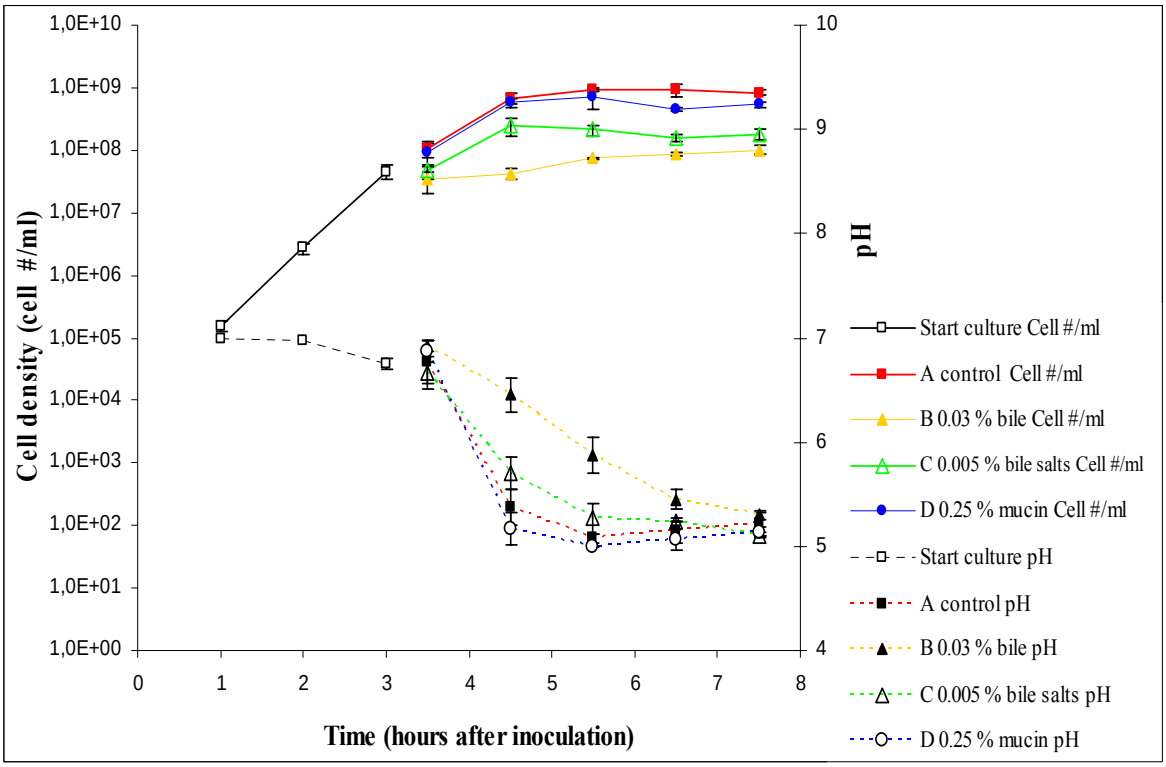


Figure 3.4 After 3 hours the start culture was shifted to new growth media containing 100 ml IR-medium and; A no additives (control), B bile 0.03 %, C bile salts 0.005 %, D mucin 0.25 % (all w/v, final concentrations). The cultures were grown at 37 ° and 175 rpm. The error bars indicate the standard deviation from a minimum of two different experiments.

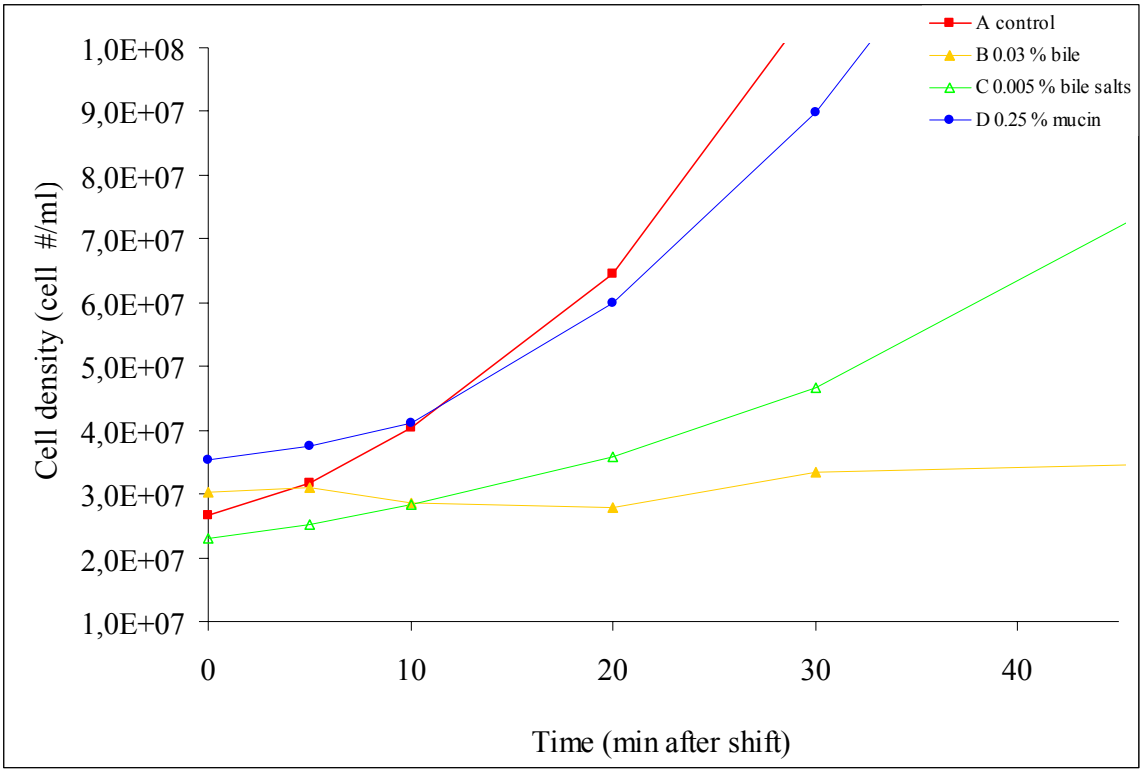


Figure 3.5 The 45 minutes time-window after the cultures were shifted to new growth media. This is not included in the previous figure (fig 3.4). The dots represent the average of at least 3 different experiments. The standard deviation is left out for the sake of clarity. Note that the scale is *not* logarithmic.

3.2 *In silico* identification of candidate virulence genes

3.2.1 Candidate virulence genes in *B. cereus* ATCC 14579

To identify candidate virulence genes three criteria for selection were defined; involved in pathogenesis/host cell adhesion, associated with the cell wall, and possibly regulated in intestine. A number of genes satisfying these 3 criteria are shown in table 3.2. The genes are selected on basis of their NCBI annotation, their documented function, their similarity to known domains, and/or by their similarity to well investigated genes in other gram positive bacteria investigated with blastp or tblastn with a minimum 25 % identity cut-off.

Table 3.2 Candidate virulence genes in *Bacillus cereus* ATCC 14579 which are associated with the cell surface.

BC number	Gene name (NCBI)	Selection basis	Reference
BC 3873	Fibronectin binding protein	Similar gene in *L. monocytogenes* and *Streptococcus* spp. Known virulence factor	(Dramsi *et al.*, 2004)
BC 1771	Fibronectin binding protein	Similar gene in *L. monocytogenes* and *Streptococcus* spp. Known virulence factor	See above.
BC 0552	Internalin A	NCBI-annotation / Well known virulence factor in *L. monocytogenes* / similarity	(Cossart *et al.*, 2003)
BC 1331	Internalin A	NCBI-annotation / Well known virulence factor in *L. monocytogenes* / similarity	See above.
BC 1101	Internalin G?	NCBI-annotation / Well known virulence factor in *L. monocytogenes* / similarity	See above.
BC 4948	Internalin G?	NCBI-annotation / Well known virulence factor in *L. monocytogenes* / similarity	See above.
BC 4868	Camelysin	NCBI-annotation / Documented toxin	(Grass *et al.*, 2004)
BC 1281	Camelysin	NCBI-annotation / Documented toxin	(Grass *et al.*, 2004)
BC 5135	Enolase	Virulence factor in *Streptococcus* spp / similatrity	(Pancholi & Fischetti, 1998)
BC 2952	Cell wall binding protein, enterotoxin	NCBI-annotation / SH3 domain	
BC 0813	Cell wall binding protein, enterotoxin	NCBI-annotation / SH3 domain	
BC 0887	Collagen adhesion protein	NCBI-annotation / similarity with CW-genes in other *B. cereus strains*	
BC 2508	Collagen adhesion protein	NCBI-annotation / similarity with CW-genes in other *B. cereus strains*	
BC 2510	Collagen adhesion protein	NCBI-annotation / similarity with CW-genes in other *B. cereus strains*	
BC 1060	Collagen adhesion protein	NCBI-annotation / similarity with CW-genes in other *B. cereus strains*	
BC 5358	Collagen adhesion protein	NCBI-annotation / similarity with CW-genes in other *B. cereus strains*	
BC 3526	Collagen adhesion protein	NCBI-annotation / similarity with CW-genes in other *B. cereus strains*	
BC 5056	Collagen adhesion protein	NCBI-annotation / similarity with CW-genes in other *B. cereus strains*	
BC 3529	Microbial collagenase	NCBI-annotation	
BC 0556	Microbial collagenase	NCBI-annotation	
BC 2466	Microbial collagenase	NCBI-annotation	
BC 3161	Microbial collagenase	NCBI-annotation	
BC 3762	Microbial collagenase	NCBI-annotation	
BC 3009	Microbial collagenase	NCBI-annotation	
BC 3010	Microbial collagenase	NCBI-annotation	

Fibronectin binding protein

A needleman-wunch global alignment with BLOSUM62 matrix was preformed between the fibronectin binding protein of L. monocytogenes EGD-e strain and *Bacillus cereus* ATCC 14579. 50 % of the sequences are identical and 70 % are similar. The alignment between the sequences is given in figure 3.6

An Interpro scan also reveals that the two fibronectin binding proteins (BC3873 and BC1771) have a fibronectin binding domain (Pfam, accession number: PF07299, (Christie *et al.*, 2002; Gilot *et al.*, 1999)). However the fbp proteins do not have a known (by the databases at Interpro) cell surface localization signal.

Figure 3.6 Needleman-Wunch global alignment of *Listeria monocytogenes* EGD-e and *Bacillus cereus* ATCC 14579 fibronectin binding proteins (lmo1829 and BC3873, respectively) RED color: identical amino acid.

Internalin

The internalin A genes in *Bacillus cereus* ATCC 14579 have ~23 % identity and ~36 % similarity with the internalin A genes of L. monocytogenes EDG-e strain (Needleman-Wunch global alignment, not shown). Both BC1331 and BC0552 have several leucine rich repeats (Pfam, Accession number: PF00560). BC0552 has a LPXTG motif (HMMTigr, accession number: TIGR01167; Pfam, accession number: PF00746) for covalently linking to peptidoglycan (Ton-That *et al.*, 2004) and BC1331 has a s-layer homology domain (Pfam, accession number: PF00395; Prosite, accession number: PS01072). BC1331 has in addition an internalin domain (panther.appliedbiosystems.com; accession number: PTHR10588:SF3). BC1331 also has a IMP dehydrogenase / GMP reductase domain (Pfam, accession number: PF00478 (Zhang *et al.*, 1999))

The two internalin G sequences both have leucine rich repeats (Superfamily HMM library, accession number: 52058) and prove similar to internalin G domains (ProDom family, accession number: PD696974)

Enolase

Apart from their well investigated function in glycolysis enolases are often surface-associated and involved in binding to plasminogen (see discussion). The enolase gene scores positive to enolase-domains, but not surface-localization or plasminogen-binding (data not shown). Similar results were also obtained for enolases of the other species listed below. In figure 3.7 below is a multiple alignment of enolases from *Staphylococcus aureus* N315, *Streptococcus pneumonia* TIGR4, *Streptococcus pyogenes* M1 GAS, *Listeria monocytogenes* EGD-e, and *B. cereus* ATCC 14579. A Needleman-Wunch global alignment (BLOSUM62) reveals 75 % identical and 84 % similar amino acids residues of the enolases from *B. cereus* ATCC 14579 (BC5135) and *S. pyogenes* M1 GAS (spy0731), (data not shown).

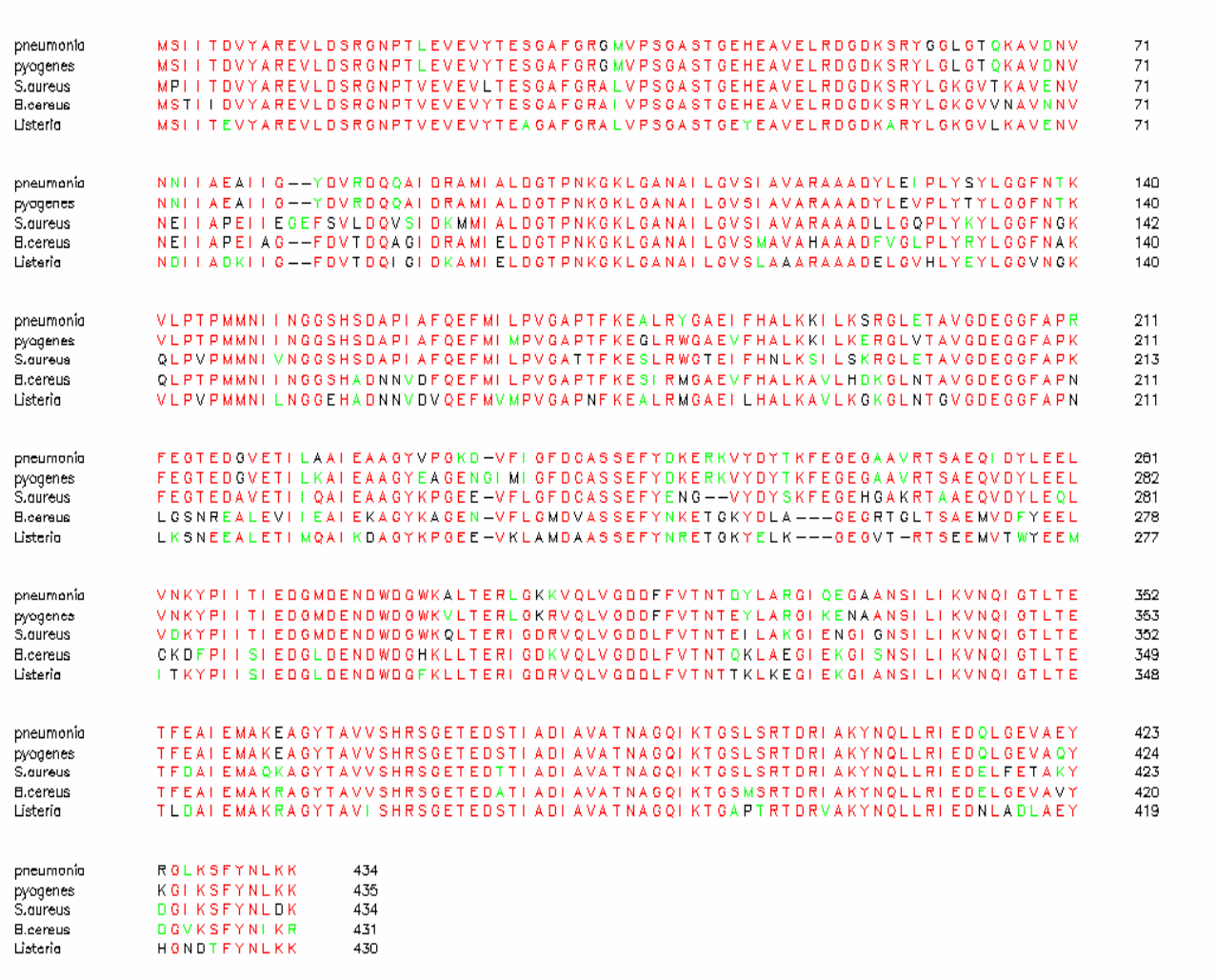


Figure 3.7 Multiple alignment of enolases from different gram positive bacteria. The bacteria strains are listed in the text above. The genes' locus tags are: sp1128, spy0731, sa0731, bc5135 and lmo2455, respectively. Red color: identical amino acid. Green color similar: amino acid.

3.3 RNA isolation and PCR primer testing

3.3.1 RNA isolation

Total RNA was isolated from the four cultures in the shift experiment (A – control, B – bile, C – bile salts and D – mucin). Samples for RNA isolation were taken at 0 – 5 – 10 – 20 and 30 minutes after the shift. The RNA from two different shift experiments was isolated by the FastPrep® - method. The RNA concentrations and protein contaminations were estimated spectrophotometrically by measuring the A_{260nm} and the ratio A_{260nm}/A_{280nm}. Total RNA concentrations varied between 0.31µg/µl and 1.66 µg/µl, giving a yield of, from 31 µg RNA to 166 µg RNA. The A_{260nm}/A_{280nm} – ratio

varied between 1.5 and 2.2 with most of the samples in the range 1.6 – 1.8. The concentrations and the A_{260nm}/A_{280nm} – ratio are given in table 3.3

Table 3.3 Total RNA concentrations and A_{260nm}/A_{280nm} – ratio of RNA isolated from two different shift experiments. The letters indicate the different shift cultures (A – control, B – bile, C – bile salts and D – mucin), and the numbers indicates the time in minutes after the shift.

	Parallel 1		Parallel 2	
Sample	Ratio A260nm:A280nm	[RNA] (µg/µl)	Ratio A260nm:A280nm	[RNA] (µg/µl)
A0	1.6	0.41	1.7	0.48
A5	1.6	0.52	1.7	0.47
A10	1.6	1.04	1.7	0.48
A20	1.6	1.24	1.7	0.53
A30	1.6	1.01	1.5	0.73
B0	1.6	0.76	1.6	0.37
B5	1.6	0.56	1.8	0.23
B10	1.6	0.64	1.7	0.40
B20	1.6	0.31	1.7	0.73
B30	1.6	0.63	1.7	0.64
C0	1.6	0.66	1.8	0.58
C5	1.6	0.84	1.9	0.68
C10	1.6	1.02	2.1	0.57
C20	1.6	0.92	2.2	1.06
C30	1.6	1.66	2.0	0.59
D0	1.6	0.47	1.8	0.43
D5	1.6	0.80	1.7	0.62
D10	1.6	0.77	1.8	0.70
D20	1.6	0.78	1.5	1.12
D30	1.6	1.31	1.8	0.60

The integrity of the isolated RNA was checked by electrophoresis on formaldehyde agarose gels and detection by ethidium bromide staining. With the exception of the mucin-containing samples, most of the samples have two intact rRNA bands and minimal DNA contamination. A picture of the gels is shown in figure 3.8. Only samples with intact rRNA were used for further analysis.

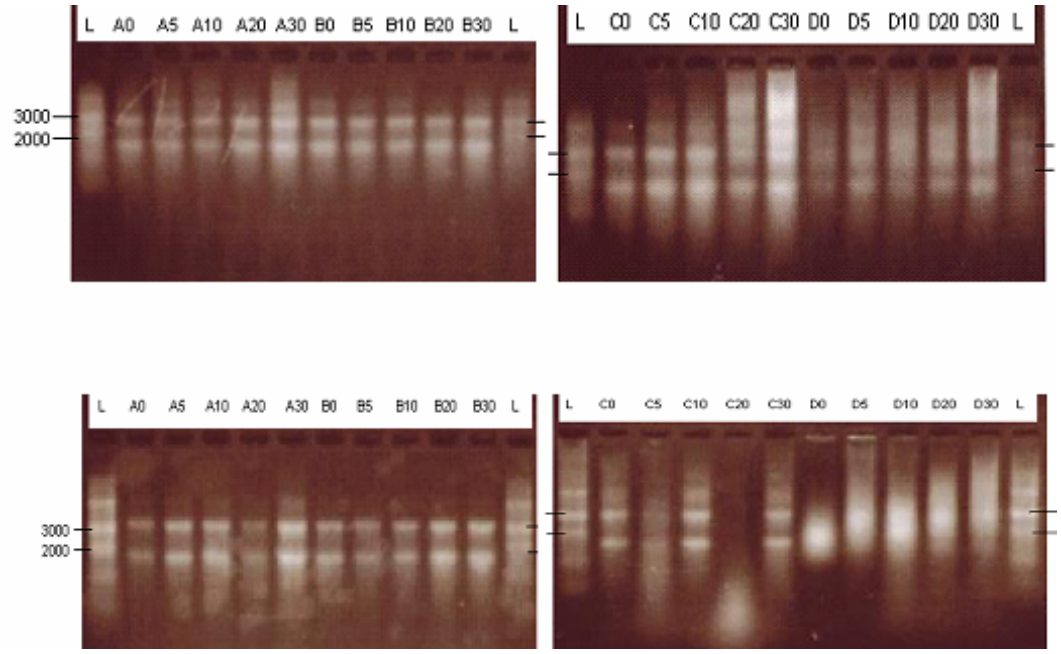


Figure 3.8 Integrity of RNA investigated by electrophoresis. The pictures show formaldehyde agarose gels of total RNA samples from two parallel experiments. The 23S and 16S rRNA bands are clearly visible and DNA contamination is minimal for most samples. L = RNA ladder, A = control, B = bile, C = bile salts and D = mucin. The number following each letter indicates how many minutes after the shift the RNA is isolated.

3.3.2 Testing of PCR primers

Oligonucleotide primers were designed with eprimer3, and synthesized by MWG. The sequences are given in table 2.2 in the methods section.

Three genes were, fbp, intA and intB, selected for investigation of mRNA levels as estimated by RealTime RT PCR analysis. The reference gene selected was gap. The primers for these four genes were tested on *Bacillus cereus* ATCC 14579 genomic DNA template. All the selected primers were found to be function and gave PCR products of correct size (~200 bp) as estimated by agarose gel electrophoresis. A picture of the agarose gel after electrophoresis and ethidium bromide staining is given in figure 3.9

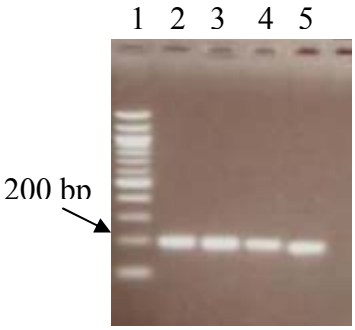


Figure 3.9 Picture of an etidium bromide stained agarose gel showing the results of PCR primers testing. The primers were tested on *Bacillus cereus* ATCC 14579 genomic DNA template. From left are (1) 1 kb ladder, (2) *intA*, (3) *intB*, (4) *fbp* and (5) *gap*. The primer sequences is given in table 2.2

3.4 Expression patterns investigated by real-time PCR

3.4.1 Reference gene

The expression pattern of the selected reference gene, gap, was investigated to see if it was equally expressed throughout the time window (0 to 30 minutes) of the shift experiment. With the exception of D0 (and to some degree C0) the changes in the expression levels are minimal. The expression changes ($2^{-\Delta Ct}$) for 10^{-1} dilutions of the specified samples are shown in table 3.4 below.

Table 3.4 Expression changes in the reference gene, *gap*. A0 is used as reference and set to 1.

Sample	Ct-value	$2^{-\Delta Ct}$ ($\Delta Ct = Ct_{A0} - Ct_{sample}$)
A0	15.04	1
A30	13.42	0.65
B0	13.99	0.97
B5	13.62	0.75
B10	13.91	0.91
B30	14.13	1.06
C0	16.01	3.91
C30	13.68	0.80
D0	20.78	106
D30	14.89	1.80

3.4.2 Amplification efficiencies of gap, fbp, intA, and intB

The amplification efficiencies were determined for each target sequence and the results are given in table 3.5.

The Ct-values of each target sequence was plotted against log 10 of the dilution and a regression line was calculated. Based on the slope of each line, the amplification efficiencies were calculated; see section 2.5.1 (7). At least 5 different regression lines were used for each target sequence amplification was calculated for each regression line. The average amplification efficiencies were used. Figure 3.10 a – d shows the regression lines of the selected genes (fbp, intA, intB, and gap). All Ct-values, both for amplification efficiencies and relative quantification can be found in appendix III.

Table 3.5 Amplification efficiencies and their standard deviation for gap, fbp, intA and intB.

Target sequence	Amplification efficiencies	Standard deviation
gap	1.04	0.026
fbp	0.99	0.035
intA	1.03	0.076
intB	1.02	0.049

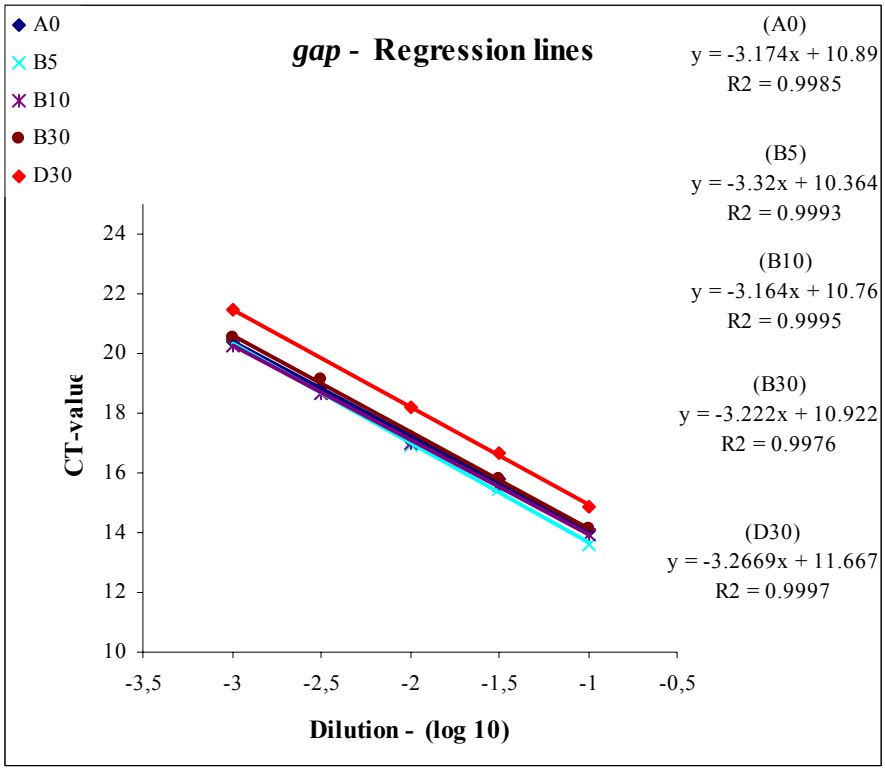


Figure 3.10a Regression lines for intB. RNA samples used are indicated in the figures. The regression lines are calculated using the least square method. The amplification efficiencies are calculated from the absolute value of the slope.

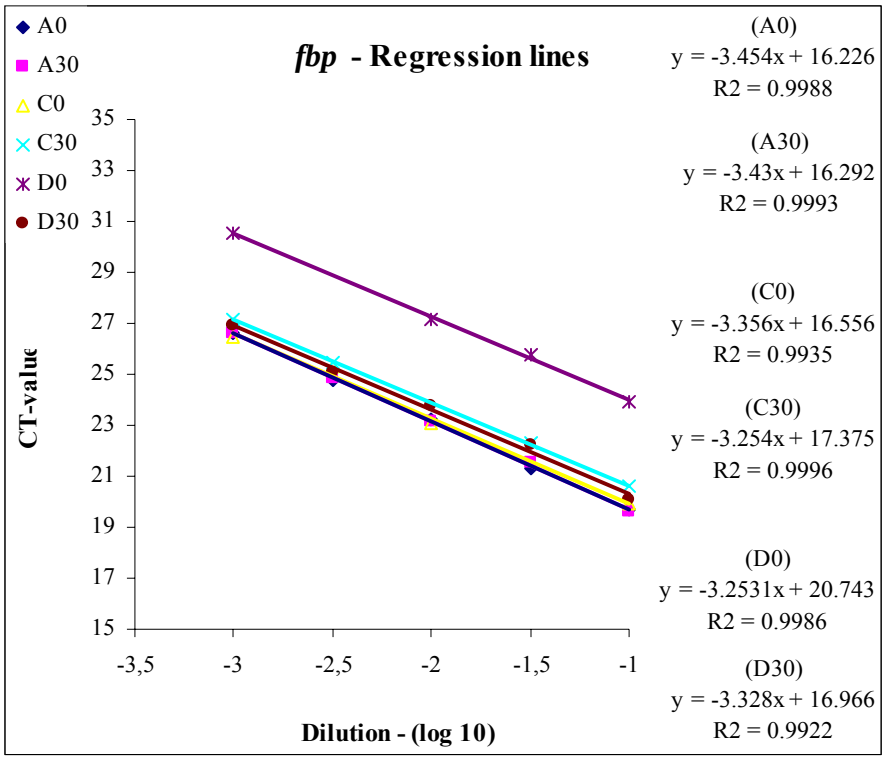


Figure 3.10b Regression lines for fbp. RNA samples used are indicated in the figures. The regression lines are calculated using the least square method. The amplification efficiencies are calculated from the absolute value of the slope.

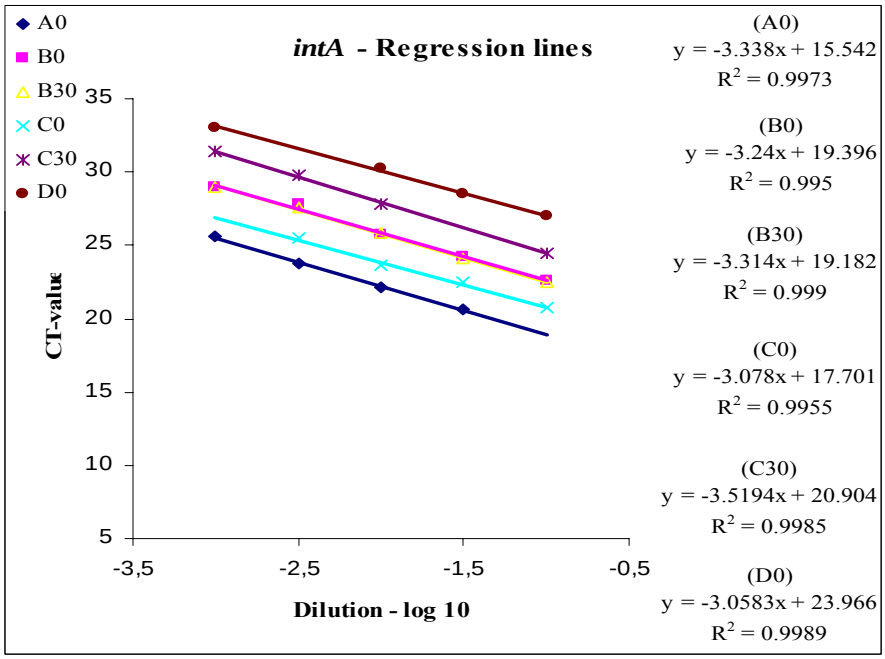


Figure 3.10c Regression lines for intA. RNA samples used are indicated in the figures. The regression lines are calculated using the least square method. The amplification efficiencies are calculated from the absolute value of the slope.

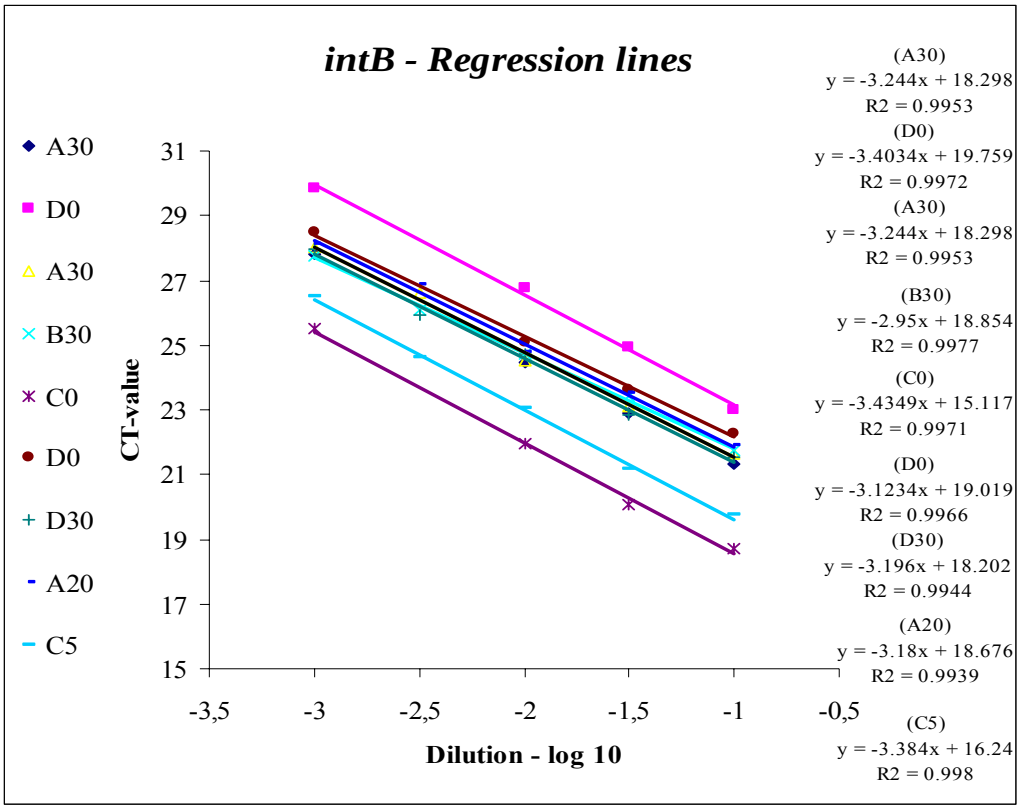


Figure 3.10d Regression lines for intB. RNA samples used are indicated in the figures. The regression lines are calculated using the least square method. The amplification efficiencies are calculated from the absolute value of the slope.

3.4.3 Relative quantification of gene expression

The Plaffl-method (section 2.4.4.3) was used to calculate the relative expression of intA, intB and fbp, when exponentially growing *Bacillus cereus* ATCC 14579 was shifted from IR medium to IR media with no additives or IR-media containing bile, bile salts and mucin (section 2.2.7). Figure 3.11 to 3.13 shows the differences in expression for each gene 0 – 30 minutes after the shift. A0 (the control immediately after the shift) is set as a reference to 1. All other samples are expressed as the fold changes compared to A0. The fbp sequence gave no significant changes due to the shift in the first experiment and further work was not carried out. IntB is down-regulated in the bile salts shift experiment. This gene was therefore investigated in 2 or 3 parallels. The control (A) and bile salts (C) were analyzed 3 times, in which one of the analysis was performed on RNA from the two different shift experiments. These samples were also investigated at 5 and 20 minutes after the shift. IntA also show some of the same pattern as intB, however it was only measured once.

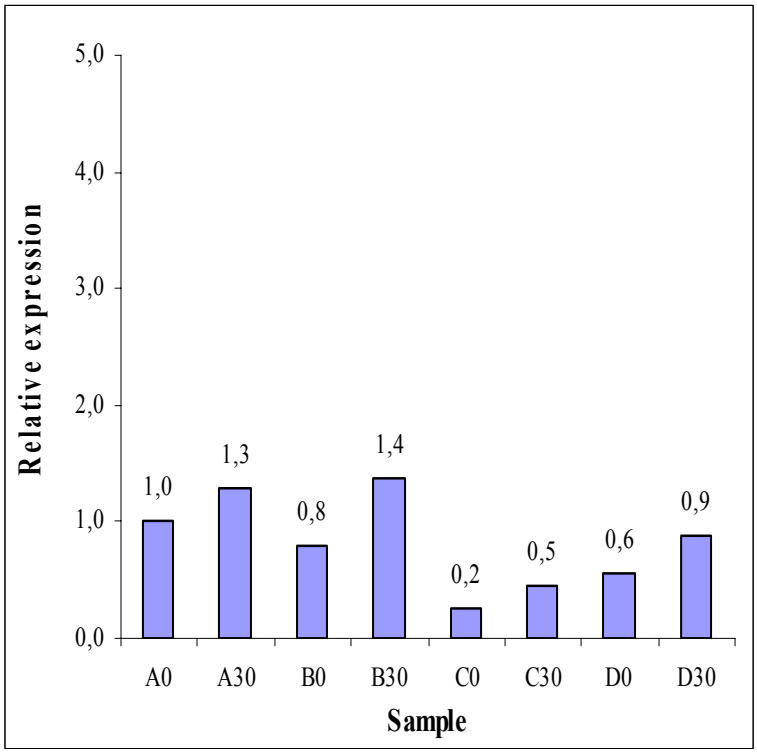


Figure 3.11 Expression pattern of fbp during shift experiment. All samples are expressed relative to A0, in which the expression is set to 1. The letter indicates the different additives (A – control, B – bile, C – bile salts and D – mucin); the following number indicates the time in minutes after the shift.

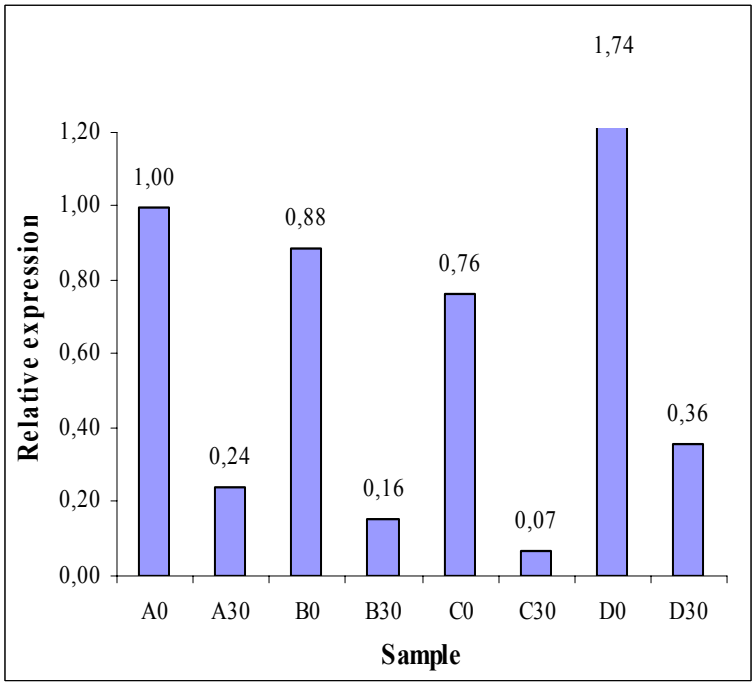


Figure 3.12 Relative expression pattern of intA during shift experiment. All samples are expressed relative to A0, in which the expression is set to 1. The letter indicates the different additives (A – control, B – bile, C – bile salts and D – mucin); the following number indicates the time in minutes after the shift.

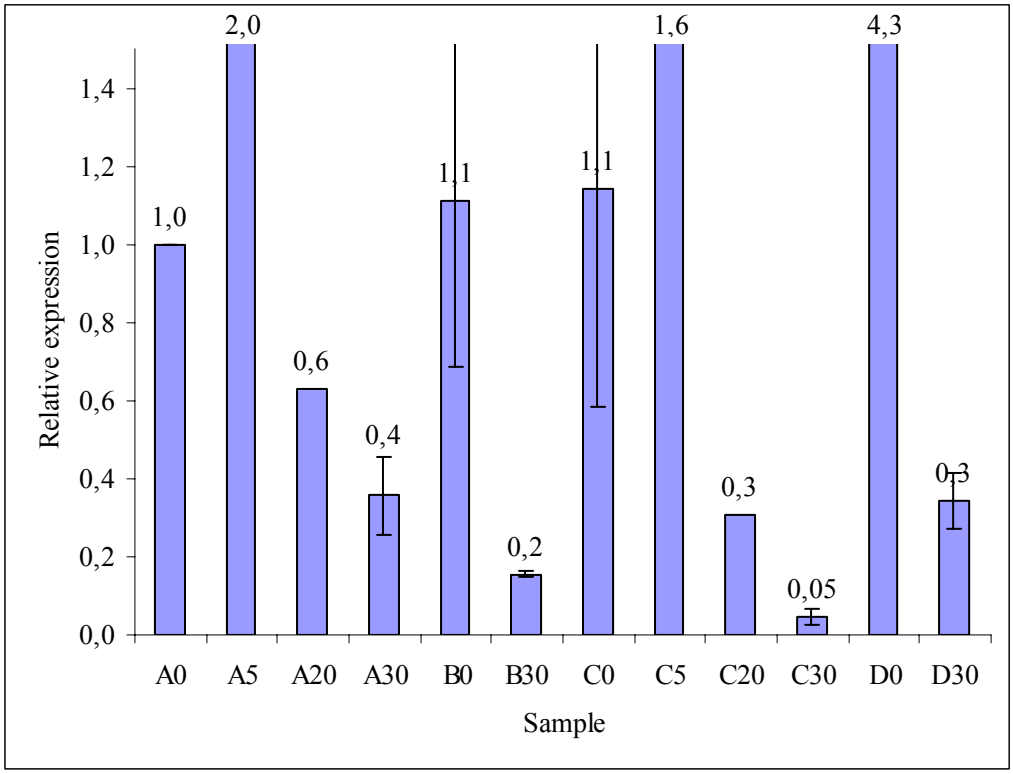


Figure 3.13 Relative expression pattern of intB during shift experiment. All samples are expressed relative to A0, in which the expression is set to 1. The letter indicates the different additives (A – control, B – bile, C – bile salts and D – mucin); the following number indicates the time in minutes after the shift. The bars indicate the average of two or three experiments; the error bars indicate the standard deviation.

3.4.4 Verification of RealTime PCR products

The melting curves showed a distinct melting temperature (T_m) for each product that was specific to that product. The T_m had a range maximum of 1.0 °C, and all parallel samples fitted this in this range. No additional peaks (indicating primer dimers, chimerical products etc.) were observed. The menting temperature is given in table 3.6.

Table 3.6 Melting temperatures of real-time PCR products. All PCR products fitted in each sequence' range.

Target sequence	T_m (range)
gap	80.2 – 81.1 ºC
fbp	79.9 – 80.9 ºC
intA	77.3 – 78.3 ºC
intB	77.5 – 78.5 ºC

The PCR products were subjected to agarose gel electrophoresis and the results showed that the size of the products corresponded to the theoretical size. See fig 3.14

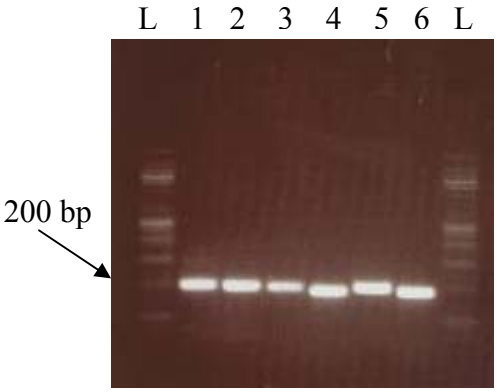


Figure 3.14 Picture of etidium bromide stained agarose gel of RealTime PCR products, from left to right; L = 100 bp ladder, lane 1: intA, lane 2: intB, lane 3: fbp, lane 4: gap, lane 5: intB (Ct = 24.33), lane 6: gap (Ct = 13.33). Note that the intensity of the two differently expressed products (lane 6 and 7) are fairly identical.

3.5 Microarray analysis

3.5.1 RNA isolation and hybridization

Control RNA samples were isolated immediately after a shift to medium containing no additives (A0) and after 30 minutes incubation (A30). Samples of RNA were isolated 30 minutes after the shift to medium containing bile or bile salts (B30 and C30), see section 2.2.7. The RNA integrity and possible DNA contamination was investigated by formaldehyde gel electrophoresis and found suitable (data not shown.) All samples (A30, B30, and C30) were hybridized against the control at 0 minutes (A0) on microarrays of the *B. cereus* ATCC 14579 genome. All runs were done in two biological replicates. The data was analyzed with using a script written in R 2.0.1. by K. Fægri at the institute of pharmacy, Oslo University.

3.5.2 Microarray and expression pattern

The results from the microarray analysis, expressed as the log2 ratios and P-values of each sample against A0 are shown in table 3.7. All signals that had a log2 ratio over ±0.59 and a P-value below 0.05 for B30 and C30 against A0 are listed in the table. All of these signals that also were present in the A30/A0 hybridization and had a P-value

over 0.05 are included in the list. There were some problems in the second hybridization of A30/A0 and C30/A0 which caused low signal intensity. Therefore C30/A0 is represented by only one biological replicate. Since the P-value is based on the number of observations this led to higher P-values and the use of only one replicate in the A30/A0 hybridization gave only a few significant observations. The A30/A0 is therefore represented by two biological replicates, but many of the genes seen in B30/A0 (and C30/A0) are missing. The groups of genes listed are based on KEGGs putative functions. Some of the genes can belong to more than one group; however, they are only listed once.

Several genes are regulated in the bile and bile salts treated cultures, but not in the control. The vast majority of genes regulated fall into categories such as metabolism, transcriptional regulators, stress response, and transport. See discussion for details.

Table 3.7 Section of the microarray results. The A30, B30, and C30 samples were each hybridized against the A0 sample. The log2 ratios and the P-values are listed. The complete list is given in appendix IV. Please note that this is a section only, and the complete list should be examined to get a better overview.

		A30/A0		**B30/A0**		**C30/A0**	
BC #	**Genename**	Log2 ratio	P-value	Log2 ratio	P-value	Log2 ratio	P-value
Transciptional regulators							
BC0518	Transcriptional regulator. Fur family	-0.17	0.039	0.97	0.017		
Toxins							
BC3104	Hemolysin BL lytic component L2			-2.31	<0.005		
Stress response							
BC4312	Chaperone protein dnaK	0.89	<0.005	1.38	<0.005	1.17[(2)]	0.016[(2)]
BC0294	Chaperonin. 10 kDa	1.29	<0.005	3.40	<0.005	2.16[(2)]	0.015[(2)]
BC0295	Chaperonin. 60 kDa	0.78	<0.005	2.97	<0.005		
BC5152	ATP-dependent Clp protease. proteolytic subunit ClpP					1.51	0.037
BC2214	Heat shock protein. Hsp20 family			3.33	0.047		
BC4314	Heat-inducible transcription repressor HrcA					1.89	0.037
BC4521	Thioredoxin	-0.82	0.019	1.24	<0.005		
BC4272	Superoxide dismutase. Mn			0.68	0.007		
Surface associated							
BC1331	Internalin. putative			-0.58	0.097		
Transport							
BC0962	Drug resistance transporter. EmrB/QacA family					2.80	0.034
BC4000	Drug resistance transporter. EmrB/QacA family			2.76	<0.005		
BC0596	Heavy metal-transporting ATPase			1.68	<0.005		
BC1948	Hypothetical Multidrug-Efflux Transporter					-0.76	0.044
Metabolism							
BC3972	Pyruvate dehydrogenase complex E1 component. beta subunit					0.75	0.038
BC3971	Pyruvate dhg complex E2 - dihydrolipoamide acetyltransferase			-1.00	<0.005		
BC3970	Pyruvate dhg complex E3 - dihydrolipoamide dehydrogenase	-0.74[(1)]	0.239[(1)]	0.28	<0.005	0.70	0.094
BC4160	Dihydrolipoamide dehydrogenase					2.34[(2)]	0.029[(2)]
BC0868	Glyceraldehyde-3-phosphate dehydrogenase. NADP-dependent			1.04	0.036		

Genes with bold BC-number are also investigated by RealTime or 2-DE. [(1)] Genes in the A30/A0 hybridization that is only confirmed with one biological replicate. [(2)] Genes in the C30/A0 hybridization that is confirmed by two biological replicates.

3.6 Protein extraction and 2-D protein gel-electrophoresis

3.6.1. Protein concentration – standard curves

Two standard curves, using BSA, for protein concentration were determined and are shown in figures 3.15 and 3.16. Protein concentrations were determined with the formulas indicated in each figure. The 1 µg to 5 µg curve was used when the absorbance in the sample was between 0.420 (A_{595nm}) and 0.600 (A_{595nm}) and the 5 µg to 20 µg curve was used when absorbance of the sample was between 0.600 (A_{595nm}) and 0.900 (A_{595nm}). A mean of the two formulas was used for samples giving an absorption between 0.580 (A_{595nm}) and 0.620 (A_{595nm}).

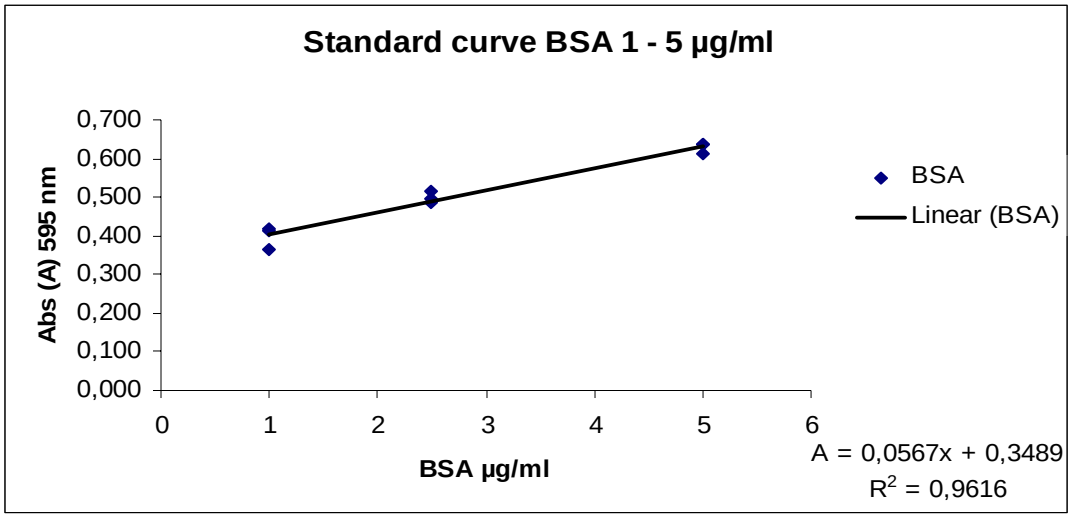


Figure 3.15 Standard curve with BSA ranging from 1 µg to 5 µg. Concentrations in protein samples were calculated with the formula given in the diagram, where A is the absorbance and x is µg/ml BSA.

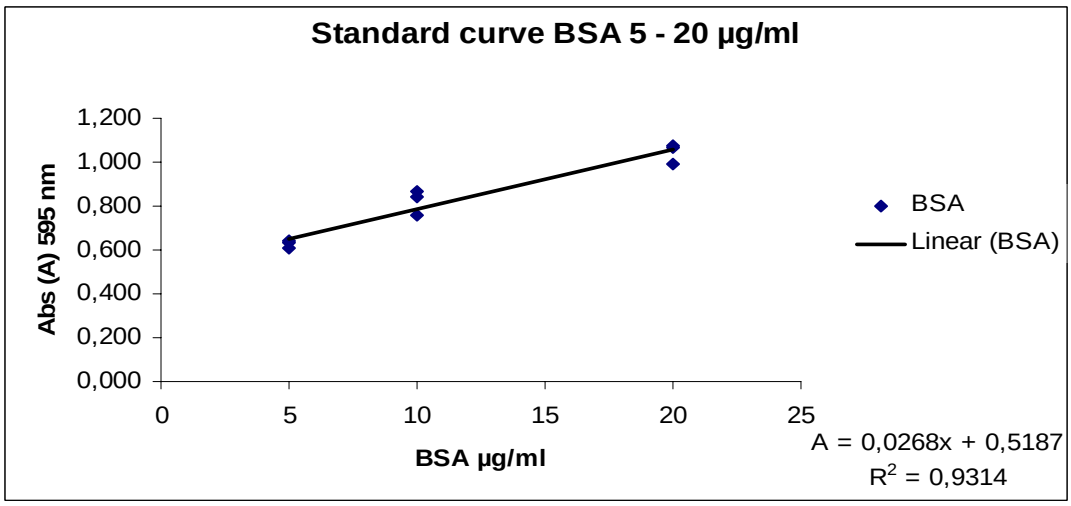


Figure 3.16 Standard curve with BSA ranging from 5 µg to 20 µg. Concentrations in protein samples were calculated with the formula given in the diagram, where A is the absorbance and x is µg/ml BSA.

3.6.2 Whole cell proteins

A whole cell protein extract of exponentially growing *B. cereus* ATCC 14579 was obtained by harvesting the cell by centrifugation at 4000 x g (rcf)., washing the cell pellet once in 20 mM Tris (pH 7.5), and resuspending the cell pellet in 20 mM Tris (pH 7.5) containing protease inhibitor or lysisbuffer (7 M Urea, 2 M Thiourea and 4 % (w/v) CHAPS). The cells were then disrupting in a FastPrep® instrument. The proteins extracted in 20 mM Tris (pH 7.5) were harvested from cultures with different cell densities and some of these were used in the aminopeptidase C assay (see 3.6.3.1).

Both protein extracts was analyzed by 2-D gel electrophoresis. Five µg protein was used in the 2-D gel electrophoresis analysis. The gels are shown in figure 3.17 a and b.

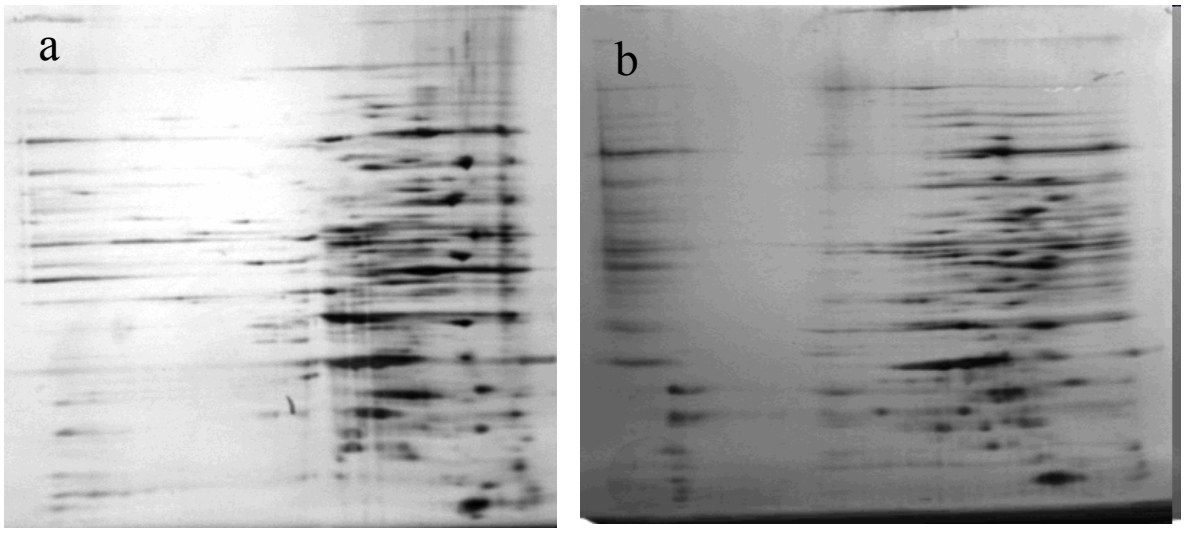


Figure 3.17 a and b 2-DE gel of total protein extract of exponentially growing *B. cereus* ATCC 14579. Proteins were extracted with the FastPrep® method. 5 µg protein was seperated by isoelectric focusing (pH 3 – 10) in the first dimension, and separated by MW in the second dimension. The gels were silver stained. pH 10 is the to left side of the gels, and pH 3 to the right side. a) Total protein extracted in lysis buffer. b) Total protein extracted in 20 mM Tris (pH 7.5).

3.6.3 Test of buffer extracts

3.6.3.1 Estimation of cytosolic contamination: aminopeptidase C activity in buffer extracts.

To test if any of the buffers did inhibit the aminopeptidase reaction 1 µl whole cell protein extract was incubated for 15 min at RT with 9 µl of the listed buffers (table 3.8) before measuring the absoration at 405 nm as described (2.6.2.2). The absorption

after 20 minutes is given in table 3.8. The CTAB buffer did inhibit the reaction to some degree. On the other hand the sample incubated in 1X PBS and 1 M Tris showed higher activity. None of the other buffers or the protease inhibitor appeared to influence the reaction.

Table 3.8 Aminopeptidase C activity of whole cell protein extract in selected buffers

Buffer	**A -595**
20 mM Tris (pH 7,5) without protease inhibitor	0,188
20 mM Tris (pH 7.5)	0,189
1X PBS (pH 7.0)	0,259
1 M Tris (pH 7.4)	0,240
1 M KSCN in 20 mM Tris (pH 7.5)	0,187
0.01 % NP-40 in 20 mM Tris (pH 7.5)	0,197
0.1 % (w/v) CTAB in 20 mM Tris (pH 7.5)	0,145
0.01 % (v/v) Triton X-100 in 20 mM Tris (pH 7.5)	0,192
1.5 M LiCl in 20 mM Tris (pH 7.5)	0,189

The buffer extracts listed in table 3.7 were tested in the aminopeptidase C assay.
The culture supernatant and the wash extract (20 mM Tris (pH 7.5)) were also tested. For each buffer extraction proteins in the remaining cell pellet were disrupted and extracted in 20 mM Tris (pH 7.5) using the FastPrep® method. In table 3.9, shown below, the aminopeptidase C activity of each buffer extract is listed together with the total protein extract from the remaining cell pellet. Whole cell protein extracts of the entire cultures (200 ml) with a cell denstity of $1.3x10^7$ and $9.3x10^7$ cell#/ml were used as positive controls. Any increase in absorption over 20 minutes or a final absorption deviating more than 10 % (A_{595} over 0,094) from the negative control was interpreted as positive aminopeptidase C activity and consequently cytosolic contamination in the sample. By these criteria the culture supernatant and the NP-40 and Triton X-100 extracts failed the test. The results are shown in table 3.7 and figure 3.18

Table 3.9 Aminopeptidase C activity in buffer extracts measured over 20 minutes. The activity is given as the absorption at 405 nm. Increase in absorption or absorption after 20 min that are higher than 10 % from the negative are considered positive for aminopeptidase C activity. The number in the left column corresponds to those given in table 3.8 and figure 3.19. A number followed by (a) indicates the buffer extract and a number followed by (b) indicates the total protein extracts from the remaining cell pellet after buffer extraction.

		Minutes incubated:	**0**	**5**	**10**	**15**	**20**
	Extraction buffer* (and negative/positive controls)	**Abbreviation**	**Absorption A - 595 nm**				
	Negative control **	Negative	0.089	0.088	0.086	0.086	0.085
	Culture supernatant	Supernatant	0.098	0.098	0.097	0.097	0.097
1	20 mM Tris (pH 7.5) (Wash prior to extraction)	Wash	0.092	0.091	0.092	0.092	0.092
2a	1X PBS (pH 7.0)	PBS	0.093	0.092	0.092	0.091	0.091
2b	Cytosolic fraction after PBS extraction		0.221	0.246	0.280	0.308	0.340
3a	1 M Tris (pH 7.4)	Tris	0.090	0.090	0.090	0.090	0.090
3b	Cytosolic fraction after Tris extraction		0.339	0.386	0.421	0.471	0.494
4a	1 M KSCN in 20 mM Tris (pH 7.5)	KSCN	0.094	0.093	0.094	0.094	0.094
4b	Cytosolic fraction after KSCN extraction		0.328	0.352	0.370	0.417	0.462
5a	0.01 % NP-40 in 20 mM Tris (pH 7.5)	NP-40	0.117	0.124	0.130	0.134	0.138
5b	Cytosolic fraction after NP-40 extraction		0.193	0.218	0.241	0.274	0.289
6a	0.1 % (w/v) CTAB in 20 mM Tris (pH 7.5)	CTAB	0.093	0.093	0.093	0.092	0.092
6b	Cytosolic fraction after CTAB extraction		0.234	0.261	0.295	0.311	0.338
7a	0.01 % (v/v) Triton X-100 in 20 mM Tris (pH 7.5)	Triton X	0.101	0.100	0.100	0.101	0.102
7b	Cytosolic fraction after Triton X-100 extraction		0.233	0.255	0.291	0.316	0.351
8a	1.5 M LiCl in 20 mM Tris (pH 7.5)	LiCl	0.094	0.093	0.093	0.093	0.093
8b	Cytosolic fraction after LiCl extraction		0.312	0.348	0.381	0.417	0.446
	Positive control of extract from 1.3*10^7 cell#/ml	Positive 1	0.139	0.147	0.153	0.158	0.165
	Positive control of extract from 9.3*10^7 cell#/ml	Positive 2	0.712	0.755	0.784	0.807	0.834

* All the extraction buffers contain protease inhibitor.

** The negative control is the mean of 5 runs, and the standard deviation was ± 0.061 (A).

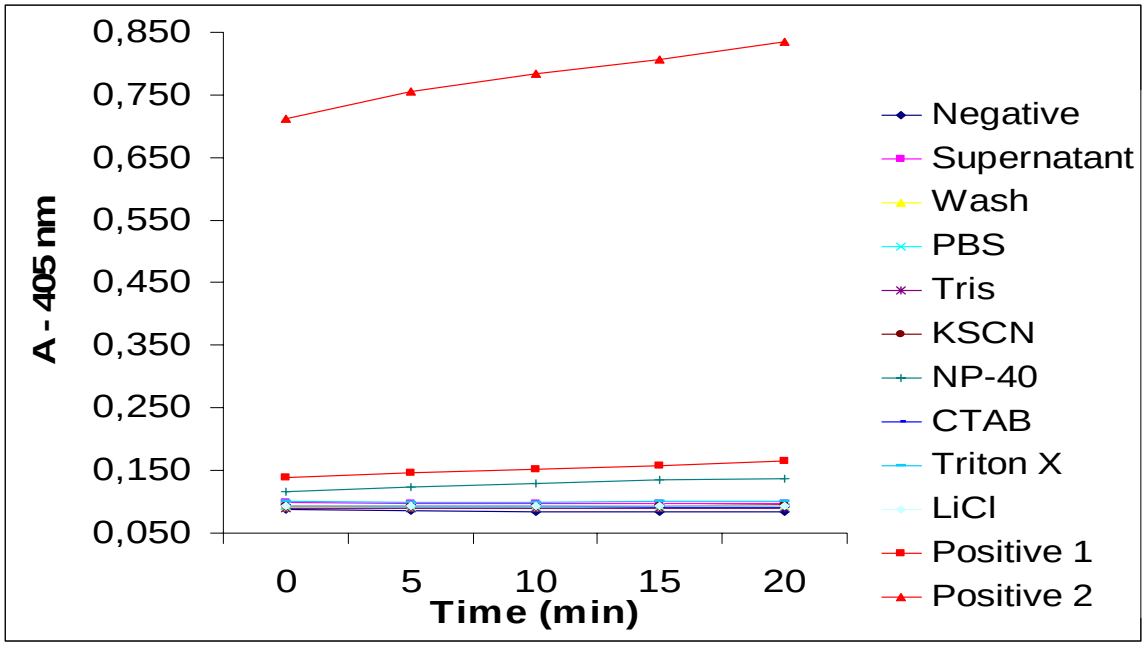


Figure 3.18 Aminopeptidase C activity of tested buffer extracts (see table 3.7 above) over 20 minutes. The activity is given as the absorption at 405 nm. Increases in absorption or absorption after 20 min that deviate more than 10 % from the negative are considered positive for aminopeptidase C activity.

3.6.3.2 Extraction buffers effectively measured by: protein yield, aminopeptidase C activity and SDS polyacrylamide gel electrophoresis

Bacillus cereus ATCC 14579 was grown in IR-media for three hours (37 °C, 175 rpm) and 100 ml culture was shifted to 100 ml fresh IR-media and harvested immediately by centrifugation. The cell pellet was washed and resuspended in different extraction buffers which are listed below in table 3.8. The bacteria cell density at the time of harvesting was measured by flow cytometry and the aminopeptidase C assay was performed on fresh extracts. The samples were concentrated by ultrafiltration and the protein concentration was measured. A summary of the results are found in table 3.10

Table 3.10 Summary of results for testing of extraction buffers. The cell densities at time of harvest, protein concentrations, total protein yield, and result of the aminopeptidase C test is given in the table

	Extraction buffer[(1)]	Cell density (Cell #/ml)	Aminopeptidase C assay	[Protein] yield (µg/ml)[(2)]	Total protein yield (µg)[(2)]
1	20 mM Tris (pH 7.5) - Wash step - 1 minute	$4.3x10^{7}$[(3)]	Pass	243[(4)]	27[(4)]
2	1X PBS (pH 7.0)	$2.2 x10^{7}$	Pass	291	29
3	1 M Tris (pH 7.4)	$5.1 x10^{7}$	Pass	423	51
4	1 M KSCN in 20 mM Tris (pH 7.5)	$6.6 x10^{7}$	Pass	337	37
5	0.01 % NP-40 in 20 mM Tris (pH 7.5)	$6.1 x10^{7}$	Fail	2860	286
6	0.1 % (w/v) CTAB in 20 mM Tris (pH 7.5)	$2.9 x10^{7}$	Pass	324	39
7	0.01 % (v/v) Triton X-100 in 20 mM Tris (pH 7.5)	$3.8 x10^{7}$	Fail	382	42
8	1.5 M LiCl in 20 mM Tris (pH 7.5)	$7.7 x10^{7}$	Pass	308	28

[(1)]All buffers contains protease inhibitor. [(2)] After concentration of samples.
The wash step had a rather dense protein concentration, thus three extractions were combined: [(3)] The cell density is the mean of three extractions. [(4)] The protein yield are from three combined extractions (washes.)

SDS-PAGE was performed with 20 µl of the concentrated buffer extracts and 5 µl of the total protein extract from the cell pellet remaining after buffer extraction. The SDS-PAGE gels are shown in figure 3.19. The washes from LiCl and KSCN extracts were also analyzed by 2-Delectrophoresis and pictures of the gels are shown in figures 3.20a, b, and c, respectively. Protein (5 µg) was applied to IPG strips (pH 3 – 10) and focused by IEF. The proteins in the IPG strips were then separated according to molecular weight on Bis-tris gels (4 – 12 %). The 2-DE protein gels were silver stained. For comparison of the protein pattern in the buffer extracts with the whole

cell proteins the 2-DE gel of the total protein extract (3.6.2, fig. 3.17 b) are included in fig 3.20 d.

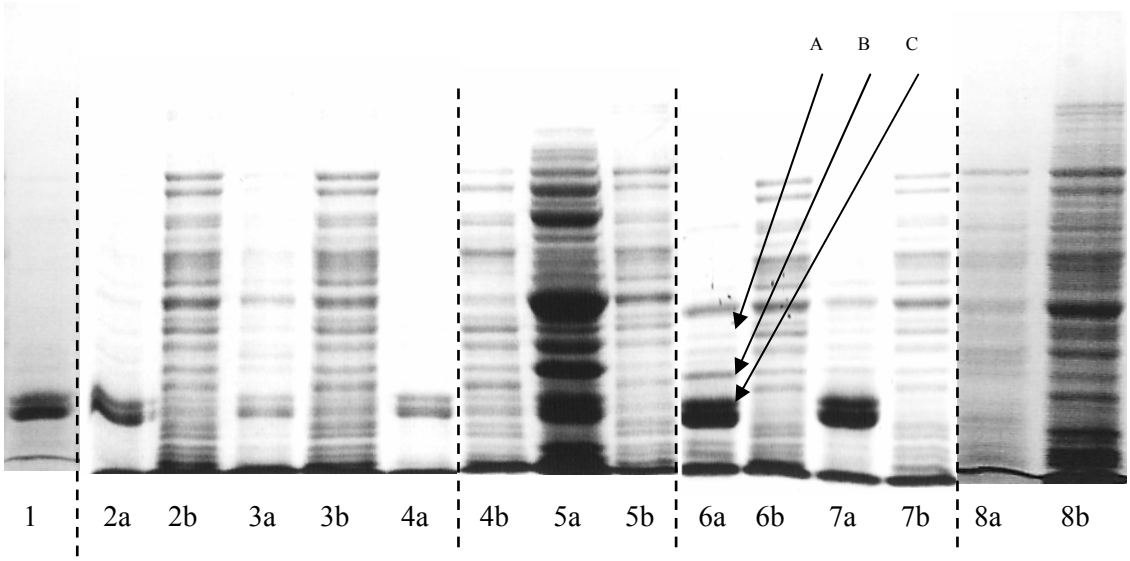


Figure 3.19 SDS-PAGE gels from the tested buffer extracts. Each buffer extraction (a) is aligned with its remaining total protein extract (b) to the right, except for number 1 which is the wash extraction. 20 µl (wash or buffer) or 5 µl (cytosol) of the protein extracts were loaded in each lane. The gels were Coomassie stained. The dashed lines delineate different gels. The letters A, B and C indicates the bands that were investigated at the proteomics course (section 2.7 and 3XX). The list of the buffers is given in table 3.8

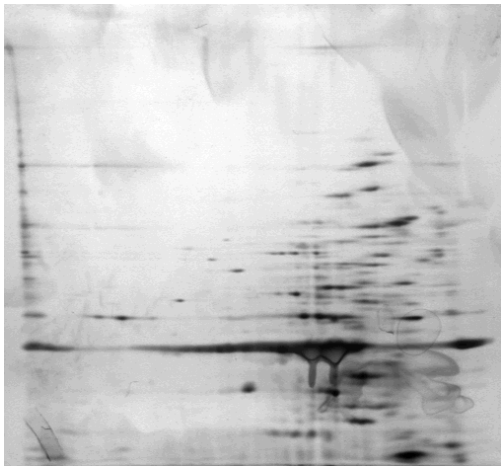


Figure 3.20a Picture of 2-DE gel of the wash extract (20 mM Tris (pH 7.5) – 1 min). See table 3.8 and above text for details.

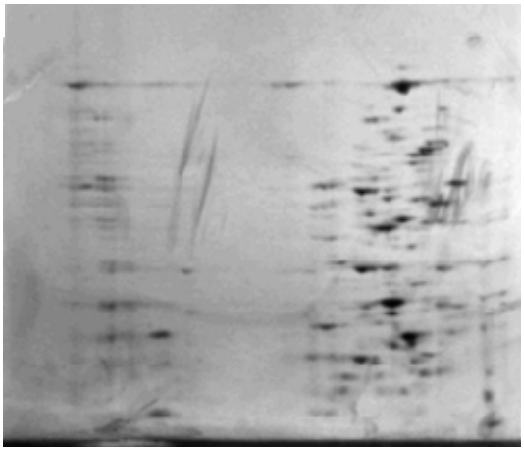


Figure 3.20b Picture of 2-DE gel of the LiCl (1.5 MLiCl in 20 mM Tris (pH 7.5) – 15 min). See table 3.8 and above text for details.

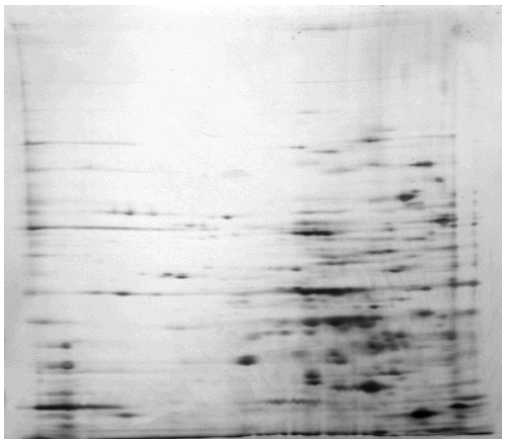
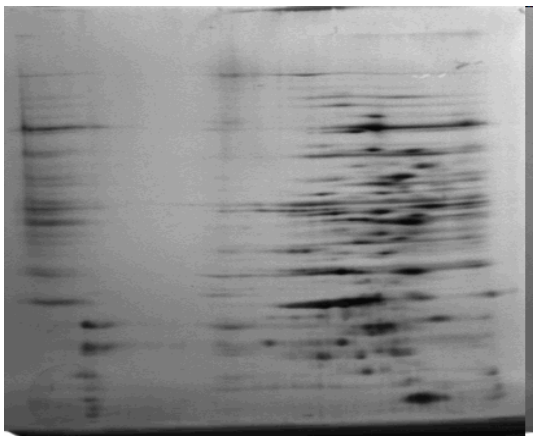


Figure 3.20c Picture of 2-DE gel of the KSCN extract (1 M KSCN in 20 mM Tris (pH 7.5) 15 min). See table 3.8 and above text for details.



Figur 3.20d Picture of 2-DE gel of the total protein in 20 mM Tris (pH 7.5) This picture is - identical to the picture given in fig 3.17 b. See section 3.6.2 and above text for details.

3.6.4 Shift experiments, 2-D electrophoresis and Protein Mass Fingerprinting

3.6.4.1 Protein extraction and 2-D electrophoresis of bile and bile salts stimulated *Bacillus cereus*

Bacillus cereus ATCC 14579 was grown and shifted to new media containing bile and bile salts to give final concentrations of 0.031 % (w/v) and 0.005 (w/v), respectively, as described in 2.2.7.

A buffer extraction procedure, selected on the basis in results described in section 3.6.3.2, using 1.5 M LiCl in 20 mM Tris (pH 7.5) containing protease inhibitor was used. The entire bacterial culture (200 ml) was harvested and proteins were isolated as described for the control culture (A) at 0 and 30 minutes after the shift and from bile (B) and bile salts (C) cultures 30 minutes after the shift. The experiment was done in three parallels and all extracts was analyzed by 2-D electrophoresis.

The 2-D gels were silver stained and analyzed with the ImageMaster™ 2DE Platinum 6.0. The 2-DE gels from B30 (bile) and C30 (bile salts) were compared to A0 (control). Protein spots that had increase or decrease in volume % in either B30 or C30 were listed. Those protein spots showing similar pattern when comparing A30 to A0 were excluded to make sure the changes in volume % observed were due to the shifts to bile or bile salts media and not just a change caused by further growth.

A summary of the results with regard to cell densities at time of harvest, protein yield and the number of spots identified in each parallel is shown in table 3.11. None of the samples showed any detectable aminopeptidase C activity.

Table 3.11 A summary of the results for the buffer extraction of proteins after the shift. Cell density, protein yield and number of different spots on 2-DE gels from LiCl protein extracts after shift experiments. The entire culture (200 ml) in each parallel was used for extraction. The number following each culture indicates the three different parallels.

Sample /parallel	Cell density (cell #/ml)	Protein yield (µg)	Number of protein spots detected
A0 1	$2.1x10^{7(1)}$	$34^{(2)}$	82
A0 2	$1.9 x10^{7(1)}$	$34^{(2)}$	77
A0 3	$2.8 x10^{7(1)}$	$36^{(2)}$	82
A30 1	$7.1 x10^{7}$	34	65
A30 2	$9.1 x10^{7}$	50	65
A30 3	$9.8 x10^{7}$	47	63
B30 1	$2.9 x10^{7(1)}$	$36^{(2)}$	68
B30 2	$2.6 x10^{7(1)}$	$43^{(2)}$	66
B30 3	$3.6 x10^{7(1)}$	$39^{(2)}$	65
C30 1	$5.3 x10^{7}$	22	72
C30 2	$6.0 x10^{7}$	17	71
C30 3	$6.2 x10^{7}$	20	67

Because of low protein yield in A0 and B30 proteins form two extractions were combined for each parallel [(1)] Mean cell density of two experiments. [(2)] Sum protein yield of the experiments.

The 2-DE gels were normalized, compared and spots were matched using the ImageMaster™ 2DE Platinum 6.0. Synthetic gels were made to facilitate comparison. 20 spots were identified as being differentially regulated in the different samples. Two of these were identified by PMF (3.6.4.2) and are assigned by name; enolase and dhla (=dihydrolipoamide dehydrogenase). Spots, their average volume %, and the standard deviation between the three parallels in each sample are shown in Table 3.12. The log2 ratio between all cultures at 30 minutes and control at 0 minutes samples (log 2 (A30/A0), log2 (B30/A0), and log2 (C30/A0) is also given.

Eight spots in bile treated sample (B30) and seven spots in bile salts treated sample (C30) were identified to be up-regulated compared to both controls (A0 and A30). Five in B30 and eight in C30 show down-regulation.

The 2-DE gels are shown in figures 3.21 to 3.24. A representative gel is shown for each sample (A0, A30, B30 and C30). These 2-DE gels without the spot markers can be found in appendix IV along with the other gels and synthetic gels.

Table 3.12 List of spots on the gels showing increase/decrease in the volume %. The mean volume % and the standard deviation between three replicates is given for each spot in each gel. Each spot is compared to its matched spot in the control gel (A0) and the log2 ratio is given. **Vol %**s marked with **bold** indicates significant up-regulation and *vol %*s marked with *italic* indicates significant down-regulation when compared to the control at both 0 and 30 minutes (A0 and A30). The spot numbers match those in the gels. The approximate MW and pI are also listed. NI indicates that the spot was not indentified in that gel, and thus the arrow (↓) means high degree of down-regulation.

Spot #	MW[1]	pI[1]	Control - 0 min (A0) Vol%	St.dev	Control -30 min (A30) Vol%	St.dev	Log2 ratio	Bile treated - 30 min (B30) Vol%	St.dev	Log2 ratio	Bile salts treated- 30 min (C30) Vol%	St.dev	Log2 ratio
1	54	4.7	0.7	0.15	1.0	0.45	0.36	0.5	0.22	-0.45	**1.9**	0.77	**1.31**
2[2]	46	4.7	1.6	0.41	1.3	0.79	-0.29	**2.8**	0.43	**0.77**	0.5	0.03	*-1.85*
3	22	4.9	2.0	0.46	1.8	1.03	-0.20	1.7	0.87	-0.30	**4.4**	1.18	**1.10**
4	38	4.7	0.3	0.21	NI			**1.2**	0.58	**1.84**	**1.1**	0.40	**1.72**
5	55	4.9	1.2	0.24	1.1	0.19	-0.13	1.4	0.53	0.20	**3.0**	0.69	**1.31**
6[2]	50	5.2	2.1	0.49	4.0	1.03	0.95	**7.2**	0.86	**1.79**	**6.5**	0.39	**1.66**
7	66	4.9	7.9	1.13	5.2	0.43	-0.60	*2.7*	0.30	*-1.56*	*3.7*	0.68	*-1.08*
8	22	8.8	0.2	0.06	NI			**1.5**	0.62	**2.95**	*NI*		
9	15	5.6	0.2	0.10	0.5	0.17	1.48	**0.8**	0.61	**2.23**	0.5	0.10	1.58
10	29	5.1	2.2	0.62	4.5	0.55	1.06	*0.6*	0.15	*-1.89*	*1.1*	0.24	*-0.92*
11	19	5.2	1.2	0.38	2.3	1.08	0.90	2.6	1.85	1.08	3.0	1.02	1.30
12	61	4.7	1.2	0.22	NI			0.7	0.19	-0.74	0.2	0.24	-2.39
13	51	4.8	0.2	0.11	NI			NI			**0.6**	0.13	**1.72**
14	73	5.8	0.9	0.12	NI			NI			**2.8**	0.31	**1.62**
15	18	4.8	1.4	0.63	1.8	0.22	0.33	*0.7*	0.22	*-0.98*	*0.4*	0.12	*-1.71*
16	52	5.3	0.7	0.10	0.8	0.36	0.17	**1.5**	0.67	**1.09**	*0.4*	0.07	*-0.67*
17	53	9.5	0.5	0.12	0.6	0.07	0.33	**0.9**	0.36	**0.98**	0.4	0.26	-0.43
18	28	4.8	0.7	0.13	0.8	0.09	0.11	*NI*		↓	*0.4*	0.10	*-0.99*
19	15	10	1.3	0.34	0.8	0.26	-0.71	**6.0**	3.39	**2.18**	0.7	0.37	-0.83
20	39	5.0	2.5	1.25	3.1	0.78	0.31	1.0	0.66	*-1.35*	*1.7*	0.66	*-0.54*

[1] Aproximate MW and pI. Spots 2 and 6 were identified to be enolase and dihydrodhlaoamide dehydrogenase by protein mass fingerprinting, repectively.

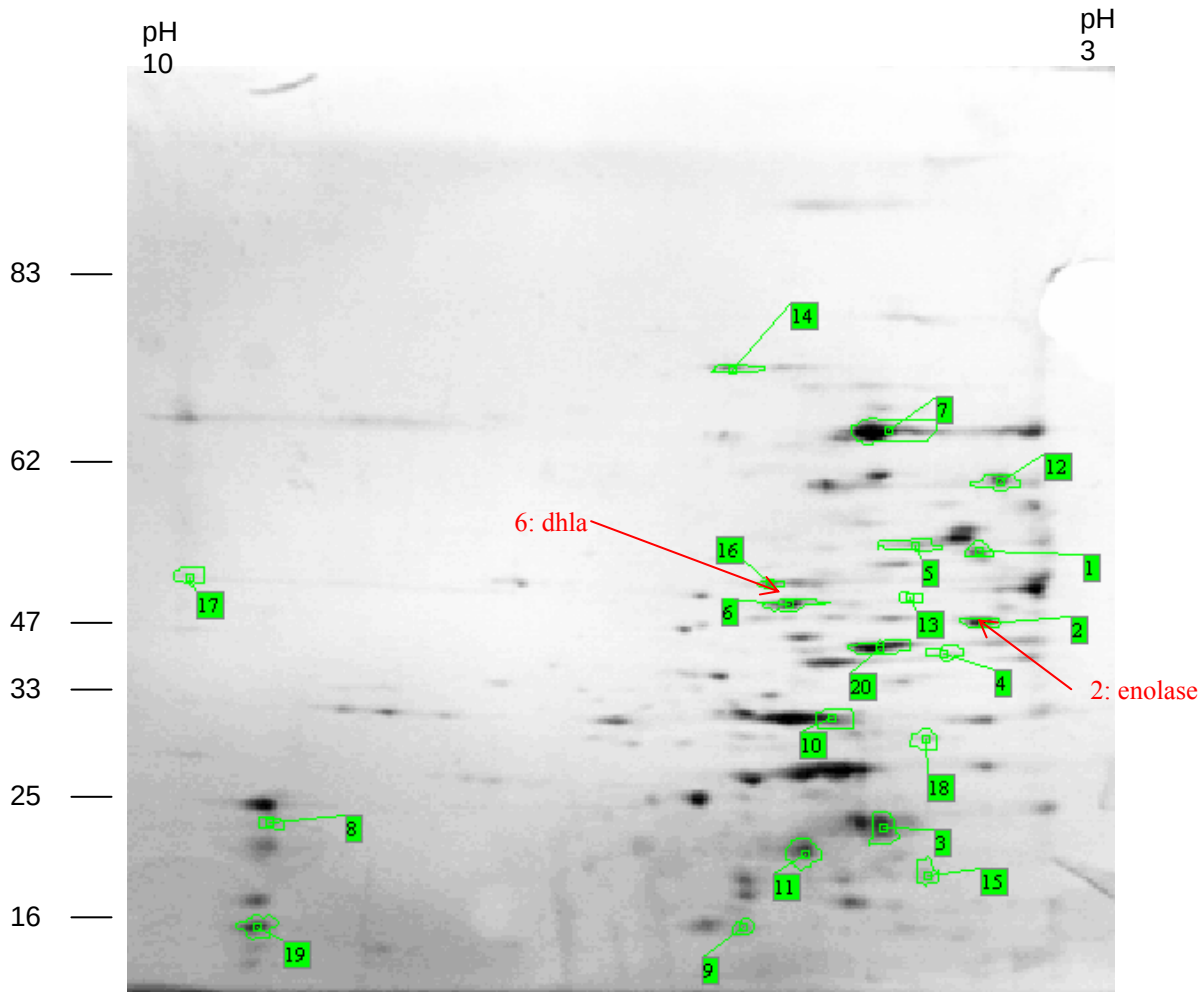


Figure 3.21 Picure of 2-DE gel of LiCl extract of *B. cereus* harvested from the control culture after 0 minutes (A0). 5 µg protein was separated by isoelectric focusing (pH 3 – 10) in the first dimension, and separated by MW in the second dimension. The gel was silver stained. Marked spots indicate spots that are differently expressed in one or both of the bile/ bile salts cultures. (dhla = dihydrolipoamide dehydrogenase)

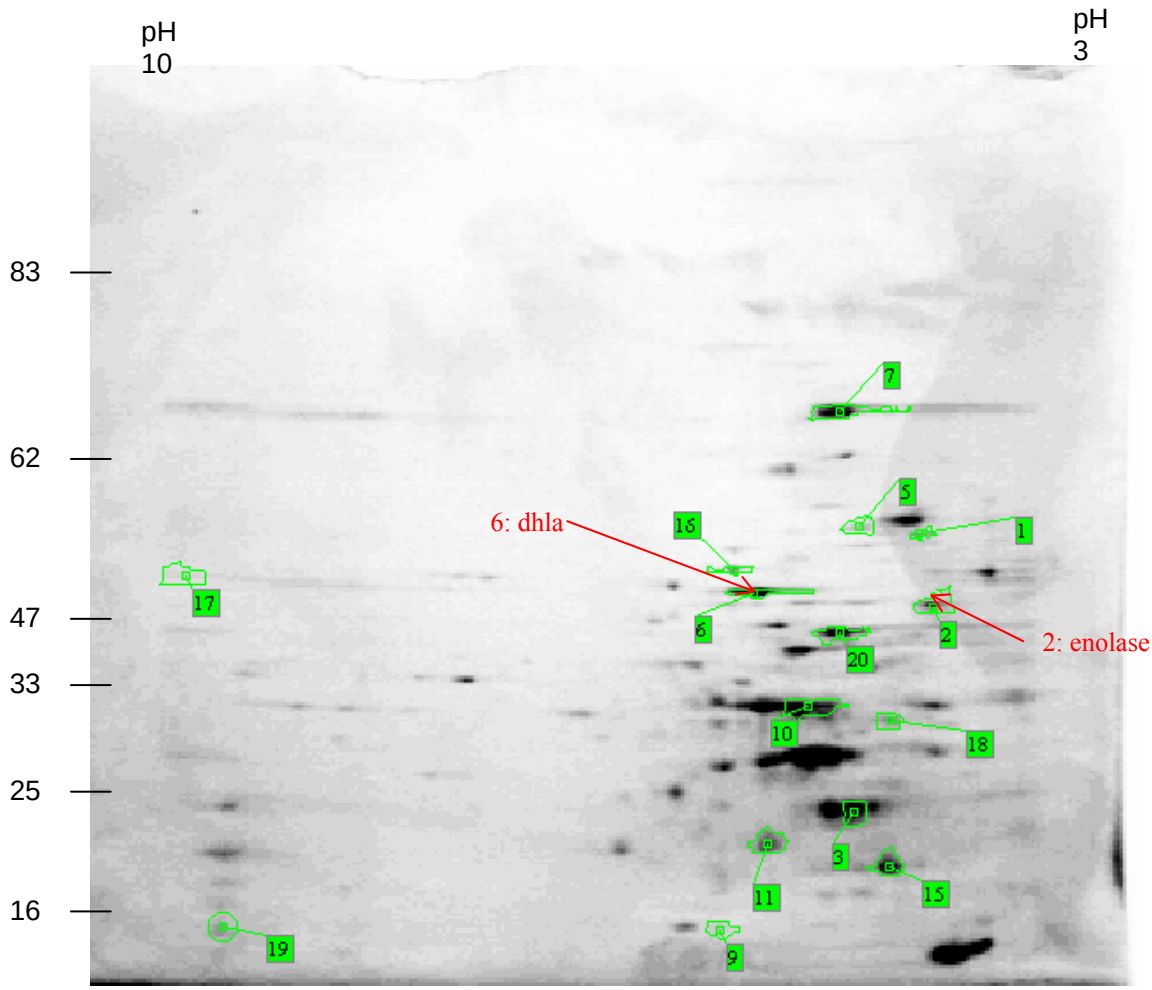


Figure 3.22 Picture of 2-DE gel of LiCl extract of *B. cereus* harvested from the control culture after 30 minutes (A30). 5 µg protein was separated by isoelectric focusing (pH 3 – 10) in the first dimension, and separated by MW in the second dimension. The gel was silver stained. Marked spots indicate spots that are differentially expressed in one or both of the bile/ bile salts cultures. (dhla = dihydrolipoamide dehydrogenase)

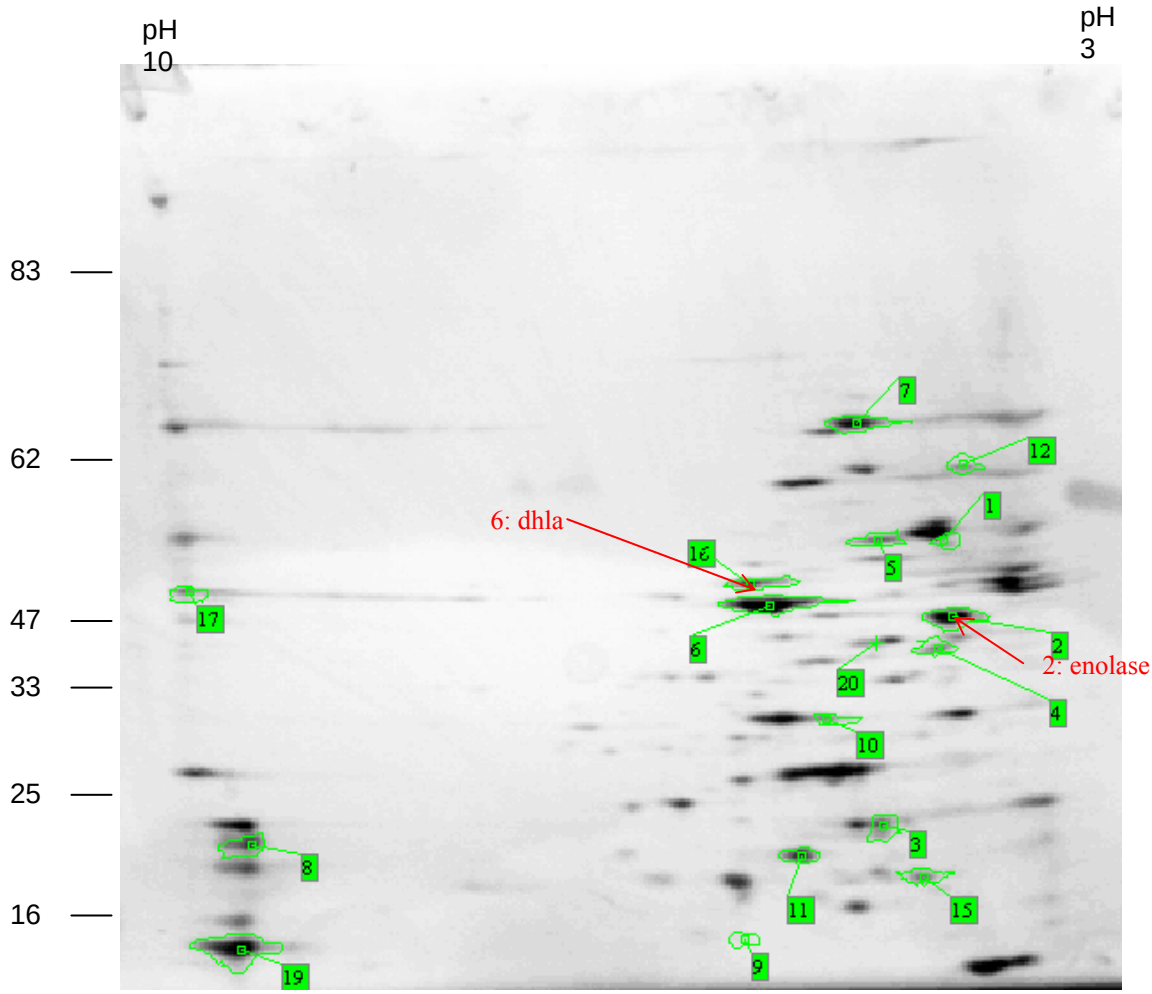


Figure 3.23 Picture of 2-DE gel of LiCl extract of *B. cereus* harvested from the bile (0.25 %) treated culture after 30 minutes (B30). 5 µg protein was separated by isoelectric focusing (pH 3 – 10) in the first dimension, and separated by MW in the second dimension. The gel was silver stained. Marked numbers indicate differentially expressed proteins in one or both of the bile/ bile salts cultures when compared to A0. Spot numbers 2, 4, 6, 8, 9, 16, 17, and 19 have an increased volume % after 30 minutes bile treatment compared to both A0 and A30. Spot number 7, 10, 15, 18, and 20 shows a decrease in the spot volume compared to A0 and A30. (Spot 18 is not identified in this gel and is therefore not marked, but can be seen in gel A0 (fig. 3.21)) (dhla = dihydrolipoamide dehydrogenase)

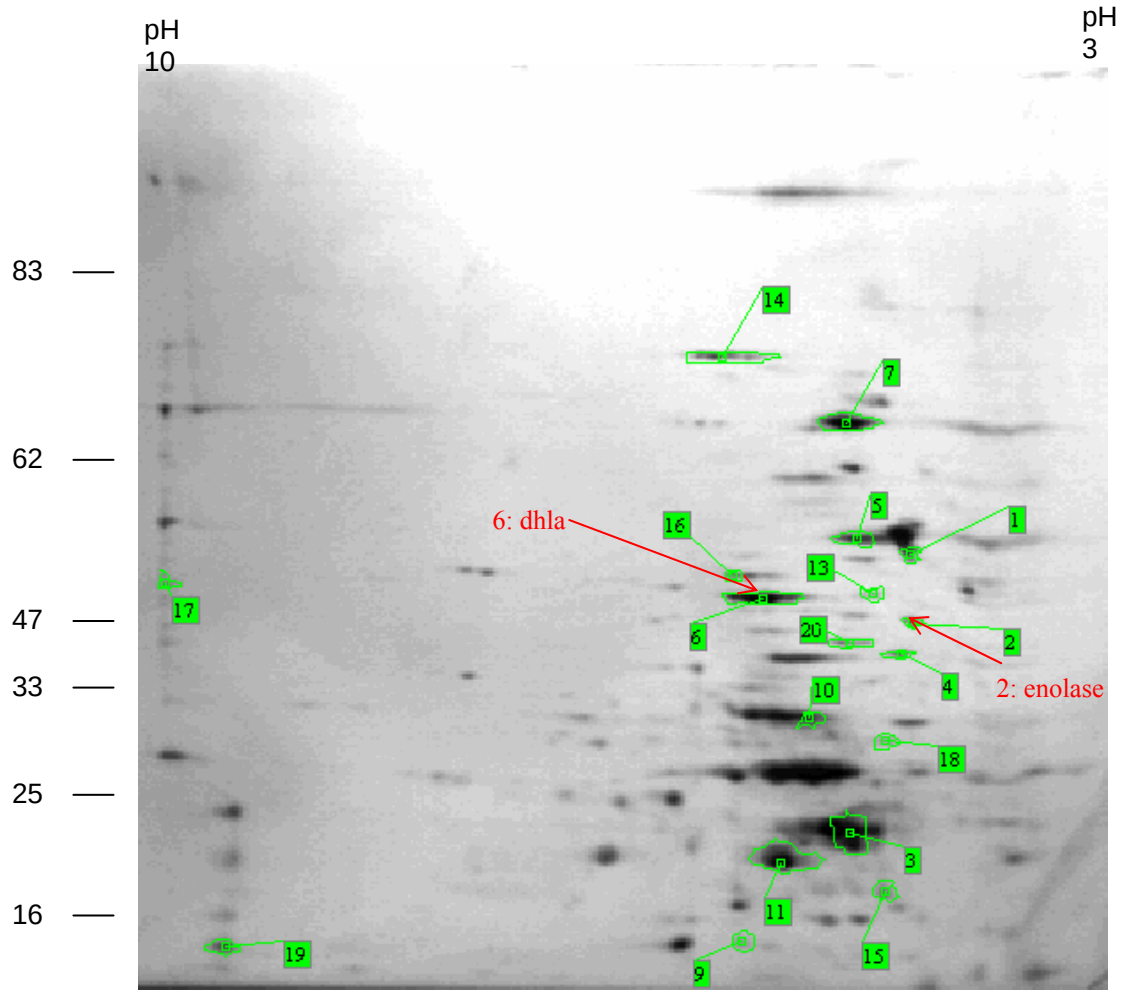


Figure 3.24 Picture of 2-DE gel of LiCl extract of *B. cereus* harvested from the bile salts (0.005 %) treated culture after 30 minutes (C30). 5 µg protein was separated by isoelectric focusing (pH 3 – 10) in the first dimension, and separated by MW in the second dimension. The gel was silver stained. Marked numbers indicate differentially expressed proteins in one or both of the bile/ bile salts cultures when compared to A0. Spot numbers 1, 3, 4, 5, 6, 13, and 14 have an increased volume % after 30 minutes bile salts treatment compared to both A0 and A30. Spot number 2, 7, 8, 10, 15, 16, 18 and 20 shows a decrease in the spot volume compared to A0 and A30. (Spot 8 is not identified in this gel and is therefore are not marked, but can be seen in gel A0 (fig. 3.21)). (dhla = dihydrolipoamide dehydrogenase)

3.6.4.2 Protein Mass Fingerprinting

Eight spots; 4 from B30, and 4 from C30 where excised, trypsin digested and analyzed by MALDI-TOF MS. The mass spectra were recorded for masses ranging from 1 kDa to 4 kDa. The lower parts of the spectra (700 Da to 1kDa) were excluded due to high background noise. The masses obtained in the spectra were exported to the Mascot program and the protein was identified. This was repeated for the same spots in parallel gels. Unfortunately, probably due to low peptide concentration and/or exclusion of the lower part of the spectra, only two protein spots were positively

identified. The identified protein spots were; spot 2: Enolase (BC5135) and spot 6: Dihydrolipoamide dehydrogenase (BC3970). The mass spectra are shown below in figures 3.25 a and b, and 3.26 and the results from the Mascot identification is given in table 3.13.

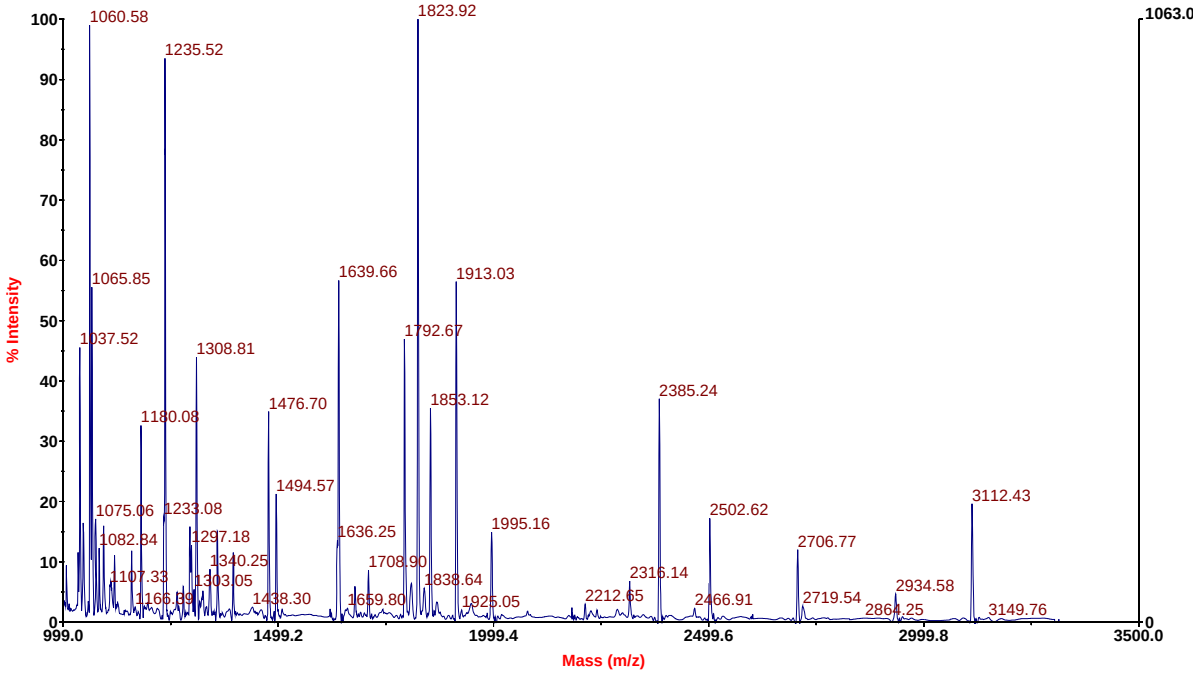


Figure 3.25a MALDI-TOF mass spectrum of spot 2 on gel B30 – 1. The peaks in the spectrum were identified as belonging to tryptic peptides of the *Bacillus cereus* ATCC 14579 protein enolase (BC5135).

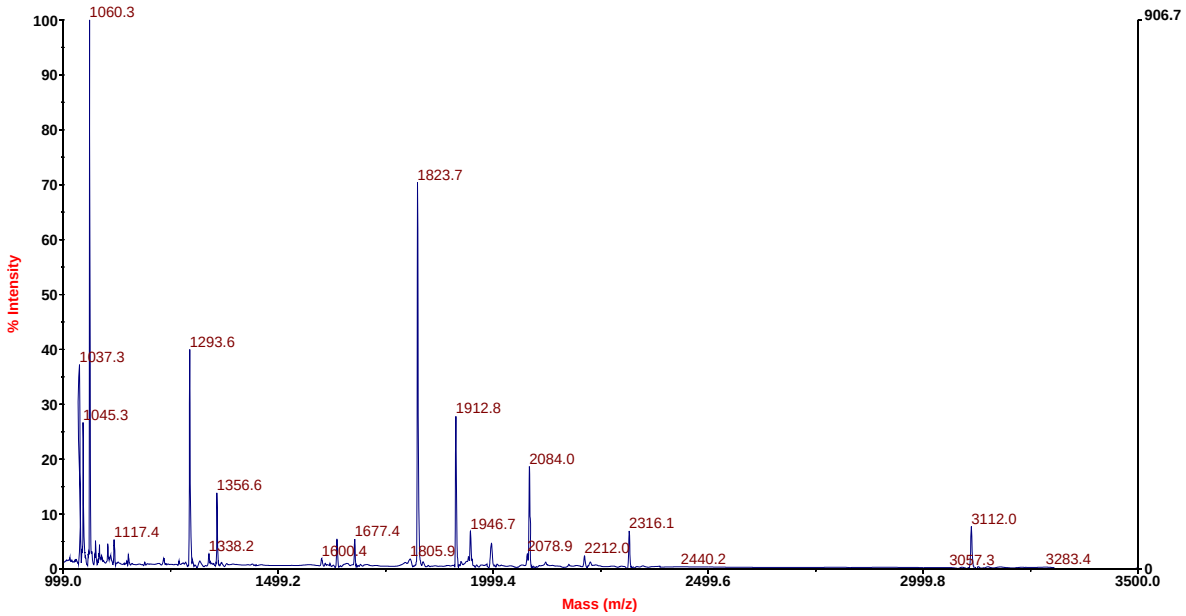


Figure 3.25b MALDI-TOF mass spectrum of spot 2 on gel B30 – 2. The peaks in the spectrum were identified as belonging to tryptic peptides of the *Bacillus cereus* ATCC 14579 protein enolase (BC5135).

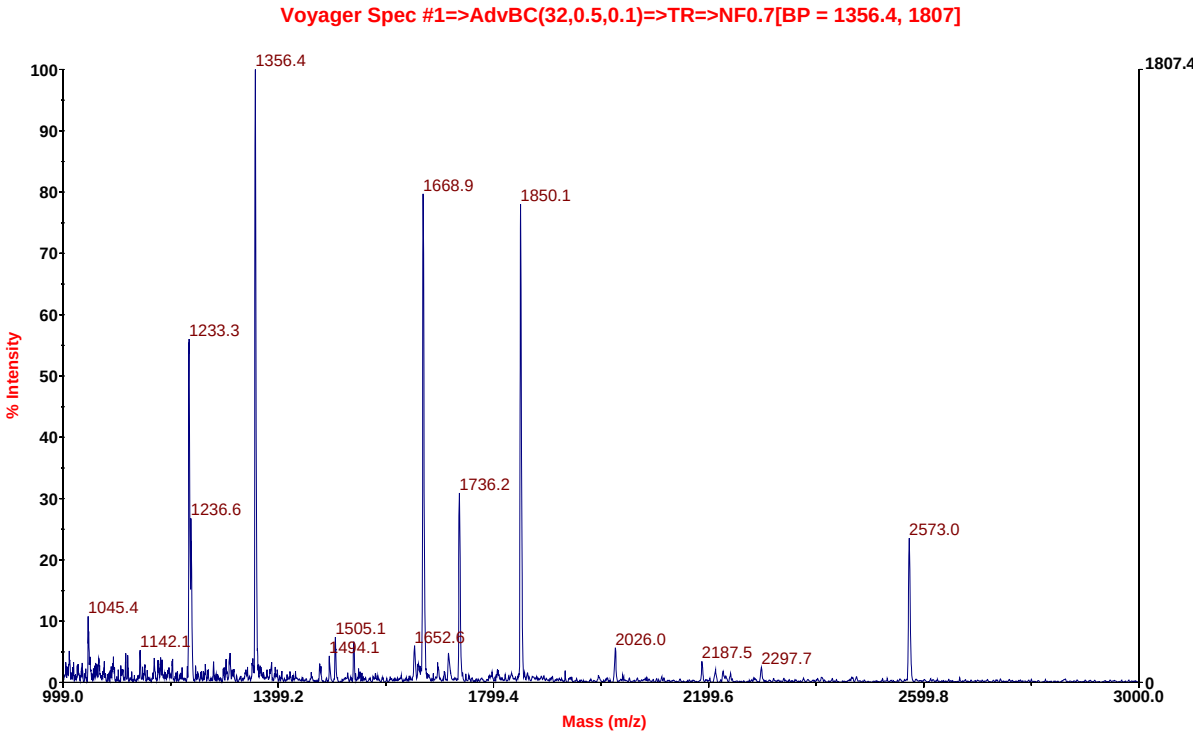


Figure 3.26 MALDI-TOF mass spectrum of spot 2 on gel C30 – 2. The peaks in the spectrum were identified as belonging to tryptic peptides of the *Bacillus cereus* ATCC 14579 protein Dihydrolipoamide dehydrogenase (BC3970).

Table 3.13 Spots identified by PMF. All spots had a MOWSE score above the threshold-value; 77 (defined by the Mascot program). No other proteins gave scores above the threshold-value. The spot localizations on the gels match the theoretical MW and pI.

Spot #	Protein name	BC #	MOWSE score	Mass values matched[2]	MW[1]	pI[1]	Excised from gel #
2	Enolase[3]	BC5135	80[3]	16	46.4	4.66	B30 - 1
2	Enolase	BC5135	84	18	46.4	4.66	B30 - 2
6	Dihydrolipoamide dehydrogenase	BC3970	92	13	49.7	5.25	C30 - 2

[1]Theroretical MW and pI. [2]Mass values matched to theoretical peptides. Some of the masses matched the same peptide. [3]The score for this spot was obtained by increasing the peptide tolerance to 2.5 Da

3.6.5 Practical proteomics in York

A CTAB extraction (section 2.6.1.3) was analyzed by 2-DE on 18 cm IPG strips. A picture of the gel is given in fig 3.27. The three gel bands (A, B, and C) indicated in figure 3.19 was analyzed by both MALDI-TOF MS and MALDI TOF-TOF MS/MS. The peptides from the five highest peaks in the MALDI TOF spectra were fractionated and analyzed by TOF/TOF. Below in table 3.14 is the mascot results for the MALDI-TOF MS. The peptide fractionated by MS/MS all matched to the

corresponding peptide. All these spectra cannot be included due to space limitations, but an example from one of the peptides of the flagellin gene is shown in figure 3.28. The mascot program was used to assist interpretation.

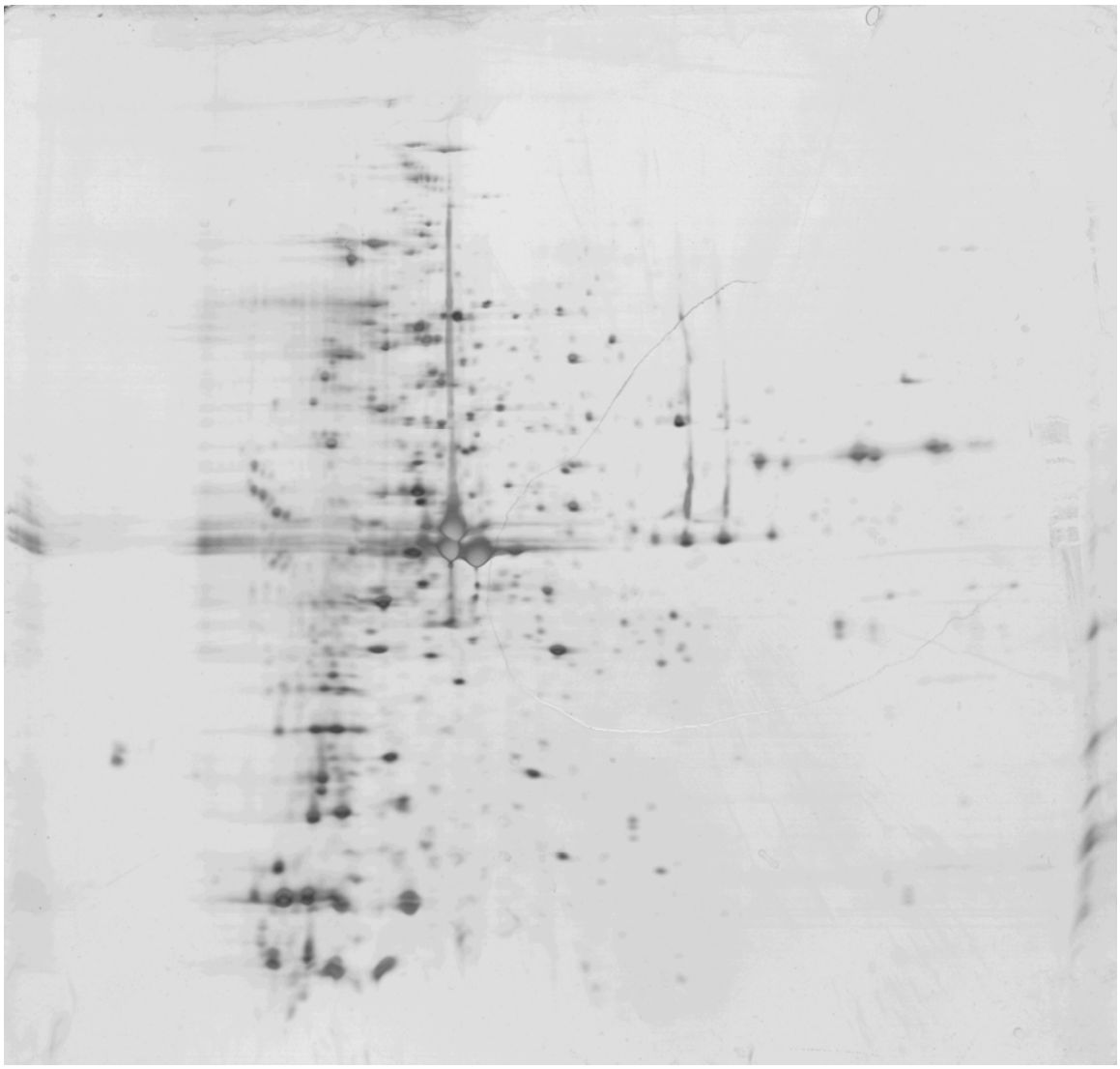


Figure 2.27 2-D gel of CTAB extract done at the practical proteomics course. ~60 µg protein was applied to 18 cm IPG strips pH 3 -10. pH 3 is the left side of the gel.

Table 14 Proteins identified from digested 1-D gel band at the practical proteomics course. The excied gel bands can be seen in figure 3.19. The parameters used in the Mascot program is the same as specified previously (2.6.5.3) except for the peptide tolerance, which was set to 0.2 for these searches.

Band #	Protein name	BC #	MOWSE score	Mass values matched[2]	MW[1] (kDa)	pI[1]
A	Anitgen	BC3699	120	18	36.2	7.71
B	Fructose-bisphosphate aldolase	BC5335	126	23	30.8	5.00
C	Flagellin	BC1658	119	29	29.4	5.21

[1]Theroretical MW and pI. [2]Mass values matched to theoretical peptides. Some of the masses matched the same peptide.

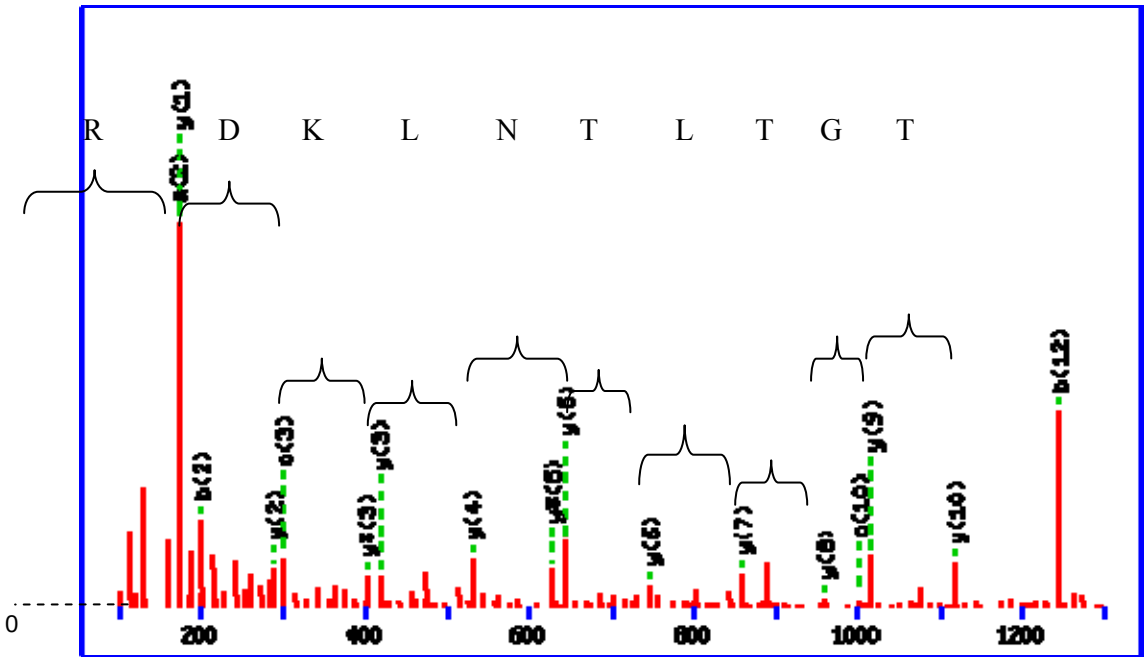


Figure 3.28 Section of MS/MS spectra of a 1419 Da peptide of flagellin (BC1658). The peptide matches the sequence SLTTGTLTNLKDR. The missing aa residues of the first 10 y-ions are annotated.

4 DISCUSSION AND CONCLUSIONS

4.1 Introduction

4.1.1 From genotype to phenotype

The complete genome sequence defines the total number and types of genes that a bacterium contains, and this may tell something about this bacteria's way of life. However, too little information exists about genes function and types of regulation to get a reliable overview of the full metabolic and physiological potential of a bacterium. The genes are subjected to regulation, which are reflected as the mRNA copy number, and quantification of mRNA levels can provide information about which genes are active and necessary for living in a given set of condition or microenvironment. The mRNA, is also subject to regulation (turn-over rate, RNAi, initiation of transcription etc) and does not adequately define those proteins that are expressed at specific times or under specific condition. After all, it is in general the proteins that are the functional units and consequently responsible for physiological changes.

In this thesis responses to conditions mimicking the human GI-tract were investigated in *B. cereus*. It was interesting to see if such conditions could induce changes, at the mRNA and protein levels, which could be correlated to virulence responses. Initially physiological responses to bile and bile salts were investigated. Then we looked for homologue genes in *B. cereus* ATCC 14579 that were known to be involved in survival and colonization of the GI-tract in other bacteria. Expression of these homologue genes was investigated in real-time RT-PCR and microarray experiments. The expression changes were also investigated at the protein level by 2D protein gel-electrophoresis.

4.1.2 Remarks

After working for one year with *B. cereus* I am left with a wealth of data and results. Some of the data and most of the results are presented in the results section because they document the entire process. However, a lot the data is of less interest when the over-all aims are taken into consideration. Consequently I have therefore chosen, in this discussion, to focus on the biological responses observed. Technical detail about methods etc. will only be discussed if they are of central relevance.

It should also be noted that the microarray chips only become available to us this autumn and were not originally a part of this thesis. What is presented here is only the start of the microarray project. Although I did the analysis and participated in the experimental design and shift experiments, the RNA isolation and hybridization were performed by Ine Robertsen and Solveig Ravnum. However, the results are highly relevant to my work, and I think it would be wrong to not include them. With this in mind I have therefore focused on the other subjects in this work, even though they do not generate the volume of data generated by microarray analysis.

4.2 Simulating *in vivo* conditions *in vitro*

No mammalian model organism is available for studies of *B. cereus* infections. Therefore, we have in this thesis developed a system that could mimic some of the conditions found in the intestinal environment. Complete simulation of the intestinal environment *in vitro* is of course impossible. In the mammalian GI-tract a numerous number of different conditions are found which are radically different from the usual conditions that *B. cereus* is subjected to in natural habitats such as soil and foods. Amongst the new challenges bacteria encounter when entering the GI-tract are: an acidic environment, variation in pH, elevated osmolarity, oxygen starvation, nutrient competition (Chowdhury *et al.*, 1996), immune response and exposure to a number of different potentially toxic compounds (degradative enzymes, detergents etc.) In this work the some of the conditions found in the mammalian GI-tract are simulated using bile (and bile salts) and mucin in the culture media. Bile is a specific component of the GI-tract and mucin is present on the surfaces of all cavities in the body, including the GI-tract (see introduction). Bile is restricted to the small intestine, while mucin is present throughout the GI-tract in various types and abundance. Bile and mucin also

are effective barriers against pathogenesis (see introduction) and it is likely that responses are induced in enteric bacteria when they are encountered. It has been demonstrated that a number of bacteria (*Salmonella*, *Shigella*, *Enterococcus* and *Campylobacter* among others) use bile as a localization signal for the GI-tract and alter expression of virulence genes (Pegues *et al.*, 1995; Pope *et al.*, 1995; Rivera-Amill *et al.*, 2001; Van Velkinburgh & Gunn, 1999; Waar *et al.*, 2002).

In this work we have developed a system that simulates some of the conditions found in the GI-tract by adding bacteria suspensions to culture media containing different concentrations of bile, bile salts and mucin (section 2.2.7 and 3.1.4). Due to the toxic effect of bile (and bile salts) the bacteria suspension was added to an equal volume culture media with non-lethal concentration of bile/bile salts. This ensures that no bacteria are exposed to locally lethal concentration as they might if the bile (or bile salts) were added directly to the bacteria culture. The shifts are performed in exponential growth to avoid biased results, attributed to stress, which would arise when the bacteria enter the stationary phase

The mucin was dissolved and sterilized in NaOH, and pH adjusted with HCl, and then added to NaCl-free IR-media (section 2.2.1). Sterilization was achieved but the mucin did denature over time. There were also some problems in the isolation of RNA from the mucin cultures. In the real-time experiments none of the investigated genes (also those investigated by Sirnes (2005)) showed any expression changes. One explanation could be that the bacteria used the mucin solely as a carbon source which is observed in other experiments using mucin *in vitro* (Chang *et al.*, 2004). For these reasons the shift to mucin containing medium experiment was only investigated using real-time PCR.

4.3 Cellular effects of shift experiments

No cellular response to a shift to mucin containing medium were observed, consequently it will not be discussed here.

A number of studies report that gram positive and gram negative enteric pathogens have an elevated or increased tolerance to bile (Begley *et al.*, 2002; Brook, 1989;

Flores *et al.*, 2003; Prouty & Gunn, 2000; Van Velkinburgh & Gunn, 1999). Pre-exposure to sub-lethal concentrations can also increase the tolerance.

B. cereus ATCC 14579 cannot survive physiological bile and bile salts concentrations, and the tolerance is not increased by pre-exposure to bile salts (see sections 3.1.2 and 3.1.3). Bile salts (LB-agar plates with 0.02 %) completely inhibited growth with an observed MIC between 0.01 and 0.02 % (table 3.2). Bile salts concentration in the intestine range from 0.2 to 2 % (Dawson, 1998). Exposure to 0.375 % bile led to a rapid killing of bacteria, and 0.0375 % led to a reduced growth rate (see figure 3.2 and 3.3). Therefore it seems that *B. cereus* ATCC 14579 vegetative cells cannot survive in the human GI-tract. There may be other reasons to these observations. Firstly, the *in vitro* simulation does not exactly represent the intestinal environment and there could be other parameters that will effect survival. The bile used in these experiments is purified bovine bile which is of another composition to that of human bile (although the human bile is supposedly more toxic to bacteria). The bile acids (salts) used are unconjugated cholic acid and deoxycholic acid, and consequently more hydrophobic and toxic than the secreted conjugated bile acids. It is possible that *B. cereus* ATCC 14579 is more tolerant to the bile salt composition encountered *in vivo*. In a review by Begley *et al*. (2005) about bile and bacteria, it is suggested that this explanation for the low bile salts tolerance observed *in vitro* in *Enterococcus faecalis*, a human pathogen. However, Spinosa *et al*. (2000) reported a slightly higher tolerance for tauroconjugated deoxycholic acid (TDOC) which had a MIC of 98 µg/ml (~0.01 %) while deoxycholic acid (DOC) had a MIC at 78 µg/ml in *B. cereus* AF172711. It should, in this case, also be noted that longer adaptation periods could increase tolerance, something which was observed in experiments with *Propionibacterium* (Leverrier *et al.*, 2003); maximum adaptation was observed after 4 hours pre-treatment and no adaptation was observed after 5 minutes.

Secondly, the bacteria could be protected by the food matrix and/or the bile could be "occupied" in binding food constituents. Similar results are seen for survival of *Bacillus cereus* in acidic environments. Clavel *et al*. (2004) incubated *Bacillus cereus* in culture media containing 50 % (w/v) of different foods and monitored survival after exposure to different pHs. Although vegetative cells were rather sensitive towards

acid, *B. cereus* tolerated pH 3.6 when incubated with milk, which was 0.5 to 1 pH-point lower than that observed with other foods. The bile salts concentrations are also subjected to high variations (from 0.2 to 2 %) in the intestine. However, these concentrations can differ according to ingested foods and from person to person (Dawson, 1998). Since bile salts are reabsorbed by passive diffusion in the length of the small intestine, and by active transport in the distal ileum, the concentration of bile would decrease in the intestine. Though the results indicate that it is unlikely that vegetative cells of *B. cereus* ATCC 14579 can survive intestine via the oral route, it is possible that they could proliferate in the large intestine.

The reason for the low bile (and bile salts) tolerance observed in *B cereus* could be one or more of the above reported observations. However, to my knowledge, other commensial and pathogenic intestinal bacteria investigated, have a tolerance to bile and/or bile salts concentrations that are at least a factor of 10 higher than the tolerance observed in *B. cereus* ATCC 14579 (Begley *et al.*, 2002; Flahaut *et al.*, 1996; Prouty *et al.*, 2004; Spinosa *et al.*, 2000; Taranto *et al.*, 2003; Waar *et al.*, 2002).

Therefore, from my point of view, the only opportunity for *B. cereus* ATCC 14579 to survive in the intestine would be as spores, and they could germinate when and where the conditions allow it (e.g. in the mucus layer in the distal ileum or in the large intestine). In experiments on rats *B. cereus* F4433/73 spores have been isolated from all parts of the intestine while vegetative cells showed little or no survival (Wilcks *et al.*, 2005). *B. cereus* spores also survive acidic environments (pH 1.3) while vegetative cells at best survive pH 3.6 (which is significantly higher than the pH in the stomach) (Clavel *et al.*, 2004). Andersson *et al.* (1998) have showed that *B. cereus* spores can adhere to endothelial cells and they suggested that this *could* be one of *B. cereus* virulence mechanisms. To my knowledge the bile tolerance of *B.* cereus spores is not investigated. However, the bile salts concentration in the distal ileum and the large intestine is approximately 5 % of what is observed in the upper part of the small intestine. This would correspond to a minimum ~0.01 % bile salts (Dawson, 1998). This bile salts concentration (0.01 %) is in the range of *B. cereus* ATCC 14579s observed MIC, and most likely in this part of the GI-tract they can germinate.

Although the data strongly suggests that *B. cereus* ATCC 14579 is unable to survive the environment in the GI-tract this does not necessary mean that other *B. cereus* strains also lack the ability to survive, as bile tolerance is thought to be a strain specific (see introduction).

It is possible that diarrhea caused by *B. cereus* is not dependent on colonization of the GI-tract. The plcR-regulon (the principle regulator of enterotoxins and degradative enzymes in *B. cereus,* see introduction) is regulated, at least *in vitro*, in a quorum sensing manner, with an expression maximum at ~10^9 cells. This suggests that *B. cereus* would have to increase in number after ingestion, and not decrease as seems to be the case with *B. cereus* ATCC 14579.

4.4 Methods for detection of expression

In this section I will briefly discuss the advantages and disadvantages of the methods used for the quantification of RNA, the protein levels and the isolation of proteins.

4.4.1 Real-time RT PCR, microarray and 2D protein gel-electrophoresis

Real-time PCR and microarray

The major differences in real-time RT PCR and microarray in the quantification of mRNA is sensitivity. In real-time RT PCR genes expressed at very low levels can be detected (in theory one RNA molecule is sufficient); while in microarray more copies of RNA must be present in order to obtain reliable signals from Cy3/Cy5. At the other end of the scale, real-time RT PCR is also more reliable, and there is no upper limit for the level of detection (if necessary samples can be diluted). In microarray experiments the spots becomes saturated when the cDNA is very abundant, something which leads to bias in the observed ratios. However, for genes that are moderately expressed and regulated there is a good correlation between microarray and real-time RT PCR experiments. There is one exception: the ratios between differently regulated genes seem to be about 2 -10 fold higher in real-time RT-PCR than ratios in corresponding microarray (Reviewed by Conway & Schoolnik, 2003). Therefore one should include in the results the genes that display only small changes in regulation in microarray data, as these would probably be more visible when documented by real-

time RT-PCR. In this work all genes that had an up/down-regulation of 1.5 (log2 0.59) are included (section 2.5.3 and 3.5.2).

2-D protein gel-electrophoresis

In this thesis 2-DE and silver-staining of the gels has been used to detect shift in protein expressions (section 2.6.4 and 3.6.4). Silver staining, SYPRO Ruby staining and Cy3/Cy5 labeling (used in DiGE) are all sensitive markers, but none of them is as sensitive as radio-labeling. Though a widely used method there are some problem with silver staining: Proteins expressed at a low level are hardly detected, silver-staining lack an end-point in the development and the spots become saturated.. While the strongest spots may be saturated, prolonged developing would bias the ratio since weak spots will increase in intensity proportional with the time of development. It is also difficult to stop the developing reaction at exactly the same global intensity for different gels. The dynamic range of silver-staining has a 7 - 40-fold range of linearity, which is significantly lower than the differences in protein expression *in vivo* which ranges from a few copies to several ten-thousands copies in a single cell. This would bias the ratio of weak and strong spots. The use of volume or relative volume (vol %) for quantification removes part of this problem, since strong spots would occupy a larger area on the gel than weak ones. Gels with different global intensities can be normalized using software analysis programs, but overall there could be substantial bias in the spot ratios. Care must be taken when using computer programs to analyze expression pattern, and with silver-stained gels they should only be used as indication. All spot should also be confirmed by visual examination.

Although both 2-DE with silver stain and microarray are at best are semi-quantitative methods, the direction of expression can be inferred. The exact fold-changes of expression, are however, of minor importance, and it is rather the documentation of a response that is of interest as this implies a biological response.

4.4.2 Protein extraction

Some surface proteins may be crucial for colonization of GI-tract and virulence. Therefore a number of buffers that hopefully would extract these proteins, with minimal cytosolic contamination were tested. A number of reports concerning buffer extraction of surface proteins, are to be found in the literature (Antelmann *et al.*,

2002; Nilsson *et al.*, 2000; Schaumburg *et al.*, 2004; Tavares & Sellstedt, 2000; Wright *et al.*, 2005).

Most of the buffer extracts tested did not show any cytoplasmic protein contamination, as measured by the presence aminopeptidase C activity (see section 3.6.3.1). The NP-40 protein extract had substantial aminopeptidase activity, and it is can be concluded that the presence of this detergent in the buffer lead to cell disruption.

The assay was also tested with protein extracts which had been concentrated, but this led to variable results (data not shown). For these reasons the assay as it was described by Schaumburg *et al.* (2004) was used, although it fails to detect low aminopeptidase C activity in samples containing low protein concentrations (see below).

Analysis of the different protein extracts using SDS polyacrylamide gel protein gel electrophoresis (see section 3.6.2.2, figure 3.19) shows there was little difference between the different buffers (except NP-40) and the very low aminopeptidase C activity indicates that these buffers did not disrupt the cells as observed in the NP-40 extraction. On the other hand the buffer extracts exhibit strong similarities to the cytosolic fractions. (This is not so obvious on the pictures, but was clear in the original gels. This was also observed for the wash extract.) The 2-DE gel (when equal amounts of protein were loaded, see figure 3.20a-d) of the whole cell extraction did reveal a pattern of spots highly similar to the extracts, and it seems that the buffer extracts contains whole cell proteins from a low number of cells, but with a higher proportion of surface proteins in the buffer extracts. (The total protein 2D –gel is somewhat underfocused, but it reveals a similar pattern.) No aminopeptidase C activity was detected in the extract. This is in contrast to the pattern on the 2-DE protein gels, where one can clearly see the similarity between whole cell extract and buffer extract which is a *strong* indication of the presence of cytosolic proteins. Even though it is probably that is a higher portion of surface proteins in the buffer extracts, they are certainly not exclusively surface proteins and are therefore described as buffer extracts rather than the cell surface proteins.

Since the cells are washed, cytosolic proteins must result from lysis during the buffer treatment. The rigidity of gram positive cells suggests that this is a controlled behavior, and not a result of the relatively mild buffers. It has very recently been reported that when exponentially growing bacteria of the *B. cereus* group was transferred from growth media to buffered solution rather extensive autolysis is observed (Raddadi *et al.*, 2005). This phenomenon is probably the explanation for the presence of cytosolic proteins in buffer extracts.

Two other methods are described for the isolation of surface proteins. One method is to label proteins on intact cells with biotin-linked markers or fluorescent dyes such as Cy3/Cy5 and then harvest or detect the labeled proteins (Sabarth *et al.*, 2002). The other method is based on isolation of the cell wall by centrifugation, and then degrade the cell wall enzymatically or chemically and harvest the proteins (Mauri *et al.*, 1999). Seeing the results discussed above and if given more time, one of these methods would be preferable if one wished to specifically investigate cell surface proteins.

4.5 Function of identified and regulated genes

As previously mentioned, intestinal bacteria must survive the lethal effect of bile and to maintain their presence in the intestine they must adhere to the host endothelial cells. *B. cereus* ATCC 14579 genes that could assist in colonization of the GI-tract were investigated in a bioinformatics based analysis. The results shown in table 3.2 indicate that *B. cereus* ATCC 14579 has some genes that could be used in colonization of endothelial cells the GI-tract. Some of these genes were investigated by real-time PCR and are discussed below. Collagen adhesion proteins, cell-wall binding protein (enterotoxin) and camelysin were not further investigated and are thus not discussed. Sirnes (2005) investigated 52 genes involved in bile response in *L. monocytogenes, S. typhimurium, Lactobacillus plantarum* and *Enterococcus faecalis* with regard to similarity to genes in the *B. cereus* ATCC 14579 genome. None of the investigated genes had any significant similarity.

In addition, will the functions of the proteins identified by 2-DE and PMF be discussed here (see section 3.6.4.1 and 3.6.4.2).

Fibronectin binding protein

Fibronectin is a dimeric glycoprotein present in plasma, extracellular fluids and at cell surfaces in fibrillar (Reviewed by Romberger, 1997). The fibronectin binding protein (FBP) of *Bacillus cereus* ATCC 14579 is highly similar to those of *Streptococcus* spp. and *Listeria monocytogenes* (section 3.2.1), and the binding properties of FBP in these organisms are well documented (Gilot *et al.*, 1999; Gilot *et al.*, 2000; Joh *et al.*, 1999; Joh *et al.*, 1994). *Streptococcus pyogenes*, *Streptococcus gordonii*, *Staphylococcus aureus*, and *Listeria monocytogenes* all uses fibronectin binding protein as a virulence factor that is involved in invasion and/or adhesion of host cells (Christie *et al.*, 2002; Dramsi *et al.*, 2004; Joh *et al.*, 1999).

Internalin

Internalins are well documented proteins in *Listeria monocytogenes,* where they mediate invasion of host cells (Reviewed by Cossart *et al.*, 2003). The internalin A proteins mediate invasion by binding to E-cadherin and consists of leucine rich repeats which are thought to be responsible for the binding process (Cossart *et al.*, 2003). The internalin A proteins of *Bacillus cereus* only are ~25 % similar to those in *L. monocytogenes* so any conclusion about parallel functions seem unreasonable (section 3.2.1). However, they do have surface binding signals and leucine rich repeats (which exists in many proteins and are responsible for protein-protein interactions (Kobe & Kajava, 2001)). They *may* therefore be implicated in virulence in *B. cereus* ATCC 14579. In *L. monocytogenes* the internalin-machinery and fbp are associated and essential for full virulence (Dramsi *et al.*, 2004).

Enolase

Enolase (phosphopyruvate hydratase, section 3.2.1.) is an enzyme (EC 4.2.1.11) in glycolysis which catalyses the formation of phosphoenolpyruvat from 2-phosphoglycerate or vice versa; 2-phospho-D-glycerate ↔ phosphoenolpyruvate + H_2O. It can also catalyse the reaction 3-dehydroquinate ↔ 3-dehydroshikimate + H_2O in aromatic amino acid biosynthesis. However, this protein has also been documented as having several other (moonlighting) functions according to the organism and sub-cellular localization. It forms a structural component of lens in vertebrates (Williams *et al.*, 1985; Wistow *et al.*, 1988). It has been documented as having DNA-binding properties in yeast (Algiery & Brewer, 1992) and its is described as an endothelial

hypoxic stress protein in bovine endothelial cells (Aaronson *et al.*, 1995). It is also present at surfaces of some cancer cells, where it activates plasminogen and thus degrade the extracellular matrix in order to migrate (Lopez-Alemany *et al.*, 2003). Most interestingly, in several bacteria this protein is expressed on cell surfaces where it can bind plasminogen. This ability is so far described for Group A *Streptococci* (GAS) (Pancholi & Fischetti, 1998), *Streptococcus pneumoniae* (Bergmann *et al.*, 2001), *Bacteroides fragilis* (Sijbrandi *et al.*, 2005) and *Listeria monocytogenes* (Schaumburg *et al.*, 2004). Plasminogen is the proenzyme of the protease plasmin, which is a protease that cleaves fibrin (fibrin is a extracellular matrix protein and also present in the mucosal barrier). It has also a high proteolytic activity against basal lamina proteins, such as thrombospondin, laminin, fibronectin and fibrinogen (Reviewed by Cesarman-Maus & Hajjar, 2005). Pancholi *et al*. (2003) suggested that GAS may exploit enolases plasminogen-binding property for dual purposes i.e. initially to adhere pharyngeal cells and then for tissue invasion by the use of plamsin(ogens) proteolytic activity. The identification of antibodies against streptococcal enolase in patients infected with *S. pneumoniae* (Fontan *et al.*, 2000) also supports the idea that enolase is present on surfaces, and an important virulence factor. Interestingly, it has recently been documented that the enolase of *Streptococcus sobrinus* has immunosuppressive activity (Veiga-Malta *et al.*, 2004). However, it is not possible to tell if this protein is present on the surface of *B. cereus* and if it has the ability to bind plaminogen without further investigations, and its role in bile response remains uncertain.

Dihydrolipoamide dehydrogenase

Dihydrolipoamide dehydrogenase (EC 1.8.1.4) is the flavoprotein component of the multienzyme 2-oxo-acid dehydrogenase complexes such as pyruvate dehydrogenase and 2-Oxogluterate dehydrogenase. It binds to the core enzyme and catalyses oxidation of its dihydrolipoyl groups: protein N6-(dihydrolipoyl)lysine + NAD^+ ↔ protein N6-(lipoyl)lysine + NADH + H^+. *B. cereus* ATCC 14579 contains three paraloges of this protein; BC2776, BC3970, and BC4160. There have been some reports of dihydrolipoamide dehydrogenase-domain-proteins, and other pyruvate dhg components of microbes surfaces (Bringas & Fernandez, 1995; Dallo *et al.*, 2002; Li de la Sierra *et al.*, 1997; Moreno-Brito *et al.*, 2005).

4.5.2 Changes in gene-expression and their involvement in bile response

A number of studies describe bile responses for both commensial and enteropathogenic bacteria. In general the responses are stress responses similar to those induced by heat, acid, osmolaritly, and *specific* responses to bile (see introduction). As mentioned above Sirnes (2005) investigated the genome of *B. cereus* ATCC 14579 and found no genes involved in *specific* bile response. However the microarray-data, real-time data and proteomic data suggest that a general stress response is provoked by bile (-salts)

Microarray

The microarray data indicates a general stress response to bile /bile salts exposure in *B. cereus* ATCC 14579 (see table 3.7 and appendix IV). Typical stress genes such as the 10 and 60 kDa Chaperonins (GroESL), hrcA, DnaK, Hsp20, and ClpP which are involved in releaving stress caused by misfolded proteins are significantly up-regulated, both compared to A0 and A30. This is similar to what is seen in other bile challenged bacteria (Flahaut *et al.*, 1996; Leverrier *et al.*, 2003; Schmidt & Zink, 2000) In *B. cereus* many of these genes are under the control of the alternative transcription regulator SigmaB (Periago *et al.*, 2002). Sirnes (2005) documented by real-time RT-PCR that sigmaB is highly up-regulated (~100 fold compared to A0) in cultures exposed to bile and slightly up-regulated (~7 fold compared to A0) in bile salts cultures. The up-regulation of superoxide dismutase and thioredoxine, both involved in protection against oxidative stress, may also be considered general stress responses. Superoxide dismutase is up-regulated by bile-challenge in *P. freudenreichii* (Leverrier *et al.*, 2003) Thioredoxin is also up-regulated in bile stimulated cancer cells (Lechner *et al.*, 2002). And a transcriptional regulator, Fur (ferric uptake repressor), is also up-regulated. *B. cereus* fur deletion mutants are avirulent against insect targets, and this gene is probably crucial for tolerance towards oxidative stress (Harvie & Ellar, 2005; Harvie *et al.*, 2005).

(Multi)drug-transporters are important in bile response (see introduction) and 3 drug resistance transporters (2 in bile media and 1 in bile salts media) are highly up-regulated, something which could imply that they *can* have a function in removing

bile salt from the cells. However, next to nothing is known about the specificity of these (Multi)drug-transporters in *B. cereus*, and speculation in their involvement must be substantiated by experimental evidence.

There is a down-regulation of many ribosomal proteins and proteins involved in oxidative phosphorylation, and up-regulation of genes involved in carbohydrates, amino acid and peptide metabolism (see microarray results in appendix IV). This may indicate a trend of directing energy metabolism towards fermentation and/or production of other metabolites. This also correlates well with results from bile stressed *Bifidobacterium longum* Sanchez *et al.* (2005) where an increase in glucose consumption was observed.

It should also be noted that one generally accepted pathogenicity factor in B. cereus, Hemolysin BL lytic component L2, is strongly down-regulated in bile media (log2: -2.31).

Real-time PCR experiments

The real-time PCR analysis shows that *internalin* genes are strongly down regulated in bile salts media; BC1331 (intB) is ~25-fold down-regulated and BC0552 (intA) also show the same pattern (see section 3.4.3, figures 3.12 and 3.13). They are also down-regulated in bile media, but not to the same extent. BC1331 is also confirmed down-regulated in bile salts microarray results (although the P-value is rather high, 0.097). The down-regulation of internalin in a bile/bile salts response could be general a stress response, non-essential genes would be down-regulated, so energy can be utilized elsewhere. In *L. monocytogenes* the sigmaB is linked to regulation of internalin and bile response genes (Kazmierczak *et al.*, 2003), internalin is not positively regulated by sigmaB in *B. cereus* ATCC 14579.

The *fibronectin binding protein* gene does not appear to be significantly regulated.

2-D protein gel-electrophoresis

The enolase protein was up-regulated in bile and down-regulated in bile salts (see section 3.6.4; tables 3.12 and 3.13, and figures 3.21 – 3.24). Enolase is, to my knowledge, not identified in any studies describing responses to bile. However

Sanchez *et al.* (2005) observed up-regulation of glycolytic enzymes in bile stressed *Bifidobacterium longum*. This is also seen in the microarray data, suggesting that this metabolic shift is a common response to bile (or general stress). Enolase has been shown to be up-regulated in response to iron-depletion in a study aiming to identify virulence genes when *Bacteroides fragilis* encounters *in vivo* conditions, which implies that enolase is important in coping with different kind of stresses. Although enolase is a virulence factor in other species, such as *Streptococcus* this has not been investigated in *B. cereus*, and since the extraction of specifically surface protein was not unequivocal it would be unwise to draw any definitive conclusions as to the cellular localization of the enolase detected in the protein electrophoresis and identified by MS. On the other hand, the global change in metabolic genes and the down-regulation of other virulence factors suggests that the enolase of *B. cereus* is up-regulated as part of the metabolic response in sugar metabolism.

The up-regulation of enolase in the presence of bile, but not in bile salts could be ascribed to the lower growth-rate in media containing bile, which may suggest an increased need for energy. This might also be explained by the presence of something in the bile that the bacteria sense, but which is not present in the bile salts. Pope *et al.* (1995) discovered that a mix of the bile salts deoxycholate and chenodeoxycholate increased *Shigellas* invasiveness, while other structurally similar bile acids did not.

Dihydrolipoamide dehydrogenase was up-regulated in both bile and bile salts samples (section 3.6.4; tables 3.12 and 3.13, and figures 3.21 – 3.24). The microarray-analysis confirms up-regulation of dihydrolipoamide dehydrogenase in bile salt media (but has a high P-value, 0.094). In the bile media this gene has a log2 ratio of 0.28, which is only a slight up-regulation (and might imply a regulation at the mRNA-level, or a bias of the results). BC4160, a paralogue dihydrolipoamide dehydrogenase gene is also strongly up-regulated in bile salts media. The role of dihydrolipoamide dehydrogenase in bile response (or stress response) is, to my knowledge, not investigated. The increased expression of dihydrolipoamide dehydrogenase probably indicates a need for more energy and/or more biosynthetic capacity and/or other metabolites.

It should also be noted that the response seen to bile and bile salts in the real-time experiments, especially with regard to sigmaB (Sirnes 2005), was only observed 30 min. after the shift, which could indicate that the response is not induced by bile (-salts) itself, but by more unspecific stimuli such as an accumulation of unfolded proteins and/or oxidative stress. This also implies that *B. cereus* ATCC 14579 do not *sense* bile (e.g. by two component systems) in a direct manner like other enteric bacteria probably do.

Concluding remarks

B. cereus ATCC 14579 does not tolerate bile and bile salt concentrations encountered in vivo. Bile (-salts) stimuli at non-lethal concentrations gives a stress response and shifts its metabolic priorities (real-time, microarray and 2-DE). Virulence (and putative virulence) factors like enterotoxin, internalin and fibronectin binding proteins are not affected or reduced by bile (-salts) (real-time and microarray). All in all this work implies that the *vegetative* cells of *B. cereus* ATCC 14579 do not show a response which is shown by true enteropathogenic bacteria.

4.6 Further perspectives

This work should be properly concluded; the microarrays should be presented in three replicas and some of the gene expression patterns should be confirmed by real-time reverse transcriptase PCR. The protein electrophoresis should either be, extended to total protein or focused on surface proteins using a more appropriate method for labelling, extraction and detection.

Only one strain of B. cereus was investigated. Another *B. cereus* strain, preferable a clinical isolate, e. g. the recently sequenced *B. cereus* NVH391-98 would be a good candidate to investigate responses to bile/bile salt.

Surface proteins that are putatively involved in binding to host cells could be done. Firstly such genes should be documented to be localized at the surface e.g. by *in situ* immunolabeling and their ability to bind host cells proteins should be determined. Then of course, if such proteins are positively identified, their involvement in virulence should be investigated e. g. by use of an insect model.

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APPENDIXES

Appendix I: Protocol for RNA isolation using the FastRNA® Pro Blue Kit

Section from the detailed protocol from the FastRNA® Pro Blue Kit

1. Dilute 1 ml of an overnight bacterial culture into 14 ml of fresh media in a sterile 50 ml tube or 250 ml flask.

2. Incubate for ~4–6 hours at 37ºC with shaking at ~150–200 rpm to reach an OD600 = 0.9–1.0 (Note: 1.0 OD600 for Escherichia coli is ~1 X 109 cells per milliliter).

3. Remove 10 ml of the culture to a 15 ml conical tube and pellet the cells by centrifugation at 2,800 rpm (x 1,500 g) for 15 minutes at 4ºC (e.g., Beckman Model TJ-6 Centrifuge, I-92 Swinging Bucket Rotor) for 10 minutes.

4. Decant the supernatant and add 1 ml of RNA*pro*™ Solution to the tube. Completely resuspend the cells by pipetting or vortexing.

5. Transfer 1 ml of the resuspended cells to a blue-cap tube containing Lysing Matrix B provided in the kit. Securely close the cap to prevent leakage in the next step. NOTE: The calculated volumes will provide adequate airspace in the matrix tube to prevent sample leakage and/or tube failure. DO NOT overfill the matrix tube. To process a greater number of cells use a second matrix tube.

6. Process the sample tube in the FastPrep® Instrument for 40 seconds at a setting of 6.0.

7. Remove the sample tube and centrifuge at a minimum of 12,000 x g for 5 minutes at 4ºC or room temperature.

8. Transfer the liquid (~750 µl) to a new microcentrifuge tube. Avoid transferring the debris pellet and lysing matrix.

9. Incubate the transferred sample 5 minutes at room temperature to increase RNA yield.

10. Add 300 µl of chloroform (NO isoamyl alcohol). Vortex 10 seconds.

11. Incubate 5 minutes at room temperature to permit nucleoprotein dissociation and increase RNA purity.

12. Centrifuge the tubes at a minimum of 12,000 x g for 5 minutes at 4ºC. Samples containing large amounts of cellular mucopolysaccharides can be re-extracted with chloroform (isoamyl alcohol may be included with the chloroform [CHCl3:IAA, 24:1,

v:v]) to increase RNA purity. Alternatively, a lithium chloride precipitation may be used (see the Troubleshooting section and references 3, 4).

13. Transfer the upper phase to a new microcentrifuge tube without disturbing the interphase. If a portion of the interphase is transferred, repeat the centrifugation with the upper phase, and transfer the new upper phase to a clean microcentrifuge tube.

14. Add 500 µl of cold absolute ethanol to the sample, invert 5X to mix and store at -20ºC for at least 30 minutes.

15. Centrifuge at a minimum of 12,000 x g for 15 minutes at 4ºC and remove the supernatant. The RNA will appear as a white pellet in the tube. If the pellet is floating the sample may be recentrifuged to place the pellet at the tube bottom.

16. Wash the pellet with 500 µl of cold 75% ethanol (made with DEPC-H2O).

17. Remove the ethanol, air dry 5 minutes at room temperature (DO NOT completely dry the RNA) and resuspend the RNA in 100 µl of DEPC-H2O for short-term storage. RNA is generally stable for up to a year at -80ºC. For longer term storage RNA samples may be stored at -20ºC as ethanol precipitates. When stored as an ethanol precipitate, the RNA must be precipitated and resuspended in aqueous solution prior to use. NOTE: RNA does not evenly distribute in ethanol and can lead to inconsistent RNA amounts between samples when equal volumes are pipetted. Vortex the RNA:ethanol solution to disperse the RNA prior to removing the sample. In situations where precise amounts of RNA are required it is best to precipitate the total amount (or excess) of RNA required, resuspend the RNA in DEPC-H2O and measure the concentration by OD260 before proceeding.

18. Incubate 5 minutes at room temperature to facilitate RNA resuspension.

Appendix II: Protocols for microarray

Section of the FairPlay™ microarray labeling kit (Stratagene).

PROTOCOL

cDNA Generation

Prepare separate cDNA labeling reactions for each fluorescent dye you wish to use. This protocol produces a sufficient amount of labeled cDNA per reaction to hybridize to a total microarray surface area of approximately 10 cm^2.

1. For each labeling reaction, resuspend 10 µg of total RNA in 12.0 µl of DEPC water.

   Note *If the addition of control mRNA is desired, for example to gauge the success of the labeling reaction, add it at this step, but ensure that the final volume at the end of step 1 is 12.0 µl.*

2. Add 1 µl of 500 ng/µl oligonucleotide $d(T)_{12-18}$. Incubate at 70°C for 10 minutes. Cool on ice until ready for use.

3. Combine the following components in a sterile, RNase/DNase-free microcentrifuge tube:

   2 µl of 10× StrataScript reaction buffer
   1 µl of 20× dNTP mix
   1.5 µl of 0.1 M DTT
   0.5 µl of RNase Block (40 U/µl)

4. Add the annealed primer and RNA (from step 2) to the mixture.

5. Add 3 µl of StrataScript HC RT and incubate at 42°C for 60 minutes.

6. Add 10 µl of 1 M NaOH and incubate at 70°C for 10 minutes to hydrolyze RNA.

7. Cool to room temperature slowly; do not cool on ice.

8. Spin tube briefly to collect contents.

9. Add 10 µl of 1 M HCl to neutralize the solution.

cDNA Purification

The cDNA must be purified to remove unincorporated nucleotides, buffer components and hydrolyzed RNA. Stratagene recommends an ethanol precipitation to purify the cDNA. Incomplete removal of the Tris and EtOH will result in lower amino allyl-dye coupling efficiency. Care must be taken to ensure that the pellet is completely dry (at the end of step 6) indicating complete removal of the EtOH before proceeding to the dye coupling reaction.

1. Add 4 µl of 3 M sodium acetate, pH 4.5 to the reaction.

2. Add 1 µl of 20 mg/ml glycogen to the reaction.

3. Add 100 µl of ice-cold 95% EtOH.

4. Incubate at –20°C for at least 30 minutes. The reaction can be stored at this point for several days or up to 2 months.

5. Spin the reaction stored in EtOH with NaOAc at 13–14,000 × g for 15 minutes at 4°C. Carefully decant supernatant.

6. Wash with 0.5 ml ice cold 70% EtOH and spin at 13–14,000 × g for 15 minutes at 4°C. Carefully decant supernatant and allow to air dry. A vacuum dryer can be used to speed up the process but DO NOT overdry.

If coupling to NHS-ester containing dyes from Amersham Pharmacia Biotech (see *Additional Materials Required*) is desired, follow the protocol below (*NHS-Ester Containing Dye Coupling Reaction* and *Dye-Coupled cDNA Purification*) to couple the dye and purify the modified cDNA.

If coupling to STP-ester containing dyes from Molecular Probes (see *Additional Materials Required*) is desired, follow the user's guide provided with the dye to carry out this reaction. Use the reagents provided with that kit to resuspend, couple and purify the cDNA. Following coupling and cDNA purification, proceed to *Analysis of Fluorescence-labeled cDNA (Recommended)*.

NHS-Ester Containing Dye Coupling Reaction (For Use with Amersham Pharmacia Biotech Dyes)

Note *Stratagene does not recommend scaling-down the following dye coupling protocol. Performing the reaction using reduced volumes results in a significantly reduced coupling efficiency.*

1. Resuspend cDNA pellet in 5 µl of 2× coupling buffer. If pellet was overdried it will be difficult to get the pellet back into solution. Gently heat at 37°C for 15 minutes to aid in the resuspension process.

   Note *A visible precipitate may be seen in the 2× Coupling Buffer. Incubate the buffer at room temperature or 37°C to resolubilize the precipitate before use.*

2. The first time a tube of dye is used, resuspend in 45 µl DMSO. Use the high-purity DMSO provided in the kit. Do not substitute another DMSO. Vortex gently to ensure the pellet is completely solubilized. The unused dye can be aliquoted in single use aliquots and stored at –20°C in the dark for several months.

   Note *DMSO is hygroscopic and will absorb moisture from the air. Water absorbed from the air will react with the NHS ester portion of the dye and significantly reduce or eliminate dye:cDNA-coupling efficiency. To reduce absorption, allow the dye to reach room temperature before opening and store the DMSO at room temperature. Do not leave either the dye or DMSO uncapped when not in use. During storage, tightly cap the resuspended dye and store at –20°C in the dark.*

3. Add 5 µl of dye to the cDNA. If the dye was stored at –20°C prior to use, allow the dye to reach room temperature before opening the container.

4. Mix by gently pipetting up and down.

5. Incubate for 30 minutes at room temp in the dark.

Dye-Coupled cDNA Purification

The following dye-coupled cDNA purification method has been optimized for use with the DNA-binding solution and microspin cups provided in this kit. Briefly, in the presence of a chaotropic salt (introduced by the DNA-binding solution, included in this kit), the dye-coupled cDNA binds to the silica-based fiber matrix seated inside the microspin cup. Washing steps are employed to remove buffer salts and uncoupled fluorescent dye from the bound cDNA. Finally the cDNA is eluted from the matrix using a low-ionic strength solution. Use of the DNA-binding solution and microspin cups provided in this kit in conjunction with alternative purification protocols is not recommended.

1. Add 90 µl of 1× TE Buffer (see *Preparation of Reagents*) to the labeled cDNA.

2. Combine 100 µl of DNA binding solution and 100 µl of 70% (v/v) EtOH. Mix well by vortexing. Make sure that the two solutions are well mixed prior to use.

3. Add the DNA-binding solution and EtOH mixture to the labeled cDNA and mix by vortexing.

4. Using a pipet, transfer the mixture to a microspin cup that is seated in a 2-ml receptacle tube. (Exercise caution to avoid damaging the fiber matrix with the pipet tip.) Snap the cap of the 2-ml receptacle tube onto the top of the microspin cup.

   Note *To ensure proper sample flow, use the receptacle tube that is provided with the microspin cups. Do not substitute another tube.*

5. Spin the tube in a microcentrifuge at maximum speed for 30 seconds.

   Note *The labeled cDNA is retained in the fiber matrix of the microspin cup.*

6. Open the cap of the 2-ml receptacle tube, remove and retain the microspin cup, and discard the DNA-binding solution containing the uncoupled dye.

7. Combine 100 µl of the DNA binding solution and 100 µl of 70% (v/v) EtOH. Mix well by vortexing. Make sure that the two solutions are well mixed prior to use.

8. Add the DNA-binding solution and EtOH mixture to the microspin cup. Snap the cap of the receptacle tube onto the top of the microspin cup.

9. Spin the tube in a microcentrifuge at maximum speed for 30 seconds.

10. Open the cap of the 2-ml receptacle tube, remove and retain the microspin cup, and discard the DNA-binding solution/EtOH mixture.

11. Add 750 µl of 75% ethanol to the microspin cup (see "Microspin Cup Wash Buffer" in *Preparation of Reagents*). Snap the cap of the receptacle tube onto the top of the microspin cup.

12. Spin the tube in a microcentrifuge at maximum speed for 30 seconds.

13. Open the cap of the 2-ml receptacle tube, remove and retain the microspin cup, and discard the wash buffer.

14. Repeat steps 11–13.

15. Place the microspin cup back in the 2-ml receptacle tube and snap the cap of the receptacle tube onto the microspin cup.

16. Spin the tube in a microcentrifuge at maximum speed for 30 seconds. On removal from the centrifuge, make sure that all of the wash buffer is removed from the microspin cup.

17. Transfer the microspin cup to a fresh 1.5-ml microcentrifuge tube[ll] and discard the 2-ml receptacle tube.

18. Add 50 µl of 10 mM Tris base, pH 8.5 directly onto the top of the fiber matrix at the bottom of the microspin cup (see "Microspin Cup Elution Buffer" in *Preparation of Reagents*).

19. Incubate the tube at room temperature for 5 minutes.

    Note *Maximum recovery of the labeled cDNA from the microspin cup depends on the pH, the ionic strength, and the volume of the elution buffer added to the microspin cup; the placement of the elution buffer into the microspin cup; and the incubation time. Maximum recovery is obtained when the elution buffer is ≤10 mM in concentration with pH 7–9, when not less than 50 µl of elution buffer is added directly onto the fiber matrix at the bottom of the microspin cup, and when the tube is incubated for 5 minutes.*

20. Snap the cap of the 1.5-ml microcentrifuge tube onto the microspin cup and spin the tube in a microcentrifuge at maximum speed for 30 seconds.

21. Open the lid of the microcentrifuge tube and recover the flow through containing the purified labeled cDNA.

[ll] 1.5-ml flat snap cap microcentrifuge tubes from Continental Laboratory Products, Inc. are recommended.

22. Elute additional labeled cDNA by pipetting the flow through back onto the fiber matrix of the same microspin cup.

23. Re-seat the spin cup on the same 2-ml receptacle tube that contained the liquid from the first-pass elution.

24. Incubate the tube at room temperature for 5 minutes.

25. Snap the cap of the 1.5-ml microcentrifuge tube onto the microspin cup and spin the tube in a microcentrifuge at maximum speed for 30 seconds.

26. Open the lid of the microcentrifuge tube and recover the flow-through containing the purified labeled cDNA.

27. Harvest one final elution from the microspin cup by repeating steps 22–26.

28. Open the lid of the microcentrifuge tube and recover the flow through containing the purified labeled cDNA.

29. To reduce the volume of the labeled cDNA, place the labeled cDNA in a centrifuge and apply a vacuum. Continue to apply a vacuum until the volume has been reduced to approximately 14 µl.

    Note *If a higher concentration of labeled cDNA is desired, the volume may be reduced further by continued centrifugation under vacuum. Scale down the volumes of other hybridization solution components accordingly (see the* Hybridization *section).*

30. Adjust the volume of the labeled cDNA to a final volume of 14 µl by adding dH_2O.

Section from UltraGAPS™ Coated slides instruction manual (Corning).

Pre-Hybridization

Prehybridization should be done immediately preceding the application of the target cDNA onto the arrays. This step has the purpose of blocking the unused surface of the slide and removing loosely bound probe DNA. It is recommended that all target DNAs be characterized prior to the start of pre-hybridization. The preparation of the hybridization solutions can be completed during the time arrays are being pre-hybridized.

1. Prepare prehybridization solution consisting of 5 x SSC, 0.1% SDS, and 0.1 mg/mL BSA. The volumes required to process a given number of arrays depends on type of glassware available. Use Coplin jars to simultaneously process up to 5 arrays using only 50 mL of buffer per step.
2. Warm prehybridization solution to 42°C.
3. Immerse arrays in prehybridization solution and incubate at 42°C for 45 to 60 minutes.
4. Transfer prehybridized arrays to 0.1 x SSC and incubate at ambient temperature (22 to 25°C) for 5 minutes.
5. Repeat Step 4.
6. Transfer arrays to purified water and incubate at ambient temperature for 30 seconds.
7. Dry arrays by blowing high-purity nitrogen over the array or by centrifugation at 1,600 rpm, for 2 minutes. Keep arrays in a dust-free environment while completing the preparation of the hybridization solution.

Hybridization

1. Wash the required number of pieces of Corning® Cover Glass (Cat. No. 2870-22, 2940-244, 2940-246) with nuclease-free water, followed by ethanol. Dry cover glass by blowing high purity compressed nitrogen or allow to air dry in a dust-free environment.

2. Carefully pipette the target DNA onto the arrayed surface. Avoid touching the array with the pipette tip and introducing air bubbles. Carefully lower the cover glass onto the array. Avoid trapping air bubbles between the array and the cover glass. Small air bubbles that do form usually dissipate during hybridization. Transfer array-cover glass assembly to Corning® Hybridization Chamber II (Cat. No. 40080).
3. Assemble the chamber as described in the Corning Microarray Hybridization Chamber Operating Instructions Manual. Keep the chambers right-side up and in a horizontal position at all times to prevent movement of the cover glass relative to the array.
4. Submerge chamber-array assembly in a water bath or place in a hybridization oven kept at 42°C.
5. Hybridize arrays at 42°C for 12 to 16 hours.

Post-Hybridization Washes

It is extremely important not to allow the arrays to dry out between washes, as this will result in high backgrounds. Multiple containers are needed to perform the washes in the most efficient manner. Have all containers and the volumes of washing solutions ready before starting the procedure. Note that steps 2 and 3 both require solutions prewarmed to 42°C.

1. Disassemble the hybridization chambers.
2. Immerse arrays in 2 x SSC, 0.1% SDS at 42°C until the coverslip moves freely away from the slide.
3. Transfer arrays to 2 x SSC, 0.1% SDS at 42°C for 5 minutes.
4. Transfer arrays to 0.1 x SSC, 0.1% SDS at room temperature for 5 minutes.
5. Repeat step 4.
6. Transfer arrays to 0.1 x SSC at room temperature for 1 minute.
7. Repeat Step 6 four times.
8. Rinse arrays in 0.01 x SSC for 10 seconds.

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9. Dry arrays by blowing clean compressed nitrogen or by centrifugation at 1,600 x *g* for 2 minutes.
10. Store arrays in the Corning® 25 Slide Holder (Cat. No. 40081). Protect arrays from overexposure to light until ready to scan.

Note: Arrays spotted on UltraGAPS™ slides can be hybridized at temperatures up to 65°C. The use of hybridization temperatures higher than 42°C, however, calls for changes in the composition of the hybridization and wash solutions described in this manual, such as exclusion of formamide, to properly adjust their stringency to the requirements of the application at hand.

ADDITIONAL INFORMATION

Customer Service and Technical Support

For a detailed troubleshooting guide, answers to frequently asked questions, and additional information about these and other products, please visit **www.corning.com/lifesciences**. For questions, further clarification about this protocol, and other technical issues and information not covered in this manual, please e-mail **clstechserv@corning.com** or call 800.492.1110 (+1.978.635.2200 outside Canada and USA).

Appendix III: Ct-values from Real-Time experiments

(See section 2.43, 2.44 and 3.4 for details)

gap, gyceraldehyd-3-posphate dehydrogenase

Table III – 1 Ct-values for calculation of the amplification efficient of *gap*. The 10^{-1} dilutions was used for calculation of expression.

A0		**A30**		**B0**		**B5**		**B10**	
Dilution (log 10)	**CT-value**	**Dilution (log 10)**	**CT-value**	**Dilution (log 10)**	**CT-value**	**Dilution (log 10)**	**CT-value**	**Dilution (log 10)**	**CT-value**
-1	14.04	-1	13.42	-1	13.99	-1	13.62	-1	13.91
-1.5	15.77	-1.5	15.22	-1.5	15.08	-1.5	15.45	-1.5	15.58
-2	17.09	-2	17.48	-2	16.70	-2	16.96	-2	17.01
-2.5	18.86	-2.5	18.89	-2.5	18.45	-2.5	18.69	-2.5	18.66
-3	20.43	-3	20.78	-3	19.68	-3	20.3	-3	20.28

B30		**C0**		**C30**		**D0**		**D30**	
Dilution (log 10)	**CT-value**	**Dilution (log 10)**	**CT-value**	**Dilution (log 10)**	**CT-value**	**Dilution (log 10)**	**CT-value**	**Dilution (log 10)**	**CT-value**
-1	14.13	-1	16.01	-1	13.68	-1	20.78	-1	14.89
-1.5	15.82	-1.5	17.95	-1.5	15.70	-1.5	22.33	-1.5	16.64
-2	17.21	-2	19.68	-2	17.12	-2	23.95	-2	18.18
-2.5	19.15	-2.5	21.30	-2.5		-2.5	25.59	-2.5	
-3	20.52	-3	23.82	-3		-3	27.18	-3	21.46

fbp, fibronectin binding protein

Table III – 2 Ct-values for quantification of relative expression of *intA*. Shown are the Ct-values for the 10^{-1} dilutions for *fbp* and *gap*. The Ct-values used for calculation of the amplification efficiencies is specified in table VII-3 on next page.

	Ct-value	
	fbp	***gap***
A0	19.72	13.73
A30	19.65	14.01
B0	19.93	13.61
B30	19.01	13.50
C0	19.92	11.97
C30	20.63	13.51
D0	23.96	17.00
D30	20.04	13.86

Table III – 3 Ct-values for calculation of the amplification efficient of *fbp*.

	A0	A30	C0	C30	D0	D30
-1	19.72	19.65	19.92	20.63	23.96	20.04
-1.5	21.33	21.54		22.28	25.75	22.25
-2	23.23	23.17	23.09		27.13	23.79
-2.5	24.74	24.81	25.27	25.43		25.09
-3	26.65	26.59	26.47	27.19	30.53	26.94

intA, internalin A

Table III – 4 Ct-values for quantification of relative expression of *intA*. Shown are the Ct-values for the 10^{-1} dilutions for *intA* and *gap*. The Ct-values used for calculation of the amplification efficiencies is specified in table VII-5 below.

	Ct-value	
	intA	***gap***
A0	18.88	12.01
A30	22.01	13.11
B0	20.01	12.96
B30	22.45	12.96
C0	20.78	13.52
C30	24.42	13.70
D0	24.21	18.09
D30	22.55	14.22

Table III – 5 Ct-values for calculation of the amplification efficient of *intA*

	A0	B0	B30	C0	C30	D0
-1	18.88	22.65	22.45	20.74	24.46	26.96
-1.5	20.65	24.25	24.16	22.46		28.56
-2	22.11	25.71	25.83	23.69	27.8	30.2
-2.5	23.8	27.79	27.59	25.46	29.84	
-3	25.65	28.98	29.02		31.43	33.08

intB, internalin A

Table III – 6 Ct-values for quantification of relative expression of *intB*. Shown are the Ct-values for the 10^{-1} dilutions for *intB* and *gap*. Replicate 1 and 3 are performed on the same RNA, while replicate 2 is a biological replicate. The expression for A5 and A20 are calculated against a mean of the Ct-vaules for A0 *intB* and *gap*, while the other are calculated against the A0 values in the same replicate. The Ct-values used for calculation of the amplification efficiencies are specified in table VII-3 below.

	Replicate 1 Ct-value		**Replicate 2 Ct-value**		**Replicate 3 Ct-value**			**Replicate 1 Ct-value**	
	intB	***gap***	***intB***	***gap***	***intB***	***gap***		***intB***	***gap***
A0	19.23	12.23	19.4347	12.97	17.98	12.47	**A5**	19.93	14.55
A30	21.33	13.02	21.65	13.95	23.02	15.44	**A20**	21.88	14.86
B0	19.52	12.22	19.3	13.32					
B30	21.80	12.11	22.26	13.18					
C0	18.07	11.23	18.72	13.02	19.01	12.77	**C5**	19.74	14.05
C30	23.58	12.69	24.033	13.17	25.33	14.69	**C20**	22.2	14.17
D0	23.02	18.28	22.28	17.59					
D30	22.02	13.26	21.53	13.73					

Table III – 7 Ct-values for calculation of the amplification efficient of *intB*

Dilution - log 10	**A30**	**D0**	**A30**	**B30**	**C0**
-1	21.33	23.02	21.65	21.74	18.72
-1.5	22.88	24.93	23.12	23.43	20.07
-2	24.45	26.75	24.53	24.72	21.95
-2.5			26.62	26.1	
-3	27.79	29.86	28.01	27.78	25.49

Dilution - log 10	**D0**	**D30**	**A20**	**C5**
-1	22.28	21.53	21.88	19.74
-1.5	23.65	22.83	23.51	21.16
-2	25.07	24.7	24.76	23.06
-2.5		25.95	26.89	24.6
-3	28.5	27.96	28.14	26.48

Appendix IV: Results from the microarray experiments (See section 2.5 and 3.5 for details)

Table IV The complete list of microarray results. The A30, B30, and C30 samples were each hybridized against the A0 sample. The gene name, gene numbers, log2 ratios and the P-values are listed.

		A30/A0		B30/A0		C30/A0	
BC #	**Genename**	**Log2 ratio**	**P-value**	**Log2 ratio**	**P-value**	**Log2 ratio**	**P-value**
Transciptional regulators							
BC3095	Transcriptional regulator AnsR			-1.76	<0.005		
BC3826	Transcriptional regulator CodY					-0.59	0.037
BC4703	Transcriptional regulator. DeoR family			3.03	0.006	1.10	0.037
BC0518	Transcriptional regulator. Fur family	-0.17	0.039	0.97	0.017		
BC3792	Transcriptional regulator. GntR family					1.32	0.034
BC4603	Transcriptional regulator. GntR family	1.11	0.009			1.17	0.034
BC3069	Transcriptional regulator. IclR family					0.92	0.038
BC0657	Transcriptional regulator. MarR family			2.61	0.011	2.91	0.046
BC4081	Transcriptional regulator. MarR family			-0.75	0.006		
BC1449	Transcriptional regulator. putative			2.39	<0.005		
BC2340	Transcriptional regulator. TetR family			2.73	0.049		
BC0961	Transcriptional regulator. TetR-related family					1.27	0.034
BC3706	Transcriptional repressor GlnR			-1.57	0.049	-1.48[(2)]	0.042[(2)]
BC4672	Catabolite control protein A	0.98	0.015			1.00	0.034
Toxins							
BC3104	Hemolysin BL lytic component L2			-2.31	<0.005		
Stress response							
BC4312	Chaperone protein dnaK	0.89	<0.005	1.38	<0.005	1.17[(2)]	0.016[(2)]
BC0294	Chaperonin. 10 kDa	1.29	<0.005	3.40	<0.005	2.16[(2)]	0.015[(2)]
BC0295	Chaperonin. 60 kDa	0.78	<0.005	2.97	<0.005		
BC3539	Cold shock protein CspB			1.85	<0.005		
BC5152	ATP-dependent Clp protease. proteolytic subunit ClpP					1.51	0.037
BC2214	Heat shock protein. Hsp20 family			3.33	0.047		

		A30/A0		B30/A0		C30/A0	
BC #	**Genename**	**Log2 ratio**	**P-value**	**Log2 ratio**	**P-value**	**Log2 ratio**	**P-value**
Stress response *continued*							
BC4314	Heat-inducible transcription repressor HrcA					1.89	0.037
BC4272	Superoxide dismutase. Mn			0.68	0.007		
BC0102	Negative regulator of genetic competence ClpC/MecB			2.48	0.047	1.80	0.039
BC0443	Tellurium resistance protein			2.43	0.007	1.29	0.037
BC0442	Tellurium resistance protein. putative			2.33	0.047	1.12	0.034
BC0445	Tellurium resistance protein. putative			1.54	<0.005		
BC5398	Uracil-DNA glycosylase			1.03	0.006		
Motility							
BC1665	Flagellar biosynthetic protein FliP. putative	-1.85	0.024				
BC1638	Flagellar hook-associated protein 2	-1.11	<0.005	-1.23	<0.005		
BC1645	Flagellar motor switch protein FliG					-0.82	0.036
BC1662	Flagellar motor switch protein fliM			-0.71	0.048		
BC0576	Methyl-accepting chemotaxis protein			-0.99	0.008		
Membrane-/cell wall-associated							
BC3791	Lipoprotein. Bmp family			1.43	0.027		
BC5242	Membrane protein with C2C2 zinc finger					-1.31	0.034
BC1227	Membrane protein. putative			0.68	0.006		
BC2152	Membrane protein. putative					-0.67	0.037
BC3542	Membrane protein. putative					-0.78	0.036
BC3709	Membrane protein. putative			-0.74	0.009		
BC0991	S-layer protein. putative	0.41	0.036	-1.48	<0.005		
BC4549	LPXTG-motif cell wall anchor domain protein. putative					-1.45	0.034
BC1331	Internalin. putative			-0.58	0.097		
BC4927	Cell surface protein			-1.05	<0.005		
Sporulation							
BC4435	sporulation initiation phosphotransferase. putative					-0.78	0.037
BC5327	stage II sporulation protein R. putative					-0.81	0.039

BC #	Genename	A30/A0 Log2 ratio	A30/A0 P-value	B30/A0 Log2 ratio	B30/A0 P-value	C30/A0 Log2 ratio	C30/A0 P-value
Sporulation *continued*							
BC0053	stage V sporulation protein G			1.10	0.036		
BC1612	germination protein gerN			1.98	0.010		
Cell division/DNA replication							
BC5475	Single-stranded DNA-binding protein					-0.94	0.037
BC1461	Site-specific recombinase. phage integrase family			3.53	0.049		
BCp0005	Type B DNA polymerase	1.53	<0.005	1.72	<0.005	1.22	0.034
BC4649	Septation ring formation regulator			-0.77	0.005		
BC4443	Septum site-determining protein MinC	-0.35(1)	0.035(1)	0.65	0.009		
BC0001	Chromosomal replication initiator protein DnaA			-0.79	0.041		
BC0072	Cell division protein FtsH					0.75	0.037
Transport							
BC0160	ABC transporter. ATP-binding protein			-0.82	0.033		
BC4743	ABC transporter. ATP-binding protein					-0.88	0.037
BC0873	Amino acid ABC transporter. permease protein					-1.26	0.038
BC0865	Amino acid permease family protein					-2.25	0.037
BC3463	Arsenical pump family protein			1.20	0.040		
BC0962	Drug resistance transporter. EmrB/QacA family					2.80	0.034
BC4000	Drug resistance transporter. EmrB/QacA family			2.76	<0.005		
BC4568	Drug resistance transporter. EmrB/QacA family			3.35	0.042		
BC0381	Iron compound ABC transporter. permease protein					-0.88	0.047
BC5104	Iron compound ABC transporter. permease protein			-1.20	0.007	-0.68	0.037
BC5105	Iron compound ABC transporter. permease protein						
BC5012	Major facilitator family transporter					-0.91	0.034
BC1948	Hypothetical Multidrug-Efflux Transporter					-0.76	0.044
BC0596	Heavy metal-transporting ATPase			1.68	<0.005		
BC1182	Oligopeptide ABC transporter. ATP-binding protein					1.40	0.046
BC0907	Oligopeptide ABC transporter. oligopeptide-binding protein					-1.16	0.037
BC1180	Oligopeptide ABC transporter. permease protein					1.47	0.037

BC #	Genename	A30/A0 Log2 ratio	A30/A0 P-value	B30/A0 Log2 ratio	B30/A0 P-value	C30/A0 Log2 ratio	C30/A0 P-value
Transport *continued*							
BC1181	Oligopeptide ABC transporter. permease protein			1.87	<0.005		
BC1432	Proton/glutamate symporter family protein			1.01	0.009		
BC2170	Sodium-dependent transporter. putative					0.88	0.034
BC4121	Na+/H+ antiporter NhaC			1.49	0.005		
BC5435	Transporter. AcrB/AcrD/AcrF family					-0.63	0.037
BC0318	Transporter. Drug/Metabolite Exporter family			-0.83	<0.005		
BC4544	Ferrichrome transport ATP-binding protein fhuC					-1.32	0.037
BC0631	PTS system. trehalose-specific IIBC component					1.60	0.039
BC3890	Uracil permease					1.32	0.034
BC0297	Xanthine/uracil permease family protein			0.87	<0.005		
BC5395	Xanthine/uracil permease family protein			-1.01	0.009	-0.71	0.039
BC1836	Branched-chain amino acid transport system carrier protein			2.48	<0.005		
BC4895	SapB protein			1.08	0.045		
BC3355	CBS domain protein			1.65	0.042		
BC4507	Aquaporin Z			-1.22	<0.005		
Translation							
BC0124	Ribosomal protein L7A family	0.74	<0.005	-0.96	0.040	-0.63[(2)]	0.014[(2)]
BC0125	Ribosomal protein S12			-0.69	0.022		
BC0130	Ribosomal protein S10			-0.61	0.020		
BC0134	Ribosomal protein L2			-0.68	0.030		
BC0135	SSU ribosomal protein S19P					-0.59	0.039
BC0137	Ribosomal protein S3			-0.75	0.039		
BC0143	Ribosomal protein L5			-0.64	0.021		
BC0155	Ribosomal protein L36			-1.01	<0.005		
BC4436	Ribosomal protein L27	0.56	<0.005	0.95	<0.005		
BC4263	Ribosomal protein L33	0.22	0.016	0.75	0.010		
BC4320	Ribosomal protein S20					0.64	0.039
BC5075	LSU ribosomal protein L11P			1.21	0.047		
BC5062	Tyrosyl-tRNA synthetase			1.01	<0.005		

BC #	Genename	A30/A0 Log2 ratio	A30/A0 P-value	B30/A0 Log2 ratio	B30/A0 P-value	C30/A0 Log2 ratio	C30/A0 P-value
Translation *continued*							
BC0154	Translation initiation factor IF-1			-1.26	0.013		
Metabolism							
Fermentation, TCA and oxidative phosphorylation							
BC3372	6-phosphogluconate dehydrogenase family protein					0.95	0.037
BC4600	Phosphofructokinase					0.59	0.039
BC5135	Enolase	-0.33	0.430				
BC0632	Trehalose-6-phosphate hydrolase					1.23	0.042
BC3972	Pyruvate dehydrogenase complex E1 component. beta subunit					0.75	0.038
BC3971	Pyruvate dhg complex E2 - dihydrolipoamide acetyltransferase			-1.00	<0.005		
BC3970	Pyruvate dhg complex E3 - dihydrolipoamide dehydrogenase	-0.74(1)	0.239(1)	0.28	<0.005	0.70	0.094
BC3774	Pyruvate ferredoxin oxidoreductase. alpha subunit. putative			0.63	0.022		
BC4160	Dihydrolipoamide dehydrogenase					2.34(2)	0.029(2)
BC0868	Glyceraldehyde-3-phosphate dehydrogenase. NADP-dependent			1.04	0.036		
BC5335	Fructose-bisphosphate aldolase. class II	-0.58(1)	0.0311(1)	1.25	<0.005		
BC5002	Acyl-CoA dehydrogenase			1.02	0.020		
BC4594	Citrate synthase CitZ			1.52	<0.005		
BC4517	Succinate dehydrogenase. flavoprotein subunit	1.23	<0.005	1.98	<0.005	1.64	0.039
BC4516	Succinate dehydrogenase. iron-sulfur protein			1.33	<0.005	1.31	0.034
BC4592	Malate dehydrogenase			2.71	0.042	1.39	0.039
BC4593	Isocitrate dehydrogenase. NADP-dependent			2.90	0.038		
BC0665	Transaldolase. putative			0.92	<0.005		
BC3885	Dihydroorotate dehydrogenase. electron transfer subunit			-2.45	<0.005		
BC1939	Cytochrome d ubiquinol oxidase subunit II					-1.40	0.034
BC4792	Cytochrome d ubiquinol oxidase. subunit I			-0.65	0.014		
BC5294	NADH-quinone oxidoreductase chain K			-0.74	0.011		
BC5306	ATP synthase F1. beta subunit	0.31	<0.005	-0.62	<0.005		
BC5307	ATP synthase F1. gamma subunit			-1.19	<0.005		

		A30/A0		B30/A0		C30/A0	
BC #	**Genename**	**Log2 ratio**	**P-value**	**Log2 ratio**	**P-value**	**Log2 ratio**	**P-value**
Metabolism							
Proteins, peptides, and amino acids.							
BC1120	CAAX amino terminal protease family protein			3.13	0.047		
BC0293	CAAX amino terminal protease family protein	0.47[(1)]	0.031[(1)]	1.98	<0.005		
BC0360	Aminopeptidase AmpS			0.78	0.031		
BC4678	Aminopeptidase. putative			1.53	0.007		
BC4198	Proline dipeptidase	-0.25	0.034	-0.88	0.049		
BC4614	Proline dipeptidase					0.72	0.039
BC4693	Peptidase. M42 family			0.70	0.011		
BC4377	Peptidase. U32 family	-0.85[(1)]	0.048[(1)]	-1.79	0.006	-0.59	0.037
BC0289	O-sialoglycoprotein endopeptidase					0.59	0.038
BC1338	Oligoendopeptidase F. putative			1.12	0.006		
BC1233	*p*-aminobenzoate synthase glutamine amidotransferase. comp. II			1.82	0.008		
BC0153	Methionine aminopeptidase	-0.32	<0.005	-1.10	<0.005		
BC3962	Lysine decarboxylase			0.94	0.027		
BC4135	L-serine dehydratase. iron-sulfur-dependent. alpha subunit			-0.60	0.008		
BC3094	L-asparaginase			-1.68	<0.005		
BC4162	Leucine dehydrogenase	0.77	0.030	1.62	<0.005	-2.42[(2)]	0.015[(2)]
BC4224	Glycine cleavage system P protein. subunit 2			1.38	0.012	1.36	0.034
BC4225	Glycine cleavage system P-protein. subunit 1			1.79	<0.005	1.85	0.034
BC4226	Glycine cleavage system T protein			1.58	<0.005		
BC2439	Aminoacyl-histidine dipeptidase					0.95	0.040
BC3889	Aspartate carbamoyltransferase					1.25	0.034
BC1746	Aspartate--ammonia ligase			1.09	<0.005		
BC1491	Glutamate dehydrogenase			0.70	0.007		
BC2942	Chorismate mutase/phospho-2-dehydro-3-deoxyheptonate aldolase			2.46	0.009		
BC3887	Carbamoyl-phosphate synthase. small subunit	-1.32	0.008	-1.44	0.005		
BC1234	Anthranilate phosphoribosyltransferase			2.16	0.020		
BC2241	Aldehyde dehydrogenase family protein			1.78	0.014		
BC4003	5-met-tet-hydropteroyltriglutamate-cysteine methyltransferase			2.90	0.018		
BC4159	3-methyl-2-oxobutanoate dehydrogenase. alpha subunit					1.94[(2)]	0.031[(2)]

		A30/A0		B30/A0		C30/A0	
BC #	**Genename**	**Log2 ratio**	**P-value**	**Log2 ratio**	**P-value**	**Log2 ratio**	**P-value**
Metabolism							
Peptidoglycan-metabolism							
BC0677	Undecaprenol kinase family protein			0.59	0.017		
BC5334	UDP-N-acetylglucosamine 1-carboxyvinyltransferase 2			0.71	0.006		
BC3912	UDP-N-acetylmuramoylalanine--D-glutamate ligase					-0.61	0.037
BC3913	Phospho-N-acetylmuramoyl-pentapeptide-transferase			-0.70	0.023		
BC5278	Chain length regulator (capsular polysaccharide biosynthesis)			-2.13	0.036		
BC1974	Polysaccharide deacetylase. putative			-0.77	0.009		
Metabolism							
Other metabolitic genes							
BC4938	Pyridine nucleotide-disulphide oxidoreductase					1.05	0.044
BC0013	Inosine-5'-monophosphate dehydrogenase	-0.24	0.050			0.90	0.034
BC0697	Quinol oxidase. subunit I	-0.74	<0.005	-0.68	<0.005		
BC1290	Phosphatidylglycerophosphatase B					-0.93	0.034
BC3882	Orotate phosphoribosyltransferase	-2.31	0.011				
BC1353	NrdI protein			-1.07	<0.005		
BC4045	Nitroreductase family protein			0.85	0.019		
BC4329	Nicotinate (nicotinamide) nucleotide adenylyltransferase			-0.72	0.007		
BC4853	Naphthoate synthase			0.89	0.011	-1.13[(2)]	0.014[(2)]
BC4856	Menaquinone-specific isochorismate synthase					0.63	0.037
BC0071	Hypoxanthine phosphoribosyltransferase					0.79	0.038
BC2101	Formate--tetrahydrofolate ligase					1.50	0.038
BC0344	Delta-1-pyrroline-5-carboxylate dehydrogenase. putative			2.93	0.048	2.04	0.034
BC0884	Alpha-acetolactate decarboxylase					1.03	0.034
BC0668	Alcohol dehydrogenase. zinc-containing			2.37	<0.005		
BC0152	Adenylate kinase	-0.12	<0.005	-1.09	0.009		
BC3616	Aconitate hydratase 1					1.60	0.041
BC0883	Acetolactate synthase			2.81	0.047	1.64	0.034
BC1173	3-oxoacyl-(acyl-carrier-protein) synthase III			-1.46	0.032		

		A30/A0		B30/A0		C30/A0	
BC #	**Genename**	**Log2 ratio**	**P-value**	**Log2 ratio**	**P-value**	**Log2 ratio**	**P-value**
Metabolism *Other metabolitic genes, continued*							
BC1760	3-oxoacyl-(acyl-carrier-protein) synthase III. putative					0.79	0.037
BC4111	3,4-dihydroxy-2-butanone 4-phosphate synthase			1.19	<0.005		
Other functions							
BC4521	Thioredoxin	-0.82	0.019	1.24	<0.005		
BC1305	YkgG family protein			0.79	0.049		
BC2932	Sulfatase			2.29	<0.005		
BC5087	Putative lantibiotic precursor peptide			3.41	0.030		
BC5088	Putative lantibiotic precursor peptide			2.80	<0.005		
BC5090	Putative lantibiotic precursor peptide			2.90	<0.005		
BC1711	Short chain dehydrogenase					-0.70	0.037
BC4393	Rrf2 family protein			-0.64	0.006		
BC4210	Rhodanese-like domain protein					-1.00	0.034
BC1436	PspA/IM30 family protein			5.42	<0.005		
BC4270	Penicillin-binding protein			0.82	0.008		
BC2281	Penicillin-binding protein 1A			1.23	<0.005		
BC4492	Phosphoesterase. putative					-0.82	0.034
BC3854	Phosphatase. putative					-0.86	0.034
BC1316	PhaR protein			1.71	0.016		
BC4952	NifU domain protein					0.81	0.037
BC1553	Multimodular transpeptidase-transglycosylase					-0.67	0.037
BC4854	Hydrolase. alpha/beta fold family					0.63	0.038
BC1165	Hydrolase. haloacid dehalogenase-like family			1.25	0.026		
BC5379	HD domain protein					-0.91	0.039
BC3933	ComK regulator	0.58	0.013	0.67	0.011		
BC0303	Alpha/beta hydrolase			0.99	0.036		
BC4332	GTPase family protein			-0.75	0.037		
BC4161	Butyrate kinase					2.20	0.034
BC0104	DNA-binding protein. putative			0.72	0.006		
BC1224	Acetyltransferase			2.48	0.049		
BC0587	Acetyltransferase. GNAT family			3.02	<0.005		

BC #	Genename	A30/A0 Log2 ratio	A30/A0 P-value	B30/A0 Log2 ratio	B30/A0 P-value	C30/A0 Log2 ratio	C30/A0 P-value
Other functions *continued*							
BC1349	Acetyltransferase. GNAT family			0.65	0.013		
BC1368	2-nitropropane dioxygenase			0.60	0.006		
Hypotetical							
BCp0018	Hypothetical cytosolic protein			1.14	0.007	-0.67	0.047
BCp0019	Hypothetical protein					-0.70	0.037
BCp0001	Hypothetical protein	1.38	<0.005			-1.23[(2)]	0.032[(2)]
BCp0002	Hypothetical protein	2.27	0.019	1.89	0.022	1.24	0.034
BCp0004	Hypothetical cytosolic protein			1.77	0.030		
BC5388	Conserved hypothetical protein			0.63	0.017		
BC5326	Conserved hypothetical protein					-1.74	0.034
BC0520	Conserved hypothetical protein	0.58	0.009	1.08	<0.005		
BC0687	Conserved hypothetical protein			0.91	0.007		
BC0880	Conserved hypothetical protein					-1.67[(2)]	0.014[(2)]
BC1012	Conserved hypothetical protein			0.90	0.010		
BC1150	Conserved hypothetical protein			0.64	0.006		
BC1169	Conserved hypothetical protein			0.69	0.007		
BC1303	Conserved hypothetical protein			0.77	0.017		
BC1435	Conserved hypothetical protein			6.86	<0.005		
BC1528	Conserved hypothetical protein	-0.16	0.048	1.20	<0.005		
BC1563	Conserved hypothetical protein			-0.64	0.006		
BC2841	Conserved hypothetical protein			1.94	0.027		
BC2969	Conserved hypothetical protein					-0.87	0.034
BC3605	Conserved hypothetical protein			1.79	0.007		
BC3787	Conserved hypothetical protein			0.83	<0.005		
BC3844	Conserved hypothetical protein			-1.25	<0.005		
BC4182	Conserved hypothetical protein	0.22	0.039	-0.77	0.042		
BC4274	Conserved hypothetical protein			0.70	0.007		
BC4308	Conserved hypothetical protein					-0.74	0.037
BC4382	Conserved hypothetical protein	-0.59	<0.005	-0.69	0.006		
BC4519	Conserved hypothetical protein			1.13	0.009		

		A30/A0		B30/A0		C30/A0	
BC #	**Genename**	**Log2 ratio**	**P-value**	**Log2 ratio**	**P-value**	**Log2 ratio**	**P-value**
Hypotetical *continued*							
BC4531	Conserved hypothetical protein					-0.59	0.044
BC4547	Conserved hypothetical protein			-0.82	0.006	-1.51	0.042
BC4569	Conserved hypothetical protein			2.39	0.047		
BC4634	Conserved hypothetical protein					0.64	0.037
BC4653	Conserved hypothetical protein			1.61	0.006		
BC4752	Conserved hypothetical protein			0.67	0.006		
BC5060	Conserved hypothetical protein					0.65	0.045
BC5154	Conserved hypothetical protein					0.65	0.038
BC5158	Conserved hypothetical protein					-0.84	0.037
BC5208	Conserved hypothetical protein					-0.69	0.042
BC5289	Conserved hypothetical protein			-1.07	<0.005		
BC4328	Conserved hypothetical protein TIGR00488					-0.59	0.037
BC0248	Hypothetical protein			3.55	<0.005		
BC0250	Hypothetical protein			3.35	<0.005		
BC0739	Hypothetical protein			0.63	0.016		
BC2552	Hypothetical protein			-2.41	0.010	-1.00[(2)]	0.018[(2)]
BC2842	Hypothetical protein			1.54	0.041		
BC5243	Hypothetical protein					-0.91	0.036

Genes with bold BC-number are also investigated by real-time or 2-DE. [(1)] Genes in the A30/A0 hybridization that is only confirmed with one biological replicate. [(2)] Genes in the C30/A0 hybridization that is confirmed by two biological replicates.

Appendix V: 2-D electrophoresis gels of LiCl extracts from cultures in shift experiments (See section 2.6.3.2 and 3.6.4.1 for details)

Figure A0 – 1: 2-DE protein gel of LiCl extract of *B. cereus* harvested from the control culture immediately after the shift.

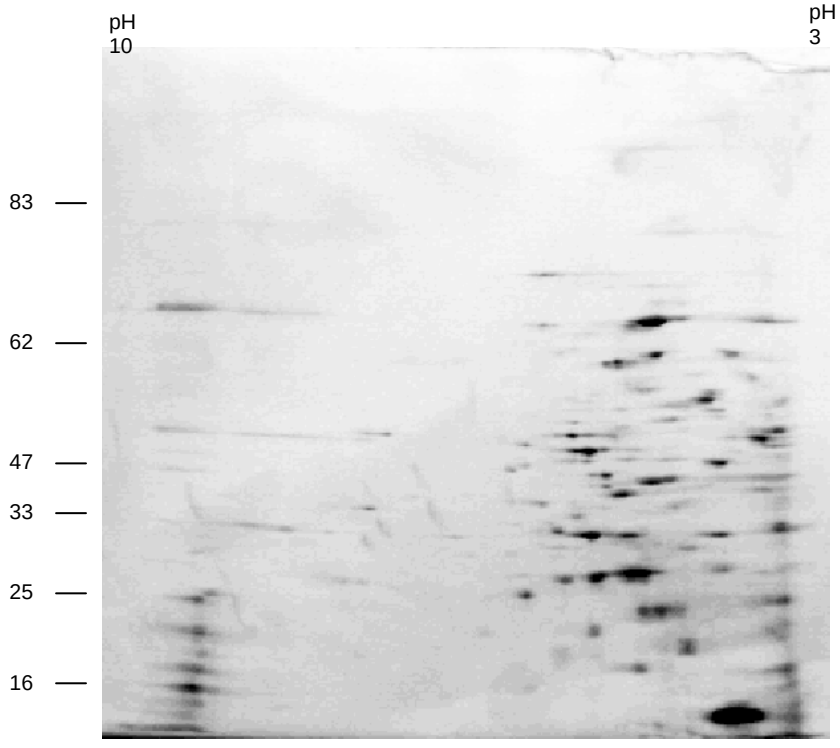

Figure A0 – 2: 2-DE protein gel of LiCl extract of *B. cereus* harvested from the control culture immediately after the shift.

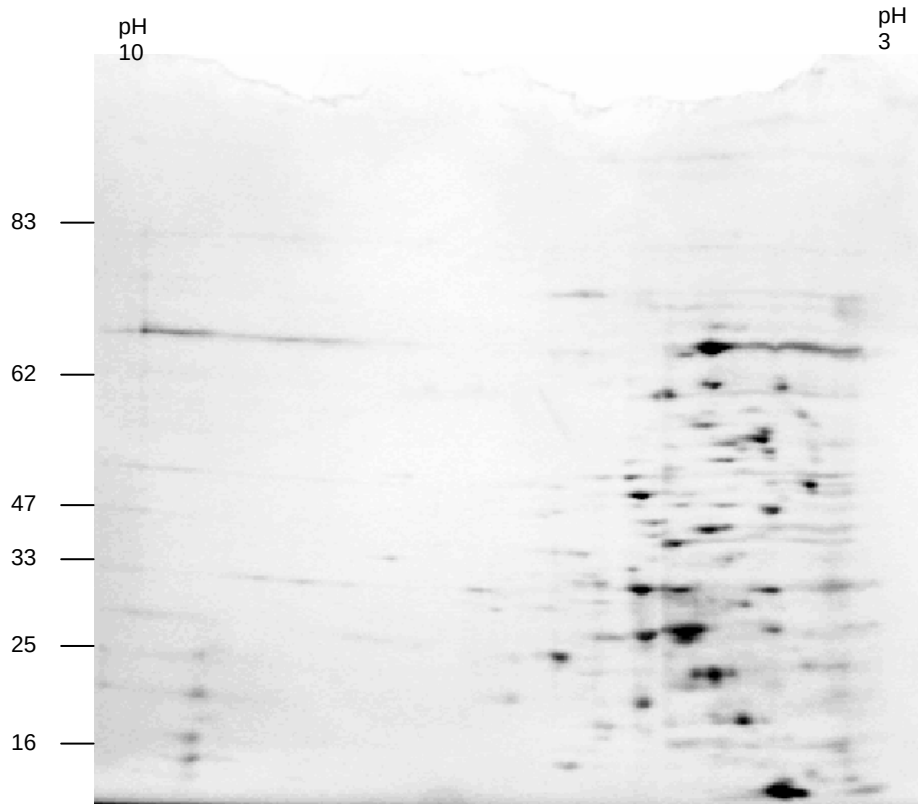

Figure A0 – 3 2-DE protein gel of LiCl extract of *B. cereus* harvested from the control culture immediately after the shift.

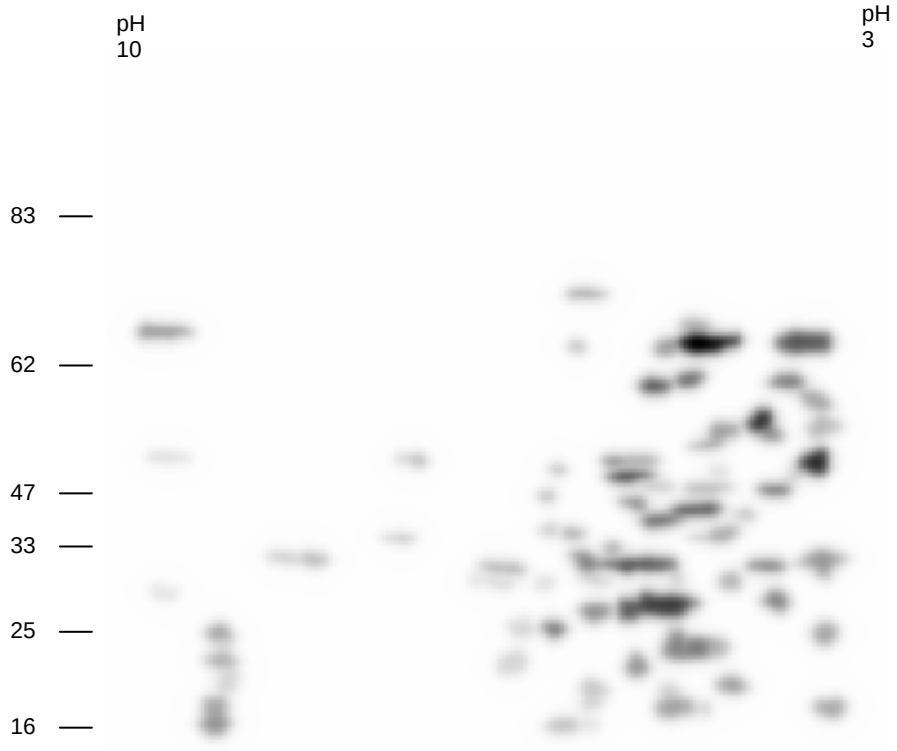

Figure A0 – synthetic: Synthetic 2-DE protein gel of LiCl extract of *B. cereus* harvested from the control culture immediately after the shift. The spot volumes are the average of A0 – 1, 2, and 3.

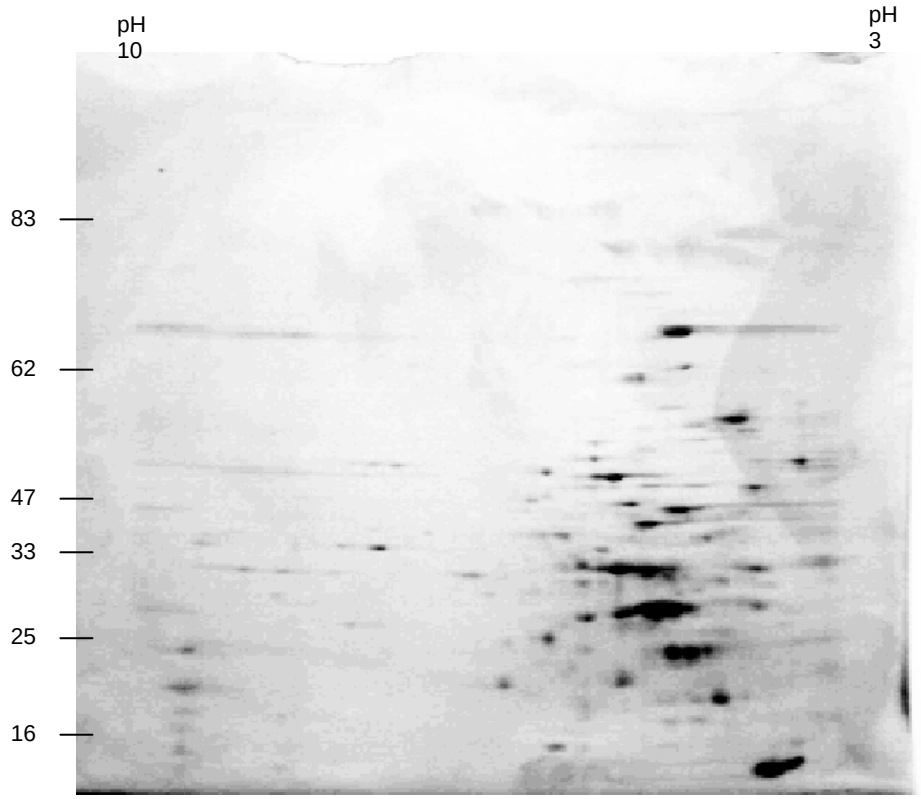

Figure A30 – 1: 2-DE protein gel of LiCl extract of *B. cereus* harvested from the control culture 30 minutes after the shift

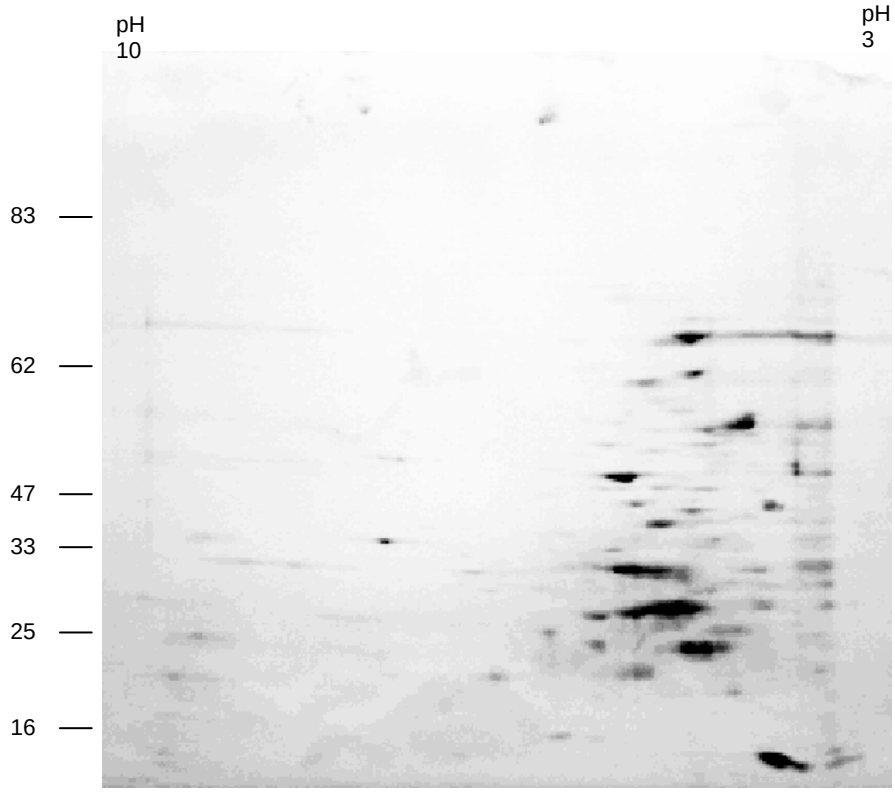

Figure A30 – 2: 2-DE protein gel of LiCl extract of *B. cereus* harvested from the control culture 30 minutes after the shift.

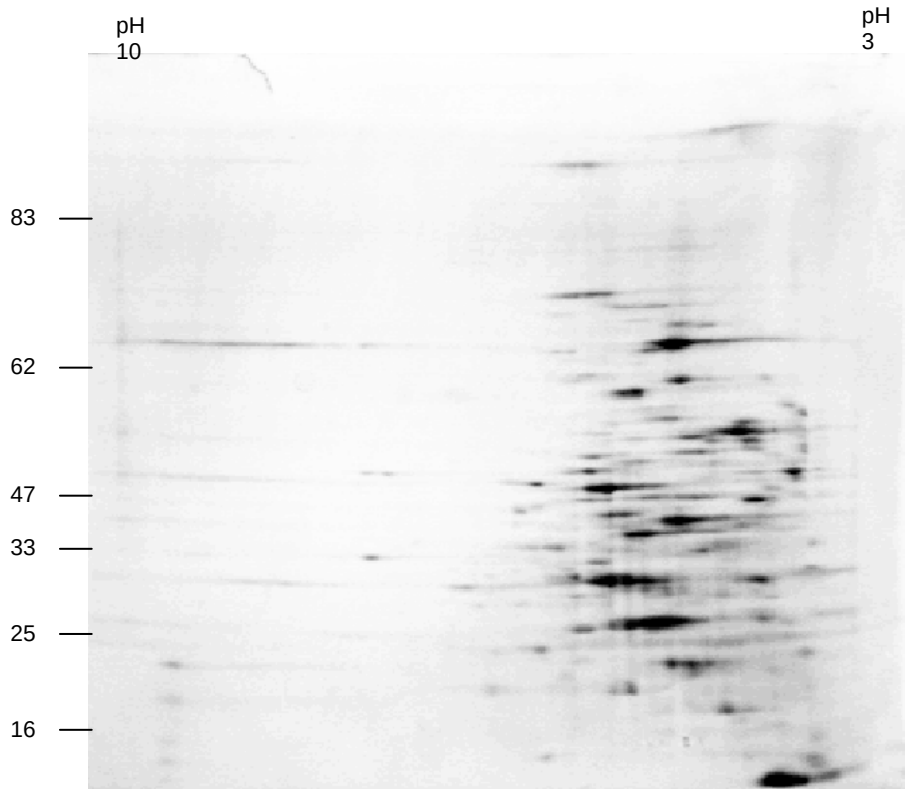

Figure A30 – 3: 2-DE protein gel of LiCl extract of *B. cereus* harvested from the control culture 30 minutes after the shift.

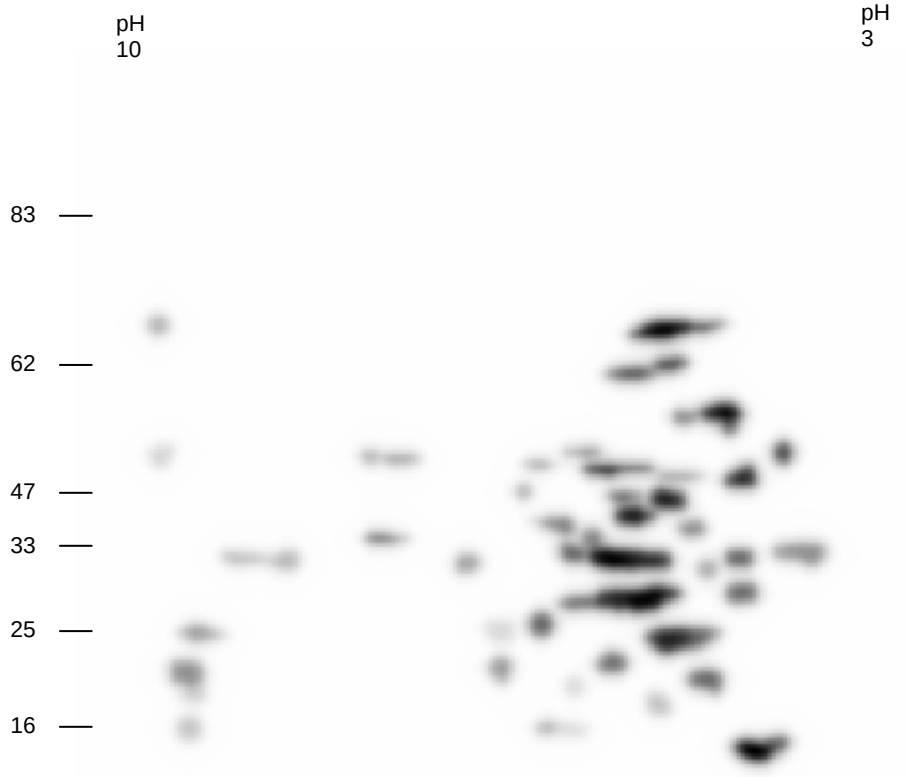

Figure A30 – synthetic: Synthetic 2-DE protein gel of LiCl extract of *B. cereus* harvested from the control culture 30 minutes after the shift. The spot volumes are the average of A30 – 1, 2, and 3.

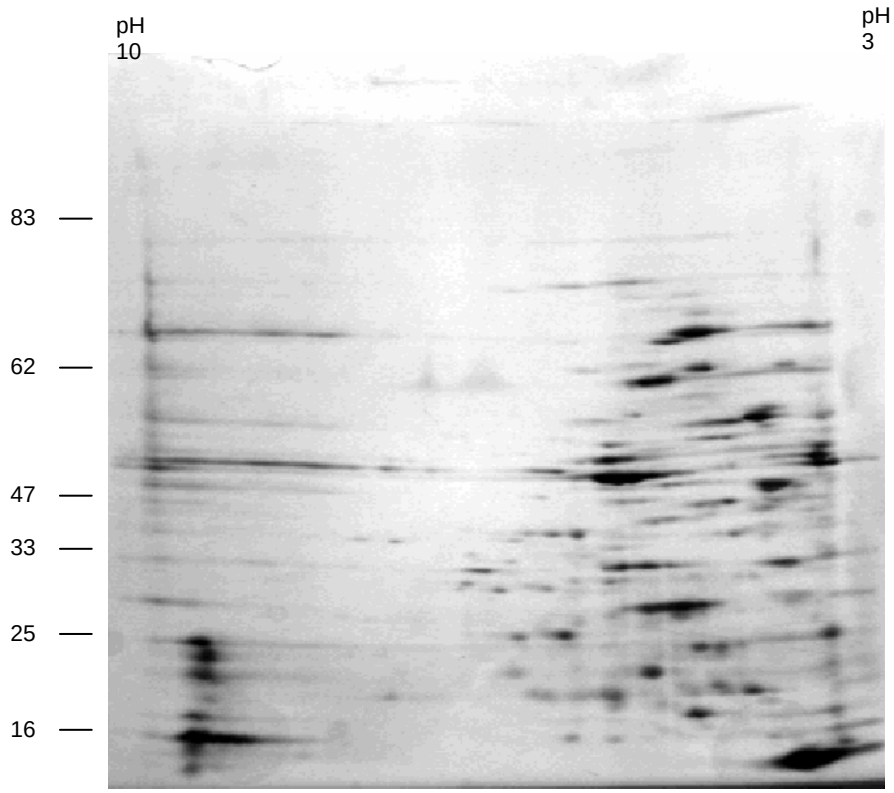

Figure B30 – 1: 2-DE protein gel of LiCl extract of *B. cereus* harvested from the bile culture 30 minutes after the shift.

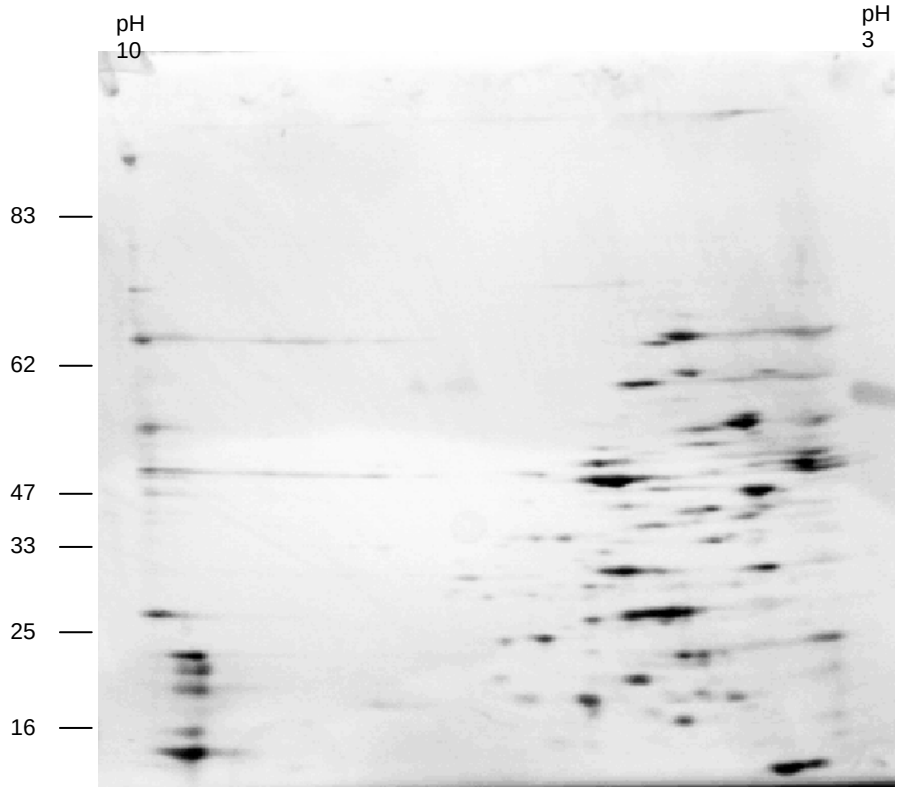

Figure B30 – 2: 2-DE protein gel of LiCl extract of *B. cereus* harvested from the bile culture 30 minutes after the shift.

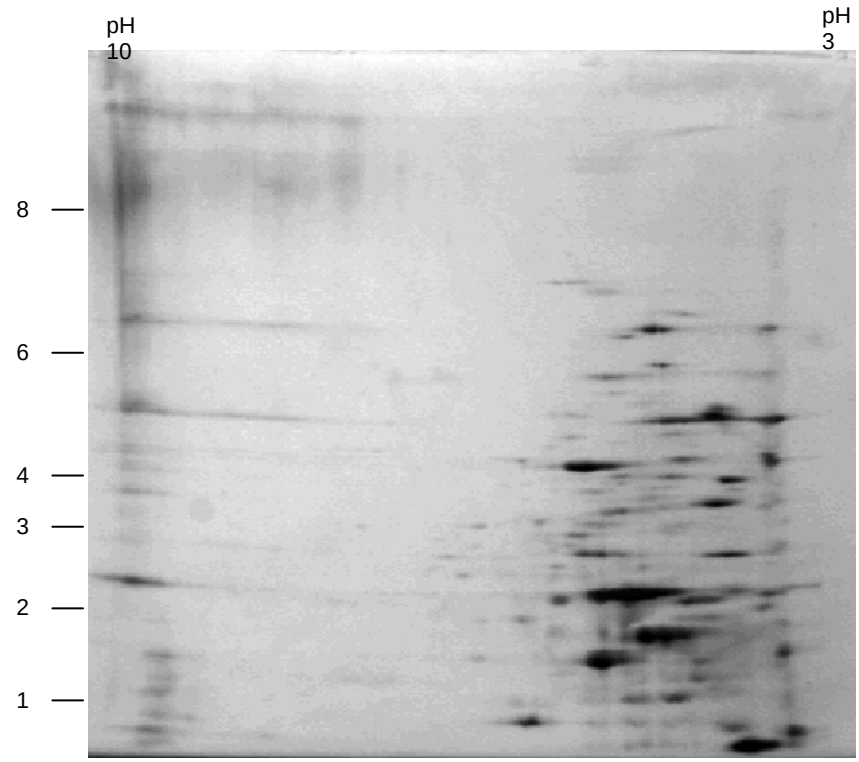

Figure B30 – 3: 2-DE protein gel of LiCl extract of *B. cereus* harvested from the bile culture 30 minutes after the shift.

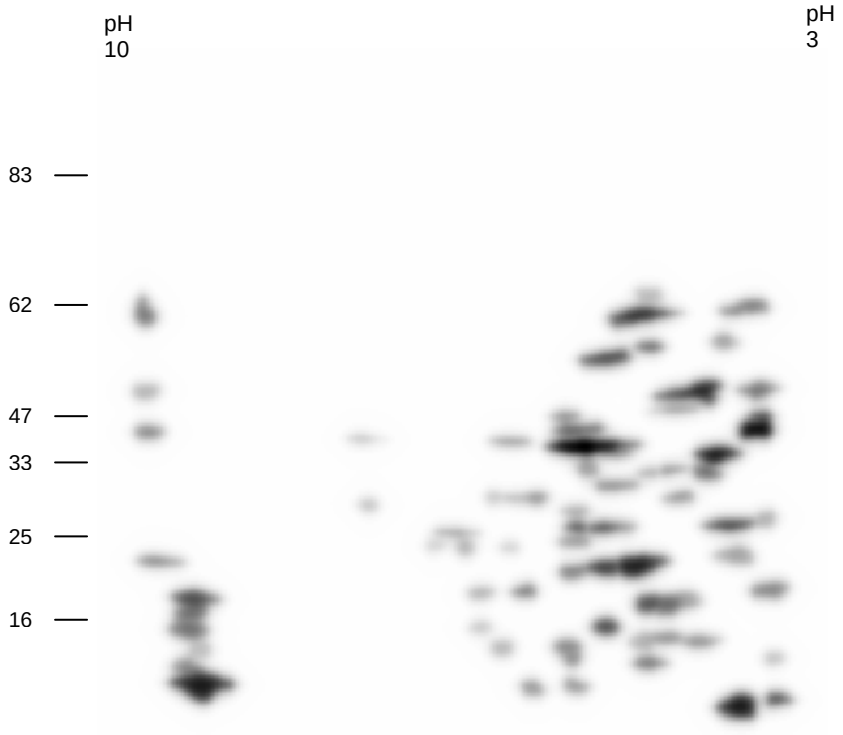

Figure B30 – synthetic: Synthetic 2-DE protein gel of LiCl extract of *B. cereus* harvested from the bile culture 30 minutes after the shift. The spot volumes are the average of B30 – 1, 2, and 3.

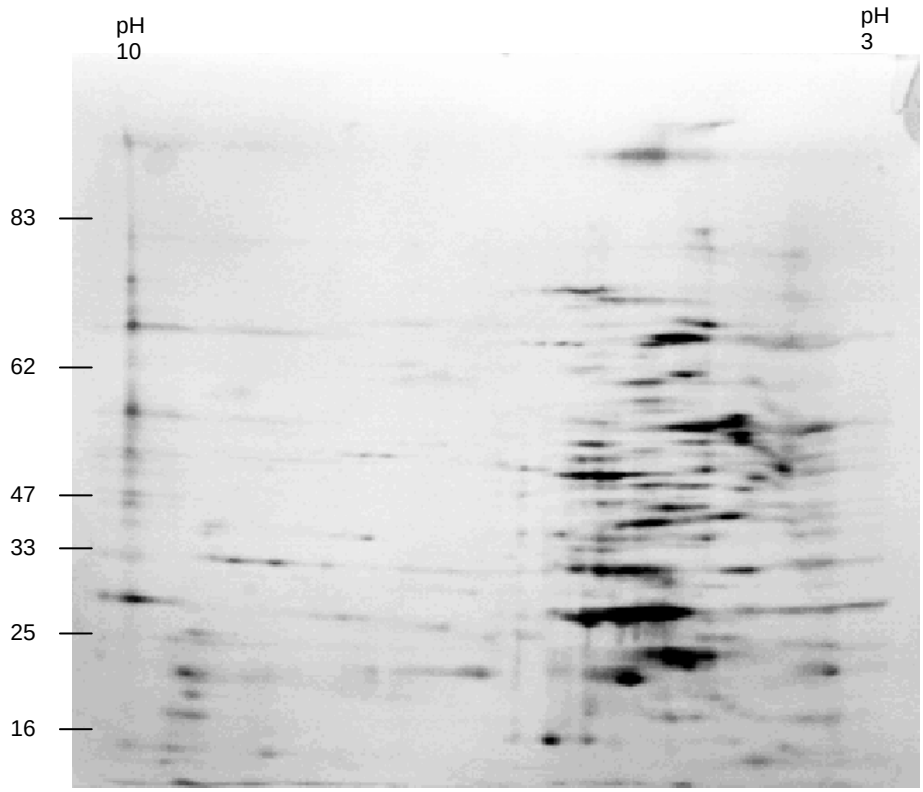

Figure C30 – 1: 2-DE protein gel of LiCl extract of *B. cereus* harvested from the bile salts culture 30 minutes after the shift.

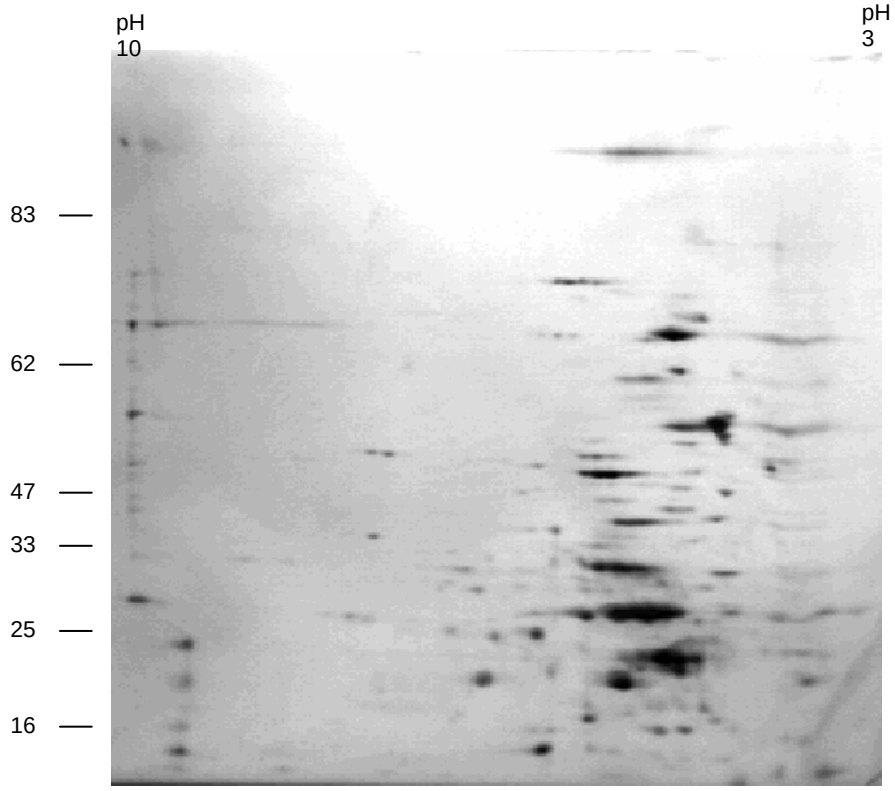

Figure C30 – 2: 2-DE protein gel of LiCl extract of *B. cereus* harvested from the bile salts culture 30 minutes after the shift.

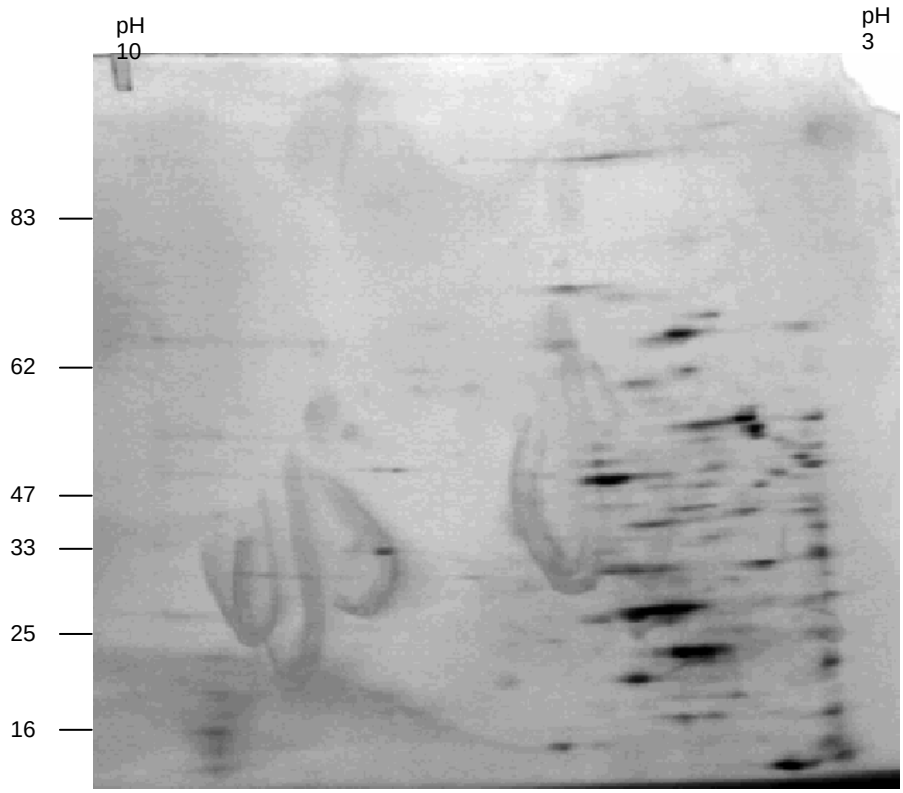

Figure C30 – 3: 2-DE protein gel of LiCl extract of *B. cereus* harvested from the bile salts culture 30 minutes after the shift.

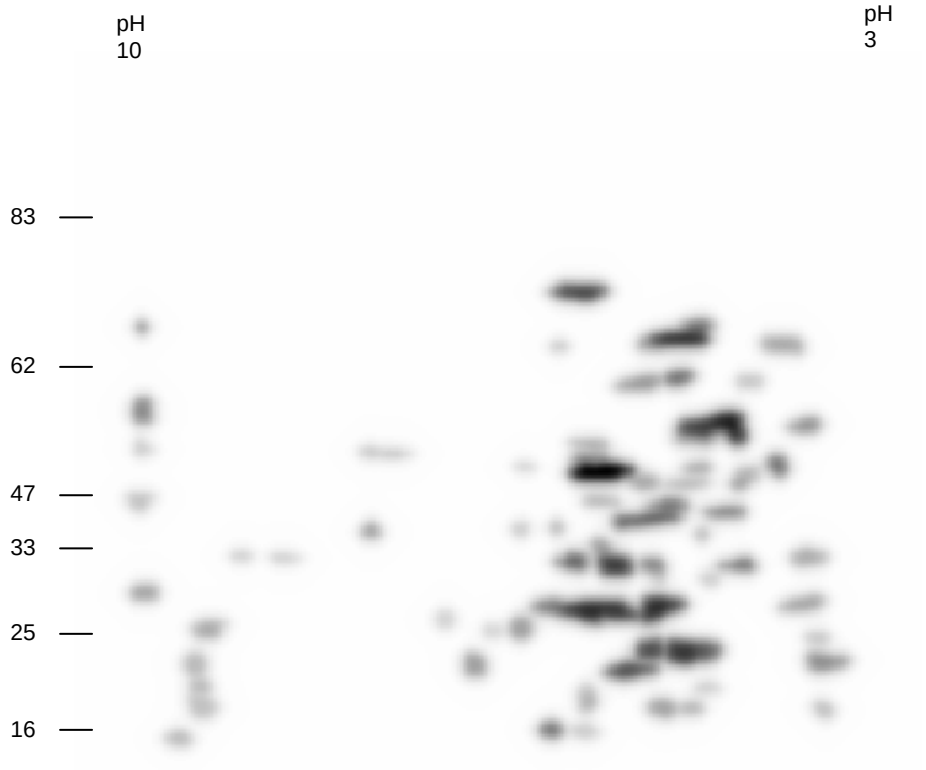

Figure C30 – synthetic: Synthetic 2-DE protein gel of LiCl extract of *B. cereus* harvested from the bile salts culture 30 minutes after the shift. The spot volumes are the average of C30 – 1, 2, and 3.