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Krista Laurila

Assessment of *sigK* Deletion on *Clostridium botulinum* Group II Type E Sporulation and Toxin Production

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Author	Krista Laurila
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The goal of this thesis study was to examine the effects of sigma factor K deletion on the sporulation process and toxin production of Beluga, a *Clostridium botulinum* group II type E strain. The many strains of *C. botulinum* differ from each other, and their genetic mechanisms are not very well known. The anaerobic bacterium produces both botulinum neurotoxin, which is the most potent toxin known to man, and spores that can survive and grow in food even after heat treatment. Botulism caused by the neurotoxin is a life-threatening and incurable disease. That is why knowledge of the species is important to prevent contraction of the disease. This project was carried out for the University of Helsinki's Faculty of Veterinary Medicine's Department of Food Hygiene and Environmental Health, in their *C. botulinum* -laboratory.

During this project, a *C. botulinum* group II type E Beluga strain, where the *sigK* gene had been removed, was studied in a laboratory for three periods of ten days, using different methods. The strain was compared to the wild type and a strain where the *sigK* had been removed from the chromosome and then added in a plasmid. Sampling and measurements were performed every 24 hours until 120 hours and the last measurements were done 240 hours from inoculation.

The laboratory work was executed using three different methods during the first weeks. The samples were photographed using a fluorescence microscope, their optical densities were evaluated by taking OD-measurements and the amounts of viable cells and heat resistant spores were investigated using MPN method. After that, ELISA tests were done to determine the samples' toxin concentrations and the microscopy pictures were examined to count the bacteria in different stages of sporulation.

Based on the results, it appears the absence of *sigK* in the Beluga strain affects negatively on the last stages of sporulation. The bacteria did not release most of their spores and they remained trapped inside of the cells instead. The maturation of the spores also seemed to have been incomplete. The population diminished noticeably over time, most likely due to the lack of viable spores. More toxin was found at different times compared to the wild type, but most of it was not secreted from the cells. The strain that was complemented with *sigK* in a plasmid reacted similarly, but the effects were not as drastic, indicating that total complementation was not achieved. The results gained from this study can be helpful in later studies regarding *C. botulinum*.

Keywords	*Clostridium botulinum*, *sigK*, sporulation, botulinum neurotoxin

Tekijä Otsikko Sivumäärä Aika	Krista Laurila *sigK*-geenin poiston vaikutus *Clostridium botulinum* -bakteerin ryhmä II tyyppi E:n itiönmuodostukseen ja toksiinin tuottoon 40 sivua + 6 liitettä 13.2.2020
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Opinnäytetyön tavoitteena oli tutkia sigmatekijä K:n poiston vaikutuksia Belugan, *Clostridium botulinum* ryhmä II tyyppi E -kannan itiönmuodostukseen sekä toksiinin tuottoon. *C. botulinum* -bakteerin useat kannat eroavat toisistaan, eikä eri kantojen geneettisiä toimintatapoja tunneta vielä kovin tarkasti. Tämän anaerobisen bakteerin erittämä hermomyrkky botuliini on myrkyllisin tunnettu toksiini, ja sen tuottamat itiöt voivat selviytyä ja kasvaa ruuassa jopa kuumennuksen jälkeen. Hermomyrkyn aiheuttama botulismi on hengenvaarallinen ja parantumaton tauti, joten lajin tuntemus on tärkeää sairastapausten ehkäisemiseksi. Projekti suoritettiin Helsingin yliopiston Eläinlääketieteellisen tiedekunnan Elintarvikehygienian ja ympäristöterveyden osastolle, *C. botulinum* -laboratorion tiloissa.

Projektissa tutkittiin laboratoriossa eri menetelmillä kolmen kymmenen päivän jakson aikana *C. botulinum* ryhmä II tyyppi E Beluga -kantaa, josta oli poistettu *sigK*-geeni. Kantaa verrattiin villityyppiin sekä kantaan, josta *sigK* oli poistettu kromosomista ja lisätty bakteeriin plasmidissa. Mittauksia tehtiin 24 tunnin välein 120 tuntiin asti ja viimeiset mittaukset tehtiin, kun viljelystä oli kulunut 240 tuntia. Työskentely suoritettiin suurelta osin 30 °C:ssa anaerobikaapissa.

Ensimmäisten viikkojen aikana laboratoriotutkimus toteutettiin kolmella eri menetelmällä. Näytteitä kuvattiin fluoresenssimikroskoopilla, OD-mittauksilla arvioitiin niiden optista tiheyttä ja MPN-menetelmällä selvitettiin elinkelpoisten solujen sekä kuumennuskäsittelyä sietävien itiöiden määrää. Sen jälkeen ELISA-testeillä määritettiin näytteiden toksiinikonsentraatioita ja mikroskoopilla otettuja kuvia tulkitsemalla laskettiin itiönmuodostuksen eri vaiheissa olevien bakteerien määriä.

Tulosten perusteella näyttäisi siltä, että *sigK*:n puutos Beluga-kannassa vaikuttaa negatiivisesti itiönmuodostuksen viimeisiin vaiheisiin. Bakteerit eivät vapauttaneet suurinta osaa itiöitä, vaan ne jäivät niiden sisään. Itiöiden kypsymisvaiheen kehitys vaikutti myös jääneen kesken. Bakteeripopulaatio pieneni ajan myötä huomattavasti, todennäköisesti elinkelpoisten itiöiden puutteen vuoksi. Toksiinia havaittiin villityyppiin verraten enemmän eri aikoina, mutta suurinta osaa siitä ei eritetty bakteerin ulkopuolelle. Kanta, johon *sigK* oli lisätty plasmidissa, reagoi samankaltaisesti, mutta vaikutukset eivät olleet yhtä voimakkaita. Tämä tarkoittaa, ettei geenin komplementaatio onnistunut täydellisesti. Saatuja tuloksia voidaan hyödyntää tulevissa tutkimuksissa *C. botulinum* -bakteeriin liittyen.

Avainsanat	*Clostridium botulinum*, *sigK*, itiönmuodostus, botuliini

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List of Abbreviations

σ^K	RNA polymerase sigma-K factor protein
ABTS	2,2'-azino-di-(3-ethyl-benzothiazoline-6 sulfonic acid) diammonium salt
BoNT/E	Botulinum neurotoxin type E
CRISPR	Clustered Regularly-Interspaced Short Palindromic Repeats
DAPI	4',6-Diamidino-2-phenylindole
ELISA	Enzyme-linked immunosorbent assay
FM4-64	N-(3-Triethylammoniopropyl)-4-(6-(4-(diethylamino)phenyl) hexa-trienyl)pyridinium di-bromide
kb	Kilobase, 2000 nucleotides in DNA or 1000 nucleotides in RNA
MPN	Most probable number
OD	Optical density
PBS	Phosphate-buffered saline
RT	Room temperature
SigK	RNA polymerase sigma-K factor protein
sigK	Gene responsible for the synthesis of RNA polymerase sigma-K factor protein
TPGY	Tryptone peptone glucose yeast extract broth

1 Introduction

Clostridium botulinum is an anaerobic bacterium, which produces dangerous botulinum neurotoxin. It can also produce endospores (hereafter called spores for simplicity), which means it can survive in extreme conditions. *C. botulinum* or its spores can be found, for example in vacuum-packed salmon or honey. The toxin is the most potent natural toxin known, so even small amounts can cause botulism, which leads to serious health issues, and even death. Infants and people with compromised immune system are especially in danger if they come in contact with *C. botulinum* or its spores. The toxin has a paralyzing effect on the nervous system and there is no known cure for botulism. Because of its paralyzing qualities, botulinum toxin can be used for medical and cosmetic purposes, when diluted and injected by a professional.

C. botulinum has been studied since it was discovered in 1895, and cases of botulism have been reported even before that [1]. Still the genetic mechanisms behind the sporulation process of *C. botulinum* are relatively unknown, and the goal of this project is to understand them a little better. This study is focusing specifically on the role of the RNA polymerase sigma factor K in the sporulation process. Different microbiological methods are used to determine, how the deletion of said sigma factor affects the release of the spore from the mother cell, and if the spore is released at all. This project was carried out in the University of Helsinki's Faculty of Veterinary Medicine, in the Department of Food Hygiene and Environmental Health.

2 *Clostridium botulinum*

Clostridium botulinum is a gram positive, rod-shaped and an obligate anaerobic bacterium. Four different groups of *C. botulinum* have been described, all of which have different phenotypic characteristics; however, they are classified as the same species for being able to produce botulinum neurotoxin. The growth temperatures of *C. botulinum* range between 3.3 and 45 °C, depending on the strain. The optimum temperatures also depend on the group, and range between 18 and 45 °C. Spores can survive and even germinate in temperatures below and over these ranges [2]. Spores can also survive in other challenging circumstances, like environments that are salty or range in pH. The

bacteria or its spores can be found for example in soil, marine sediments and in the gastro-intestinal track of fish, birds and mammals. [3; 4, p. 8–9; 5.]

2.1 Botulinum Neurotoxin

C. botulinum can produce seven different types of botulinum neurotoxin: type A, B, C, D, E, F and G. Toxin types A, B and E have been associated with human botulism. There have also been some cases where toxin types F and E produced by *Clostridium baratii* and *Clostridium butyricum* have caused human botulism. [4, p. 7.] In addition to these types of toxins, mosaic neurotoxins have been discovered. Examples of a mosaic toxins are C/D and D/C, which consist of both the toxin type C and D and possess unique characteristics that neither of their components have. [6; 7, p. 304.] The type of toxin the bacterium produces depends on the strain of *C. botulinum* in question. Below in table 1 are listed the groups and which toxin type they can produce:

Table 1. Toxin types produced by the different *Clostridium botulinum* groups.

***Clostridium botulinum* group**	**Toxin type**
I	A, B, F
II	B, E, F
III	C, D, C/D, D/C
IV	G
C. baratii	F
C. butyricum	E

As seen in table 1, some groups are able to synthesize multiple toxin types. Each type of botulinum neurotoxin is genetically different. Their molecular weights are similar, and their subunits share a similar structure. When the toxin is produced by the bacteria, it starts out as a single chain polypeptide. Before it reaches its full neurotoxic abilities, it must go through a two-step modification process in its tertiary structure. A disulphide bond is created between the toxin's heavy chain and light chain. Without this bond the toxin is practically non-functional. In mosaic toxins the heavy chain is from one toxin type and the light chain is from another. The toxin is released from the cell by bacterial lysis. [4, p. 8–10; 7, p. 304.] Botulinum neurotoxin can be destroyed by boiling for 5 minutes

or longer in a temperature above 85 °C [8]. The protein structure of toxin type E can be seen in figure 1.

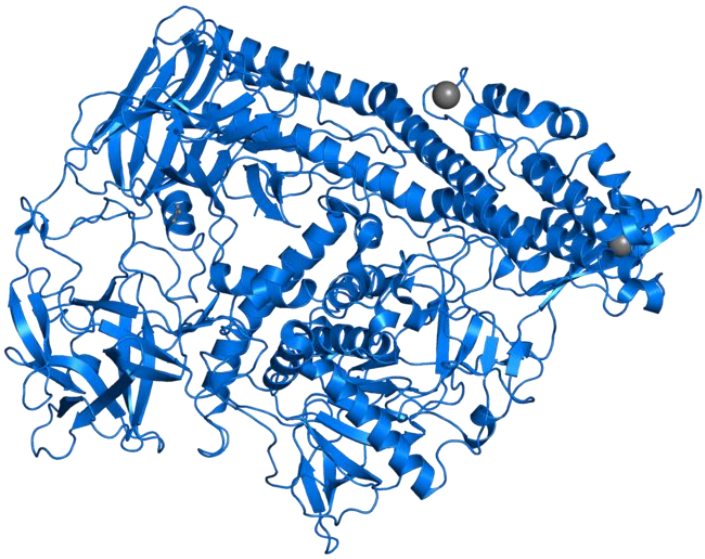


Figure 1. Botulinum neurotoxin type E. [9.]

There are different ways the neurotoxin has been utilized despite its hazards. It can be used in medical and cosmetic purposes. For example, it can be used on patients suffering from spastic muscular conditions. Some of these include torticollis, which is a neck-related muscle pain [10]; cervical and upper limb dystonia, which cause e.g. involuntary twitching and stiffness of the neck and upper limbs [11; 12]; childhood strabismus, which means the eyes are pointing in different directions [13] and apraxia of eyelid opening, which means the difficulty of voluntarily opening the eyelids [14]. [4, p. 10.] Patients suffering from Tourette syndrome can also be treated with botulinum toxin injections, which can reduce their possibly severe motor tic symptoms [15].

A common example of a cosmetic use of the toxin is the use of Botox to reduce facial wrinkles. Diluted toxin is injected in the client's specific facial muscles to relax them and make the muscles unable to contract. Since the movement of these muscles causes wrinkles, the client appears younger after the procedure, as their wrinkles get smoothed out. The effects of this procedure are temporary, and the treatment needs to be repeated when the effect wears out. [16.] One of botulinum neurotoxin's undesirable uses is chemical warfare. Because of its high potency, it can be used as an effective bioweapon. [4,

p. 10.] For this reason, facilities handling the toxin should be secured so possible bioterrorists cannot access their supplies.

2.2 Botulism

If a person encounters botulinum neurotoxin, they are in danger of developing botulism. Botulism is a non-contagious but serious disease, and in late stages lethal. There are different ways for the toxin or *C. botulinum* to enter the body: from ingestion, through a wound or through inhalation [17]. The lethal doses of botulinum neurotoxin are estimated to be only 0.03 µg orally, 0.09–0.15 µg intravenously and 0.8–0.9 µg by inhalation [18].

The most common way of contracting botulism is by ingesting contaminated food, such as vacuum-packed fish, meat or vegetables, fermented products and canned food [17]. Home-preserved and home-cooked foods, such as inadequately pasteurized vegetables are the most common sources of poisoning [4, p. 10]. There are two types of foodborne botulism: classic botulism and infant botulism [3]. Classic foodborne botulism is contracted when food consisting botulinum neurotoxin is consumed and the toxin gets assimilated in the gut. Healthy people will not be affected by spores.

Infant botulism affects babies under the age of one year. They are more susceptible to the bacteria or their spores, and the spores can grow and produce toxin in their guts, because babies that young have not yet fully developed their gastrointestinal microbiota. It is an extremely rare form of botulism, but still honey is not recommended for under one-year-olds, since it may include *C. botulinum* spores. [17.] Adults who have undergone abdominal surgery or recent antibiotic treatment, or are suffering from achlorhydria or Crohn's disease, may develop adult infectious botulism. Like in infant botulism, intestinal colonization ensues in these rare cases. [4, p. 6.]

Wound botulism occurs mostly with drug users. *C. botulinum* enters a deep wound either from the environment, or from a non-sterile needle when injecting intravenous drugs. [17.] Other causes of wound botulism can be e.g. surgical incisions, shotgun wounds and puncture wounds [4, p. 11]. The bacteria start to grow in the wound in anaerobic conditions and produce toxin, which is absorbed into blood circulation [19].

Inhalation of botulinum neurotoxin is rare, and the risk of developing botulism through inhalation is present mainly in laboratory environments, in which the toxin or bacteria are handled. The toxin gets aerosolized and can be inhaled, and then absorbed through the lungs. [4, p. 13.]

When the toxin has entered the body, and is absorbed into the blood circulation, it finds its way to the synapse where the nerve connects to the muscle. It binds to the membrane of the nerve cell and moves to the cytoplasm at the axon terminal. It blocks synaptic transmission at the excitatory synapses and as a result, the patient's muscles become paralyzed. [4, p. 15.] The first symptoms of botulism appear usually 12–36 hours after exposure. Possible symptoms include difficulty swallowing, speaking problems, blurred or double vision, vomiting and constipation. The paralysis usually starts in the face and moves down until it reaches the respiratory muscles. This causes the patient to be unable to breathe properly, and without medical assistance they will suffocate. Intensive care at the hospital is often needed and sometimes the patient must be connected to a medical ventilator, if they are already unable to breathe properly on their own. Botulism is treated with an antitoxin, which should be given in the early stages of illness, because it is only effective on circulating toxin and it will not affect any toxin that has already entered the nerves. [17.]

2.3 Sporulation Process

When examining the sporulation process of spore-producing bacteria, such as *Bacillus subtilis*, seven stages can be distinguished: axial filamentation, asymmetric division, engulfment, cortex synthesis, coat synthesis, maturation and mother-cell lysis. They are depicted below in figure 2. [20.] These stages are very similar for *C. botulinum*, so this model can be applied for the spore formation of *C. botulinum* as well [21].

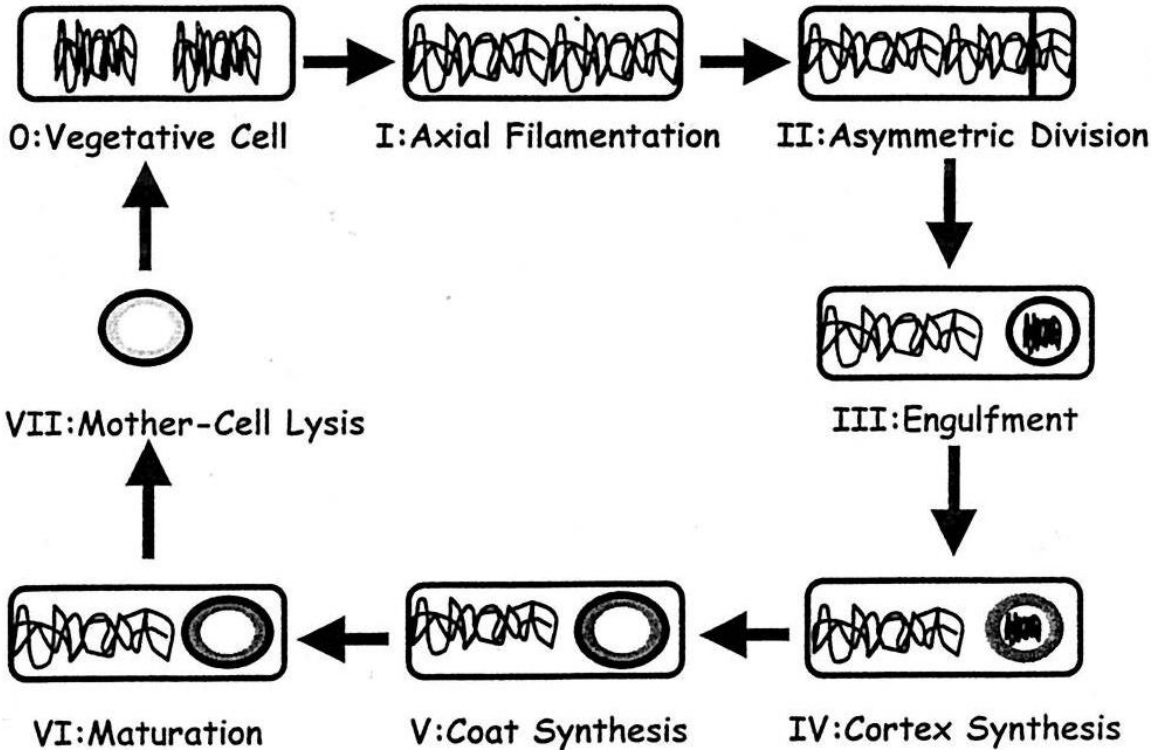


Figure 2. The seven stages of sporulation of spore-producing bacteria. [20.]

Stage 0 (figure 2) occurs when the cell is vegetatively growing and replicating its DNA. Before the DNA replication is ready, axial filamentation, the first stage of spore formation begins. During this stage, chromosomes form a structure stretching across the cell's long axis. Then during the second stage, asymmetric division, a septum is formed. It divides the cell into the mother cell and a smaller prespore. Only 30 % of the chromosome is in the prespore at this point, but the rest are being pumped inside it by a DNA translocase SpoIIIE. At stage III, engulfment, the prespore has separated into its own protoplast inside the mother cell. Two membranes envelop the prespore. During stage IV, cortex synthesis, the primordial germ cell wall and cortex are synthesized. Cortex is a specific form of peptidoglycan and is located between the prespore's two membranes. The next stage, V, is when coat synthesis occurs. The layers protecting the proteins around the prespore are called the spore coat, and they are depositioned during this stage. The prespore is dehydrated, which makes it appear bright when observed with a microscope. During stage VI, maturation, the prespore's resistance properties are developed before the mature spore is released during the final stage, mother cell lysis. [20.]

2.4 σ^K and Other RNA Polymerase Sigma Factors Involved in the Sporulation Process

There is not much yet known about the genetic mechanisms behind sporulation of *C. botulinum*. However, the genetic mechanisms of sporulation in the *Bacillaceae* family have been widely studied, especially the model organism *Bacillus subtilis* [20; 22]. There are four different RNA polymerase sigma factors that control the sporulation process of *B. subtilis* and other spore-creating bacteria. They are called sigma E, sigma F, sigma G and sigma K. Each of them has their own role in spore-formation, in either the mother cell or the prespore. σ^F and σ^G are active in the prespore, while σ^E and σ^K are active in the mother cell. Before the sporulation process begins, two sigma factors, σ^A and σ^H, and the master sporulation response regulator Spo0A, initiate the whole process. [20.] The genes that code the four sigma factors σ^E, σ^F, σ^G and σ^K are also found in *C. botulinum*, but their precise roles in its spore-formation are still unknown [22].

On *B. subtilis*, it was found that sigma F and sigma E become active after the second stage of sporulation, the asymmetric division. They are responsible for the engulfment stage and the synthesis of the other two sigma factors. σ^F initiates the synthesis of σ^G, while σ^E initiates σ^K's synthesis. σ^F is also a regulator gene and regulates all the other three sigma factors and three other sporulation-related regulator genes in one way or another. Sigma G and sigma K become active after the third stage of sporulation, the engulfment. They are responsible for the cortex and coat synthesis, spore maturation and mother cell lysis. If one step is not completed, the next will not occur. The prespore and the mother cell are communicating with each other using biochemical signals during the stages occurring after engulfment. This communication contributes to the coordination of the genetic programs during sporulation. [20.]

In a study done on *C. botulinum* ATCC 3502, which is part of *C. botulinum* group I, the role of σ^K in the stages of sporulation was studied. It was found that the absence of *sigK* gene in the strain prevented the bacteria from even beginning spore-formation. The study suggests that in the *C. botulinum* ATCC 3502, σ^K has an important role in early-stage sporulation. This was a different discovery compared to the studies on *B. subtilis*, where σ^K became active in the mother cell at a later stage, after engulfment [20]. During the late stages of sporulation in *B. subtilis*, σ^K regulates the formation of the spore coat. In

C. botulinum ATCC 3502 σ^K was found to possibly have a role in regulating the initiation of sporulation via the expression of spo0A and *sigF*.

The genetic structure of *sigK* in some *B. subtilis* strains includes an intervening 42-kb element that divides the gene into two parts. The element is called *skin*, and is thought to be an evolutionary remnant, because it does not seem to have any important roles on the functions of the bacteria. In clostridia, the *skin* is mostly absent excluding some *C. difficile* strains where it is an important element of sporulation. [22.]

3 Microscopy with Fluorescent Dyes

3.1 Fluorescent Dyes DAPI and FM4-64 as Tools of Analysis

Fluorescent dyes emit light, when excited with light [23]. They localize to specific parts of cells, which makes them helpful tools in examining the structures of cells with a fluorescent microscope. There are many different fluorescent dyes that bind for example to the nucleic acids, glycans or specific proteins [24]. In this study, two different dyes were used: DAPI, which binds to the DNA and FM4-64, which accumulates to the cell's plasma membrane. The chemical structures of the molecules are depicted below in figure 3.

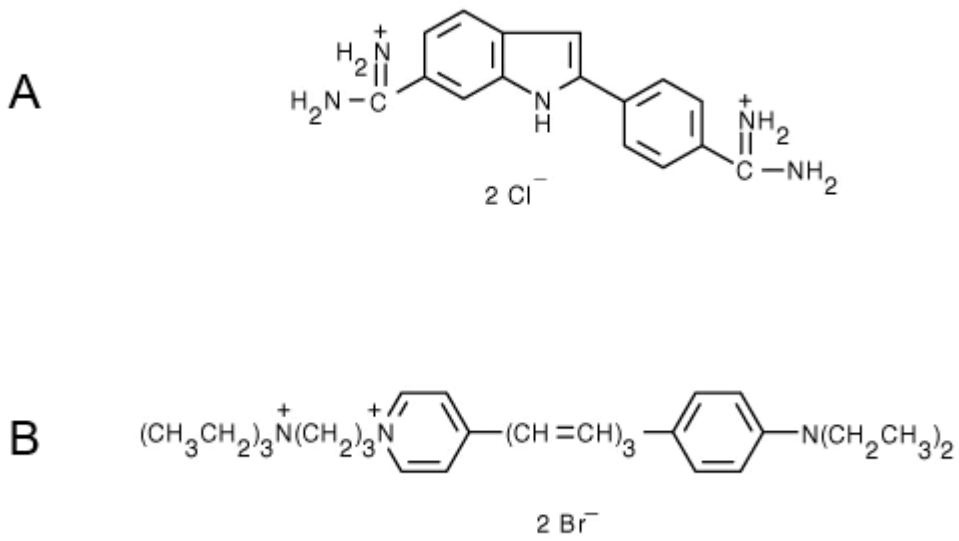


Figure 3. Chemical structures of the two fluorochromes used in this study. 4',6-Diamidino-2-phenylindole, Dihydrochloride, DAPI is shown above (A) and N-(3-Triethylammoniopropyl)-4-(6-(4-(diethylamino)phenyl) hexatrienyl)pyridinium dibromide, FM4-64 is shown below (B). Modified from [25; 26].

4',6-Diamidino-2-phenylindole, also known as DAPI, is commonly used as a fluorochrome in fluorescence microscopy. It binds to the DNA by attaching itself between the bases of DNA strands. [23.] When excited at 358 nm, it emits blue fluorescence at 461 nm. The DAPI used in this study has a dihydrochloride part and binds specifically to AT regions of DNA. [25.]

N-(3-Triethylammoniopropyl)-4-(6-(4-(diethylamino)phenyl) hexatrienyl)pyridinium dibromide is more commonly referred to as FM4-64 [27]. It is used as a fluorochrome to detect the plasma membrane of a cell. It can also be used to detect the vacuolar membranes of yeasts. It is lipophilic, so it gathers between the phospholipid layers of the cell membrane. It emits red fluorescence at 640 nm when excited at 515 nm. [28.]

FM4-64 dye was chosen for this study, because it gives important information about the stages of sporulation of bacterial cells. The early stages cannot be distinguished from each other by using only phase contrast. The septum that is formed at stage II and the plasma membrane around the early prespore from stage III forward become visible in

microscopic images when using this fluorescent dye. The later stages also become easier to spot with the additional information that the FM4-64 images give. For example, sometimes it is difficult to tell if a spore is really a spore or just an agar-crystal or an artefact. In the FM4-64 image spores usually appear similar to vegetative cells, but are short and round, whereas the crystals and artefacts are usually vaguer in shape.

DAPI dye was chosen for this study, because it gives information about the location of genetic material in the bacterial cells. When interpreting the microscopy images to determine which stage of sporulation the cells are in, it is helpful to see where in the cell DNA is located and how much of it is there. For example, when the cell is at stage II there is the same amount of DNA in both the prespore and mother cell. Because the chromosome in the prespore is packed in a more confined space, it gives a stronger fluorescent signal which can be seen in the image. It should be noted though, that at the beginning of stage II there is less DNA in the prespore, but it is being pumped inside. Another example of DAPI's usefulness in detecting the stage of sporulation is when the prespore is being enveloped with a primordial germ cell wall and cortex at stage IV. When the prespore has developed this far, DAPI cannot enter inside of it anymore. This leads to the DNA inside the prespore not being visible in the microscopic images, and instead there appears a "void" inside the cell where the prespore is. Germinating spores usually appear very bright in DAPI images which makes them easier to spot during counting. These can all be helpful indications of the sporulation-stage, if for some reason the phase contrast or FM4-64 images are unclear for some cells.

Examples of what the above-described cells might look like under the microscope can be seen in table 3 in chapter 6.2 Fluorescence Microscopy and Cell Counting.

3.2 Fluorescent Microscopy's Working Principle

Fluorescence microscopes use a high intensity light source to excite a fluorescent sample, which then emits light with a longer wavelength. The emitted light produces a magnified image. [29.]

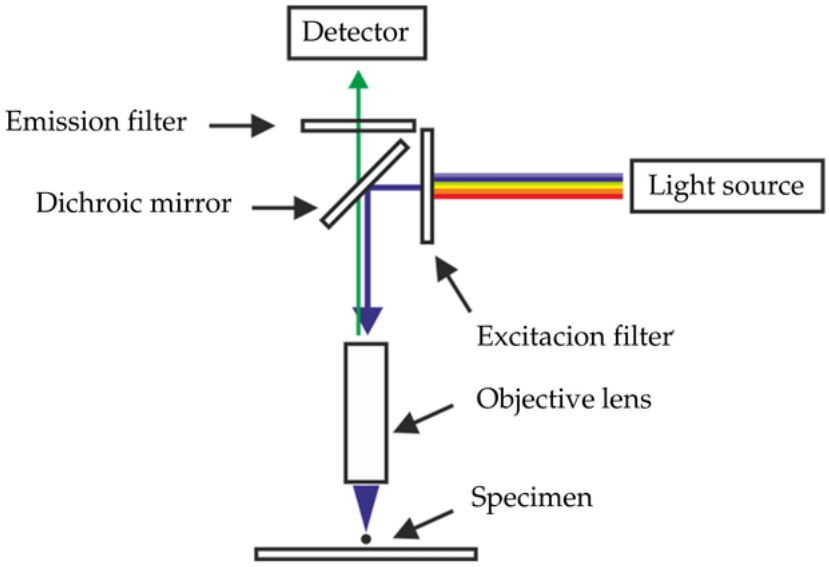


Figure 4. Diagram illustrating the basic structure of fluorescence microscopes. [30.] This figure shows an upright model, but inverted microscopes are also commonly used in fluorescent microscopy.

Figure 4 above illustrates how light travels through the different filters and lenses of a fluorescence microscope. The most common light sources used to illuminate the sample are mercury and xenon burners. First the light travels through an excitation filter, which filters out all the other wavelengths, except for the wavelength chosen according to the fluorescent material in the sample. This filtered radiation excites the electrons in the sample to a higher energy level. The electrons cannot maintain this for long, so they quickly relax to a lower level and begin to emit light at a longer wavelength than the excitation light. The fluorescence emitted by the sample goes through an emission filter, which filters out the excitation light and is then detected by a camera or is observed through an eyepiece. [29; 31.]

4 Most Probable Number -Method, MPN

The most probable number -method, also known as MPN, tells the estimated number of bacterial cells in the sample. The calculations are based on complex probability statistics, the understanding of which are not necessary for using the method as an analytical tool. It is widely used in food microbiology.

A dilution series, where the final dilutions are designed to give a negative result, is used. [32, p. 540.] For each sample, three replicates are needed. The figure below (figure 5) shows how the dilution can be done on a microwell plate:

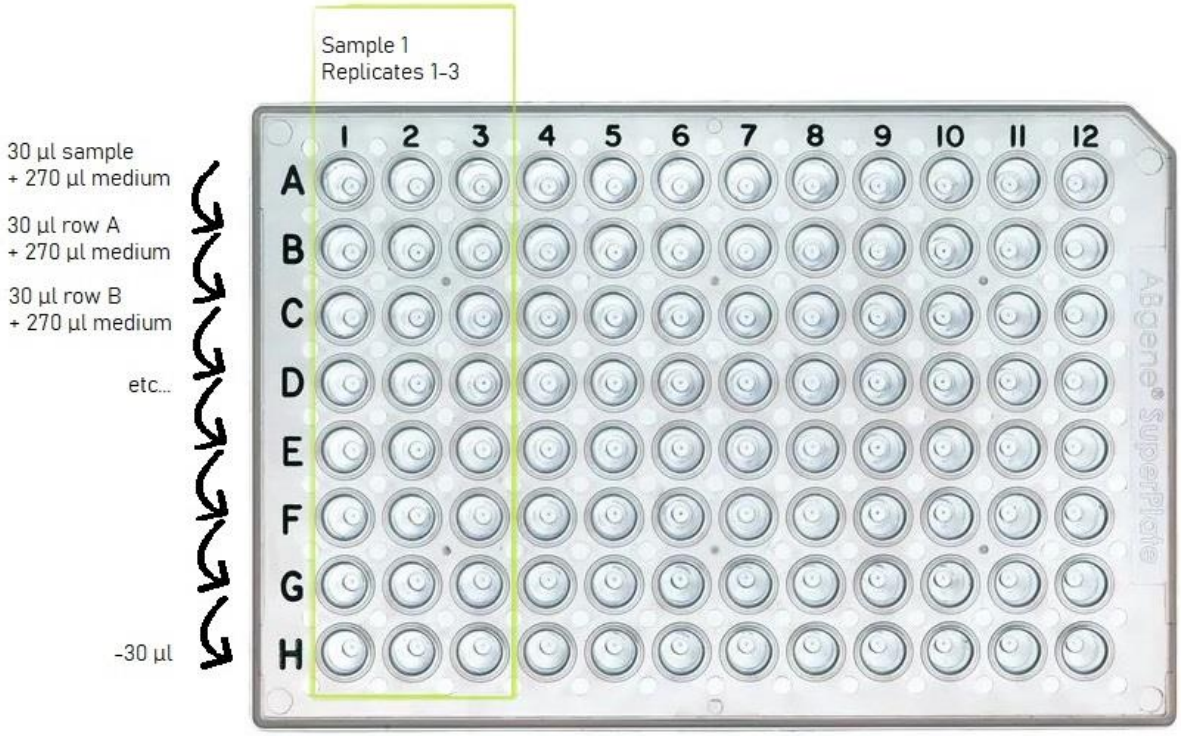


Figure 5. Demonstration of how the MPN sample is diluted on a microwell plate. Modified from [33].

In the plate, three adjacent columns are reserved for the same sample. 270 µl of medium, for example TPGY, is dispensed in all the wells used (A1-H3 in figure 5). In the first row (row A), three replicates of each sample are dispensed (wells A1, A2 and A3), 30 µl to each. Then from this row 30 µl of homogenized sample is dispensed to the next row (from row A to row B), using a multichannel pipet. This is continued until the last row (row H). After 30 µl of the sample in row G is dispensed to row H, 30 µl of suspension in row H is removed, so there is the same amount of liquid in each well. The plate is then covered and left to incubate for a minimum of 48 hours in an appropriate temperature and environment, depending on the bacterium in question.

After the incubation period, results can be read from the plate. This is done by examining in which wells growth has happened. The turbidity of the medium in the well indicates growth. Below (figure 6) is an example of how the results are calculated:

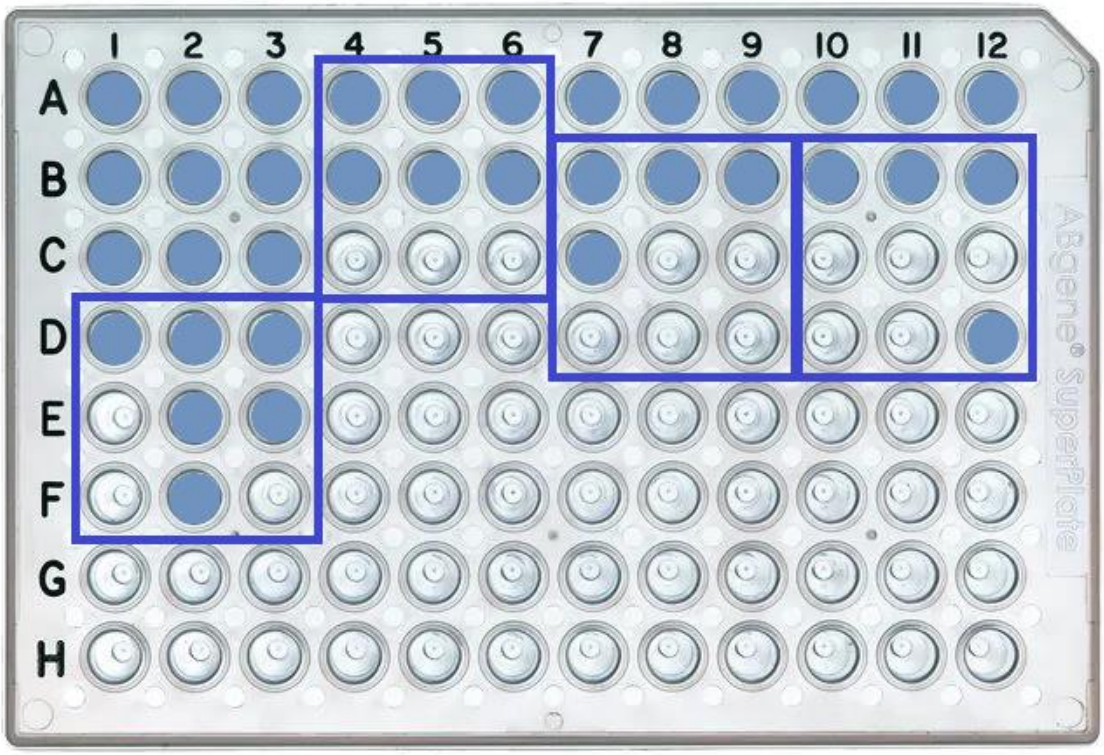


Figure 6. Example of calculating the results of an MPN plate. Modified from [33].

In the example figure (figure 6) the wells with growth in them are marked with turquoise color. When calculating the results, only three rows are taken into account per sample. This is done so the last row does not preferably have growth, and the first row has preferably three wells with growth. These rows are then turned into a three-digit number based on the number of wells with growth on each row. The first sample (columns 1-3) then gets the number 321. The second sample (columns 4-6) gets number 330 and third sample 310. The fourth sample either gets the number 301 or 330, depending on how the results are interpreted. It is rare, but possible that there is a gap-row in growth, but sometimes it could be due to contamination. If there are other similar samples in the same plate, they can serve as a clue on which interpretation to use.

The three-digit number is turned into an MPN number. The MPN numbers corresponding to each three-digit number are shown in Appendix 5. MPN Numbers. For example, 321 is turned to 1.50. The next step is to determine the power of the middle tube. The first sample's (figure 6) middle "tube" is row E. Row E is the 5th row, so the power of the middle tube is 5. [34.] The volume of the culture added to the first row is also needed. In this case it is 30 µl. The MPN is then calculated using the formula (1) shown below [35]:

$$MPN = \frac{1000 \times MPN\ number \times 10^{power\ of\ the\ middle\ tube}}{volume\ of\ culture\ added\ to\ 1st\ dilution\ (\mu l)} \quad (1)$$

The MPN number calculated is in the following form:

$$\frac{Most\ probable\ number\ of\ bacteria}{ml}$$

For example, in the first sample in figure 6, there are estimated to be 5 000 000 of bacteria cells per one milliliter.

5 Enzyme-Linked Immunosorbent Assay, ELISA

Enzyme-linked immunosorbent assay, also known as ELISA, is an antibody-antigen interaction -based method, used to detect and quantify specific particles, for example antibodies, proteins, peptides or hormones in a sample [36]. The method is sometimes called EIA, enzyme immunoassay. In this project it is used to detect BoNT/E, botulinum neurotoxin type E protein, which is the type of toxin produced by the *Clostridium botulinum* group II Beluga strain. It can also be produced by other group II strains and by *Clostridium butyricum* [4, p. 7]. There are different types of ELISAs, including direct assay, indirect assay and capture assay [36]. The ELISA type used in this study is indirect capture assay.

Capture assay ELISA is a highly sensitive and specific type of ELISA. In capture assay ELISA, a pair of antibodies, called capture and detection antibodies, are used to bind the sample's specific antigen in between themselves in a sandwich-like formation. That is why the method is sometimes called sandwich ELISA. The antibodies must bind to a

different part or epitope of the antigen, otherwise the detection is not accurate. The method begins by coating a microwell-plate with capture antibodies, unlike in direct and indirect assay ELISAs, where the plates are coated with the sample-antigen instead. Then the sample is added, and if it contains the desired antigen, it will bind to the capture antibodies. Then after the detection antibodies are added, they will also bind to the antigens captured by the capture antibodies. The detection antibodies bind to a different region of the antigens. They can have an enzyme attached to them, which makes the solution appear a specific color after binding, depending on the enzyme. The direct sandwich ELISA is illustrated below in figure 7.

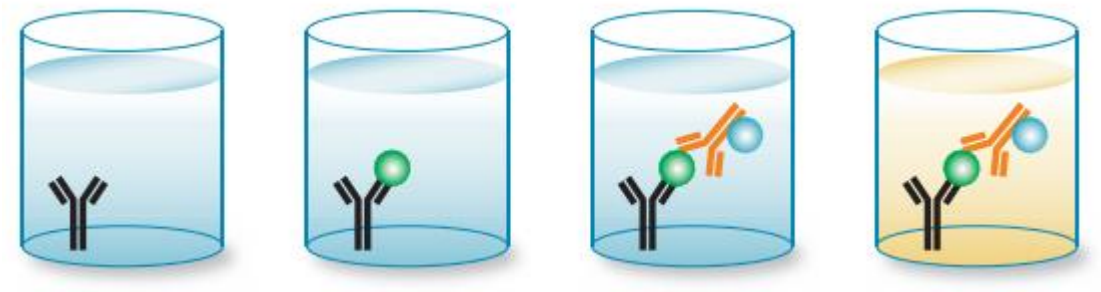


Figure 7. Direct capture assay ELISA. The capture antibody is black, and the detection antibody is orange in this figure. [37.]

Sandwich ELISA offers the choice of using either a direct or an indirect method. In direct sandwich ELISA the detection antibody is enzyme conjugated. In indirect sandwich ELISA the detection antibody is unlabeled and a secondary detection antibody, which is enzyme-conjugated, is added after the first detection antibody. [37.]

There are additional steps taken in the ELISA procedure in this study. After adding most reagents, the plate is incubated in room temperature and then washed. The washing removes any excess reagents that were not bound to the wells, antigens or antibodies. The detailed procedure is explained in chapter 6.5 ELISA. After the capture antibodies have been added, a blocking buffer is added to block any free protein-binding sites on the wells' surface. After all the antibodies have been added, a detection substrate is added. [38.] It binds with the previously added Poly-HRP antibodies, which appears green and can be measured with a spectrophotometer [39].

6 Methods

6.1 Strains, Growth Conditions and Sampling

All strains used in this work are described below. All constructed strains are derived from the *C. botulinum* group II Beluga strain. For this work, strains were grown and manipulated inside a Don Whitley anaerobic chamber, with 85% N_2, 10% CO_2, and 5% H_2 atmosphere. All strains grew at 30 °C, in anaerobic medium, either TPGY, for the growth of overnight cultures, or Meat agar, for the sporulation experiment itself. Overnight cultures are small volume cultures, usually 10 ml of liquid media in a glass tube, used to "revive" the strain and obtain a sufficient number of cells to use as the experiment starter inoculum. Strains were stored either as a spore stock at 4 °C or in 15% glycerol at -80 °C.

Three strains were used to evaluate the role of SigK in *C. botulinum*:

1. **Beluga**: Wild type *Clostridium botulinum* group II type E

2. **Beluga Δ*sigK***: *Clostridium botulinum* group II type E, where the *sigK* gene has been removed using CRISPR-Cas9 technology

3. **Beluga Δ*sigK* complemented**: Beluga Δ*sigK* strain, where the *sigK* gene has been inserted back in the strain in a plasmid.

All these strains are lab collection strains, as the Beluga strain is routinely used at the University of Helsinki's *C. botulinum* -laboratory as a group representative strain, and both genetic constructs have been prepared ahead of this work.

There were three replicates done of each of the strains, which were inoculated and sampled during a three-week period; one week for each replicate. In other words, the samples were inoculated once a week 24 hours before the first measurements: first replicates of each three different strains were inoculated for week one, second replicates were inoculated for week two, and the last replicates were inoculated for week three. The strains were inoculated into previously prepared meat agar mediums. The bottles containing the

cultures were handled inside an anaerobic chamber in 30 °C for the entire sampling period. Most of the sample preparations were carried out in the chamber, but some could be done outside of it.

Sampling was done every 24 hours for five days. After that the cultures were still left inside the anaerobic chamber until the last sampling was done after 10 days had passed from inoculation. The cultures were then destroyed by autoclaving. Below in table 2 are shown the sampling points:

Table 2. Sampling points and actions taken during them.

Time point (h)	Action
-24	Inoculation of all three strains' overnight cultures
0	Inoculation of all three strains and preparing the blank for OD-measurements
24	Sampling (microscopy, OD, MPNs, ELISA samples)
48	Sampling
72	Sampling
96	Sampling
120	Sampling
240	Sampling and destruction

During the sampling period, we used five different methods: fluorescent microscopy, measurement of optical density, MPN of viable cells and spores and ELISA. All of these were performed during the measurement points, except the ELISA tests, for which the samples were taken, prepared and stored at -80 °C until after every replicate had been sampled.

Details about mediums that were prepared and used can be found in Appendix 1. Compositions of Growing Mediums and Agarose Gel. Information about the equipment, reagents and ELISA reagents used during the study can be found in the following appendices: Appendix 2. Machines and Equipment, Appendix 3. Reagents and Appendix 4. ELISA Solutions.

6.2 Fluorescence Microscopy and Cell Counting

Samples were taken, at every timepoint described earlier (table 2), from the cultures. First the cultures were homogenized by agitating the bottles, and then 100 µl of culture was pipetted into marked Eppendorf tubes. From this point forward the collected samples were kept protected from light and oxygen as much as possible, because the fluorescent dyes added next are sensitive to light, and the cells themselves are sensitive to oxygen.

1 µl of FM4-64 and 1 µl of DAPI dyes were added and suspended into the samples using a vortex. After incubating the samples for around 2–3 minutes, they were spun down with a spinner centrifuge for 3 minutes at approximately 6 000 rpm. The supernatant was discarded, and the pellet was suspended in 1 ml of anaerobic PBS. The samples were spun down again as previously, and the supernatant was discarded. 1–10 µl of PBS was added if needed.

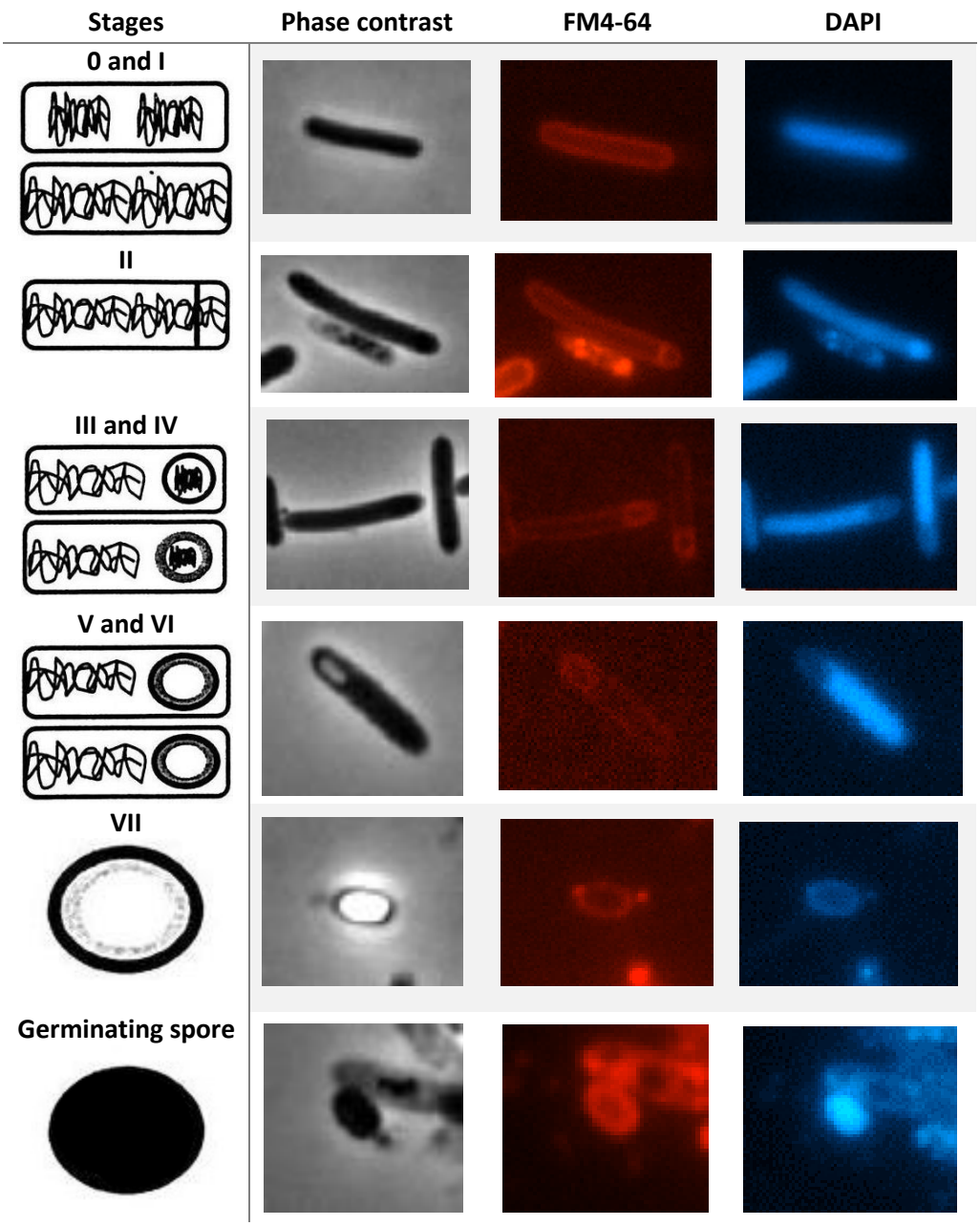
The rest of the preparations were done outside of the anaerobic chamber. Microscopy slides were prepared by adding around 800 µl of melted agarose gel evenly on a glass microscope slide. Another slide was put on top of the agar and after a few minutes, when the agar had solidified, the other slide was removed. A maximum of 3 µl of sample was mounted on the agarose-covered slide, and a cover slip was placed on top of it. The samples were still protected from excess light.

A drop of immersion oil was placed on the ready-made sample's cover slip and the sample was placed on a fluorescent microscope which was set to capture multiple wavelengths: phase contrast, FM4-64 and DAPI. A 100X objective was used when observing the samples and the microscope was covered with a hood to protect the samples from light. Each sample was photographed from at least five different "locations", and the three different wavelengths were used to get three different photographs from each photographed area.

The photographs were later analyzed using MetaMorph® software. All three pictures taken from the same location of a sample were opened next to each other, and the cells at different stages of sporulation were counted manually. When determining the stages of sporulation the cells are in at different time-points, the model from figure 2 was used

as a guide. Some of the stages are difficult to distinguish from each other, so they are counted into the same category in this study. The stages are counted as shown in the table 3 below. Table 3 also shows examples of what each stage looked like on the microscope. FM4-64 dye has colored the plasma membrane and DAPI has colored the DNA.

Table 3. Stages of sporulation under the microscope with three different filters. Illustrative drawings of the stages are modified from [20].



6.3 OD-measurement

The optical density of the samples was measured at each time point. This was done by first adding 300 µl of the culture into a disposable cuvette made of optical polystyrene, that contained 600 µl of 10 % formalin. The cuvettes' tops were covered with Parafilm® and then taken out of the anaerobic chamber for measurement.

The samples' absorbance values were measured with a spectrophotometer at 600 nm. A solution of 600 µl of 10 % formalin and 300 µl of TPGY was used as a blank. Formalin inhibits contamination of the TPGY, allowing for the use of the same blank was during the whole 10-day follow-up period of one replicate.

The final OD values were calculated by multiplying the measured OD by three, since the samples were diluted to 1/3.

6.4 Determination of Viable Cells and Spore Counts

The MPN test was performed to determine the number of viable cells and spores of *C. botulinum* in the samples at each time point. Samples for both are taken from the same culture at the same time. However, the sample for the spore counts is heated at 60 °C for 20 minutes. The spores survive this treatment, but the viable cells will die. MPN for viable cells and spore counts were performed at the same time for each sample.

The general idea of how the MPN test is performed has been explained in chapter 4 Most Probable Number -Method, MPN. The preparation of the microwell plates was done inside the anaerobic chamber where the sample-bottles were kept. The plates (96-well microwell plates) were filled with 270 µl of anaerobic, 30 °C TPGY in each well. For determination of the viable cell counts 30 µl of culture was dispensed in the wells of the first row of the plate, three replicates of each sample. Before pipetting the samples, the sample-vessels were first agitated. While pipetting, the sample was suspended to the TPGY in the well by pipetting up and down a few times. New pipette tips were used for each dilution in the dilution series. Once finished with the dilution series, the plate was

covered with a clear adhesive tape and incubated in the anaerobic chamber for a minimum of 48 hours.

Before the spore test samples were dispensed to the microwell plate, some preparation was done. 120 µl of each culture was collected into Eppendorf tubes. The tubes were taken out of the anaerobic chamber and heated in a 60 °C water bath for 20 minutes. After the heating, the spore test was carried out identically to the MPN test. Because spores are resistant to a variety of factors in their environment, including oxygen, taking the samples outside of the chamber during preparations does not affect their viability.

The results were read and calculated after 48 hours as described in chapter 4. Since the first dilution on the first row is more potent, it might sometimes appear slightly more turbid than the others even without growth. If the turbidity is not significant, the wells can be interpreted as not having growth.

6.5 ELISA

During the follow-up period, ELISA-samples were collected at each time point. 1 ml of each sample was pipetted into Eppendorf tubes and taken out of the anaerobic chamber. The tubes were centrifuged at 3 000 rpm for 10 minutes. The supernatant was separated from the pellet into another tube, and both the pellet and supernatant were stored at -80 °C.

After every sample was collected, the ELISA was performed to determine at which time points the samples had BoNT/E in them, and how much. For the test, 96-well microwell plates with special coating on the wells were used. The coating helps the reagents to stick to the wells better compared to a regular microwell plate. First the wells were coated with 50 µl primary antibody KE97, capture antibodies, at a dilution of 3.52 µg/l in PBS. If the antibody solution did not cover the wells evenly, the plate was tapped gently until they covered the whole bottom of the wells. The plate was sealed and incubated overnight at 4 °C. The plate sealing was done for every incubation period.

Next day, the wells were emptied of liquid by shaking the plate upside down over an appropriate waste container. The remaining drops were dried by tapping the plate onto

a pile of ELISA tissues. Then the wells were washed with a washing buffer of PBS and 0.1 % Tween20. Every washing during the procedure was done using this buffer. The washing was performed by pipetting 300 µl of washing solution per well, careful not to disturb the coating at the bottom and edges of the wells with the pipette tips. The plate was then emptied as described above. This was repeated four times.

Blocking of the plate was then performed by adding 200 µl of casein buffer to each well and incubating it at room temperature for one hour.

The frozen samples were melted in a RT water bath. The pellet samples were resuspended in 1 ml of sterile PBS, in order to return them to their native concentration. The samples were diluted in antigen-dilution buffer as shown in table 4, depending on the time point and sample type.

Table 4. Dilution of ELISA-samples.

Time point (h)	Dilution of pellet	Dilution of supernatant
24	1:2000	1:1000
48	1:1000	1:2000
72	1:1000	1:2000
96	1:500	1:2000
120	1:500	1:2000
240	1:500	1:2000

The dilutions shown in table 4 are too large to dilute at once, so the following dilution series (table 5) was used:

Table 5. Dilution series of ELISA-samples.

Dilution	Sample volume / dilution	Antigen-dilution buffer volume
1:10	30 µl / 1:1	270 µl
1:100	30 µl / 1:10	270 µl
1:500	60 µl / 1:100	240 µl
1:1000	30 µl / 1:100	270 µl
1:2000	150 µl / 1:1000	150 µl

The standard solutions were also prepared. Table 6 shows the concentrations of the standards, and how they were diluted. The most potent dilution (1000 ng/ml) was made

by diluting 3 µl of 0.5 mg/ml stock solution of BoNT/E into 1497 µl of antigen-dilution buffer.

Table 6. Concentrations and the dilution of the standard solutions.

STD (ng/ml)	Volume / STD	Antigen dilution buffer volume
1000	3 µl / 0.5 mg/ml stock	1497 µl
100	30 µl / 1000	270 µl
10	30 µl / 100	270 µl
1	30 µl / 10	270 µl
0.1	30 µl / 1	270 µl
0.01	30 µl / 0.1	270 µl
50	150 µl / 100	150 µl
25	150 µl / 50	150 µl
12.5	150 µl / 25	150 µl
6.25	150 µl / 12.5	150 µl
3.125	150 µl / 6.25	150 µl
1.5625	150 µl / 3.125	150 µl

After the incubation time for the blocking, the plate was again washed four times. The samples and standards were pipetted on the plate, 50 µl per well, so that both the positive and negative capture antibody rows had the same samples. For example, there were the same standards in both the first and second row, the same samples in both the third and fourth row and so on. To make this phase faster, the samples were first pipetted on a separate microwell plate, to every other row. Then it was easy to pipette each row using a multi-channel pipette. The ELISA plate was incubated at RT for two hours.

The plate was washed four times before the addition of secondary detection antibody. For the positive detection rows 50 µl of E136/5/3-QM-bio, at a dilution of 1:80 in casein buffer was dispensed to each well. For the control rows (negative capture) 50 µl of R109/3/1-QM-bio, at a dilution of 1:100 in casein buffer was dispensed to each well. The plate was incubated at RT for one hour.

The plate was again washed four times before the development phase. To each well 50 µl of PolyHRP40, at a dilution of 1:5000 in casein buffer were dispensed per well. The plate was incubated at RT for 30 minutes. This time the plate was washed eight times.

100 µl of ABTS was added per well. The plate was incubated at RT for one hour, covered with foil to protect it from the light.

The next step was measuring the OD of the samples at 405 nm and 492 nm with a spectrophotometer for microwell plates. At this point the samples with BoNT/E were already visible, indicated by a green color. The OD-measurements at 492 nm were subtracted from the measurements at 405 nm, and a linear standard curve was created. With the equation of the curve and the dilution factors, the amounts of BoNT/E in the samples were calculated.

7 Results and Discussion

The results obtained from this study are presented and discussed below. Additional tables and graphs of the results can be found in Appendix 6. Details of Results.

7.1 Microscopy Results

The average percentages of bacteria cells in different stages of sporulation for each three strains are shown in figure 8 below:

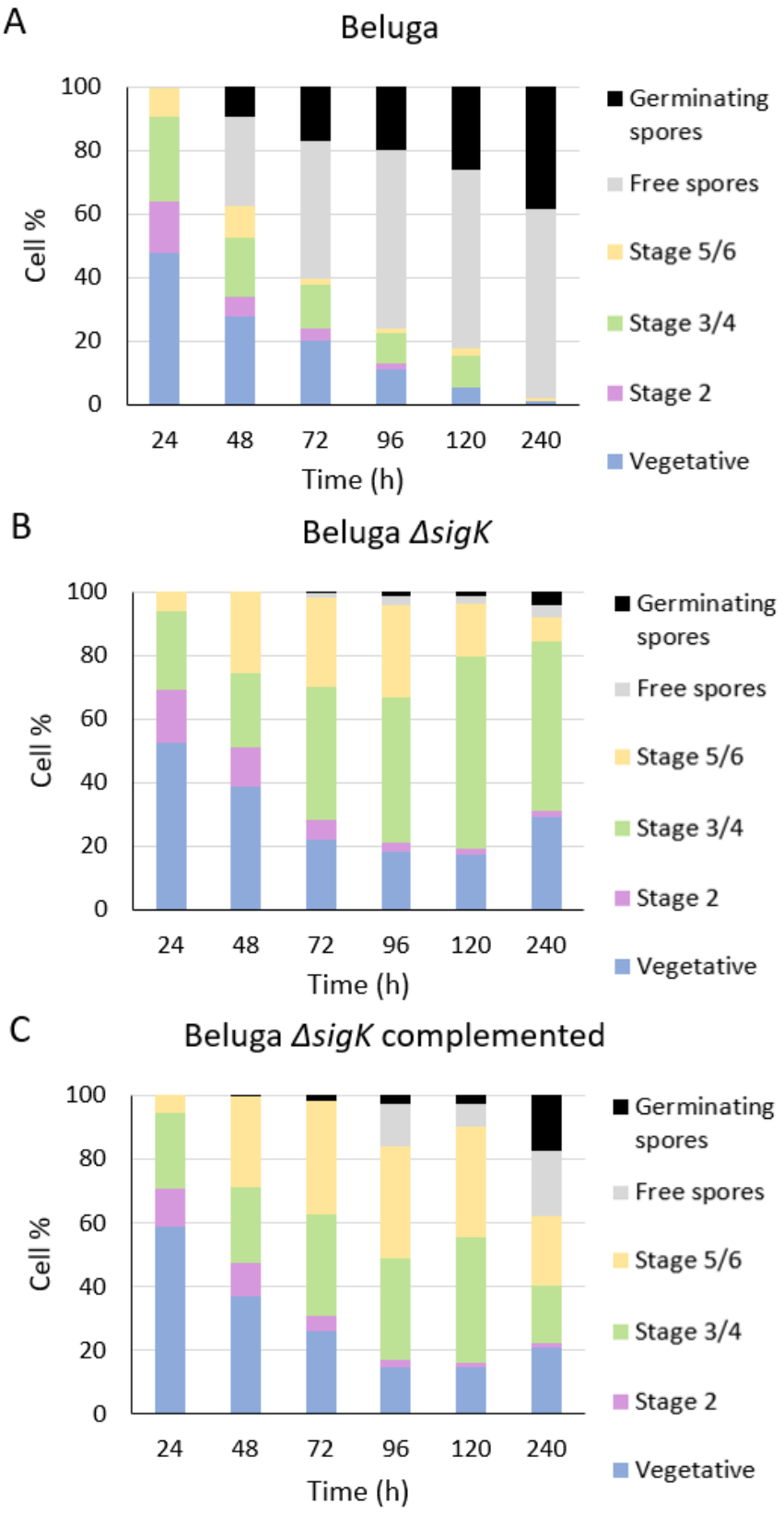


Figure 8. Cells in different stages of sporulation. The cell percentages of wild type Beluga strain are shown above (A), Beluga *ΔsigK* below that (B) and Beluga *ΔsigK* complemented at the bottom (C).

There are very little free spores in the Beluga *ΔsigK* samples (B) at any time point, less than 4 % of the total amount of cells at most (figure 8). When the amount is compared to that of the wild type (A), which has at most almost 60 % at 240 hours, it is easy to see the difference. There is a significantly larger percentage of cells at stages 5 and 6 in the Beluga *ΔsigK* strain (B) than in the wild type (A), except at 24 hours. It seems that removing *sigK* from the genome of *C. botulinum* group II type E makes the bacteria unable to release their spores and they stay trapped inside the cells. Only a few bacteria can release their spores in these samples (B). It is difficult to say why some are still able to do it. They could for example have gained the ability through mutation, or it means that the *sigK* gene is not essential for the bacteria to release the spores in some cases. If the spore has developed fully inside the mother cell, maybe some of them can survive after the mother cell has died. The spores appeared less bright in the Beluga *ΔsigK* samples when compared to the wild type's spores, though. This indicates that the maturation was incomplete.

Insertion of a plasmid carrying the *sigK* gene in the Beluga *ΔsigK* complemented strain (C) has not achieved a complete complementation. The strain cannot produce as many spores as the wild type. There are around 20 % of free spores and 17 % germinating spores at most in the Beluga *ΔsigK* complemented samples (C), which is more than in the Beluga *ΔsigK* samples (B), but also noticeably less than in the wild type samples (A). When it comes to the percentage of stages 5 and 6, a similar trend is seen than in the Beluga *ΔsigK* strain, where a big percentage of the cells are at those stages even at the later sampling points. This indicates that in the complemented strain there are also problems with mother cell lysis and spore release, though not as big as in the Beluga *ΔsigK* strain. The reason for this could have been that the complementation was plasmid-based and needed antibiotics, and that could have caused a delay and the sporulation may not have been completed during the follow up period.

7.2 OD Results

The average optical densities of each three replicates of each sample-type are illustrated in figure 9, where the standard deviations are also visible.

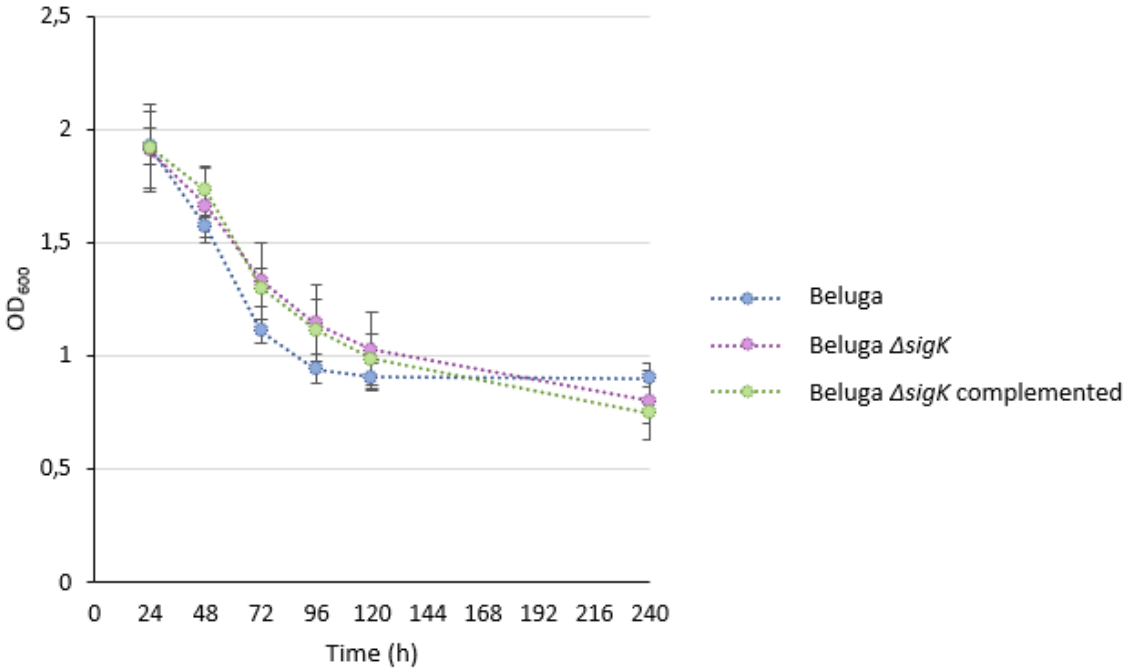


Figure 9. The average growth curves of each strain and their standard deviations at each sampling point.

As seen in figure 9, the optical densities of each sample-type at the first sampling point are around 1.9, and the ODs go down from there. By the last sampling point, the OD is only at around 0.75 to 0.9, depending on the strain. The growth curve is declining, because the bacteria have already entered the death phase when the first measurements are performed. The reason the first measurements were done at 24 hours, after the exponential growth, is because previous work on these strains have showed no difference on the results before 24 hours of growth [40].

Figure 9 shows, that the Beluga *ΔsigK* and Beluga *ΔsigK* complemented strains have almost identical growth curves. The growth curve of the wild type beluga strain differs from the two by the ODs being lower than the others, until the ten-day sampling point, when the ODs have not continued to decline almost at all unlike in the other strains.

Otherwise the growth patterns seem to be quite similar for each strain. The optical density of the wild type is declining faster, because the bacteria are lysing upon cell death. The other strains' bacteria are dying slower, because they are not releasing their spores or releasing them less, therefore the lysis occurs later. The reason why the OD of the wild type is higher at the last measuring point compared to the modified strains is, that the spores either survive or start germinating, while in the other two strains the bacteria mostly keep dying instead since there are significantly less spores released.

7.3 MPN Results for Viable Cells and Heat Resistant Spores

Figure 10 shows the average amounts of viable cells of each strain and their standard deviations. The MPNs show that the viable cells in Beluga *ΔsigK* and even Beluga *ΔsigK* complemented samples keep decreasing quite steadily compared to the wild type. The number of viable cells in wild type seems to stay about the same during the whole follow-up period and it even goes up at times.

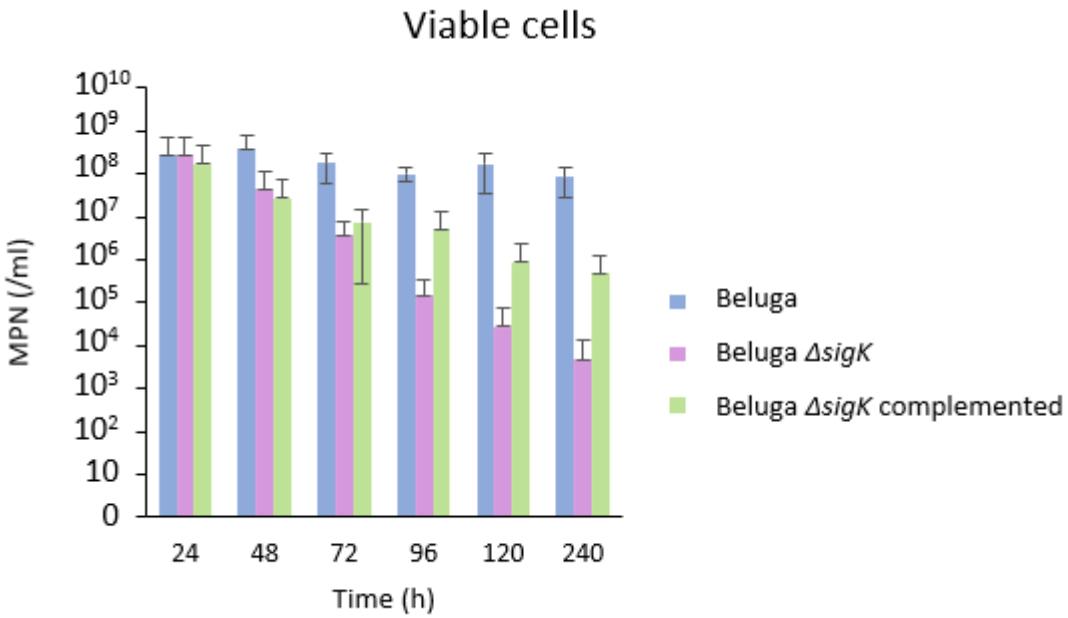


Figure 10. Most probable number of viable cells per milliliter in each strain at the follow-up time points. Note that the scale is logarithmic. It should also be noted that the spores are included in the MPNs. The error bars represent standard deviations.

The viable cells in the Beluga *ΔsigK* strain decrease the most (figure 10), and at the ten-day sampling point the MPN of the strain is only about 0.006 % of the MPN of the wild type. This would suggest, that because the bacteria cells are not able to release their spores the culture is unable to reproduce as much as the wild type. When the spore gets stuck inside the mother cell, it might not be able to germinate and become a new individual. The complemented strain shows a similar pattern, but the death rate is not as dramatic. This is probably because the strain is able to release some of its spores and they are able to germinate. The MPNs of the Beluga *ΔsigK* and Beluga *ΔsigK* complemented strains are very different, but their ODs (figure 9) are not that different from each other. The big differences in MPNs but not in ODs are probably due to the fact, that in the viable cell MPNs the heat resistant spores are included in the results.

Figure 11 shows the average MPNs of the heat resistant spores. In the Beluga *ΔsigK* samples no growth occurred after the heat treatment. This could mean that the spores observed with the microscope were either not as resistant to heat as the spores produced by strains that had the sigma K factor in their genome, or the spores might not be capable of germinating. Their poor heat resistance might have been due to not fully completing the maturation stage, which was speculated in chapter 7.1 Microscopy Results. The amounts of heat resistant spores in the Beluga *ΔsigK* complemented strain are not as high as in the wild type. The amount of heat resistant spores in the complemented strain samples remained around the same MPN the whole study. There was a peak at 72 hours but after that the MPN returned to the previous levels of around half a million. The MPNs of heat resistant spores are clearly the highest in the wild type samples. At most, there are about 550 000 000 spores at the 120-hour sampling point. The microscopy results showed no free spores at 24 hours for both the wild type and the complemented strain, but the MPN results showed that there were. That is because if the spores have matured, they will be heat-resistant even if they are still inside the mother cell. When the bacteria are heated, the mother cell will lyse but the spore will still be viable, and it will germinate. [41.]

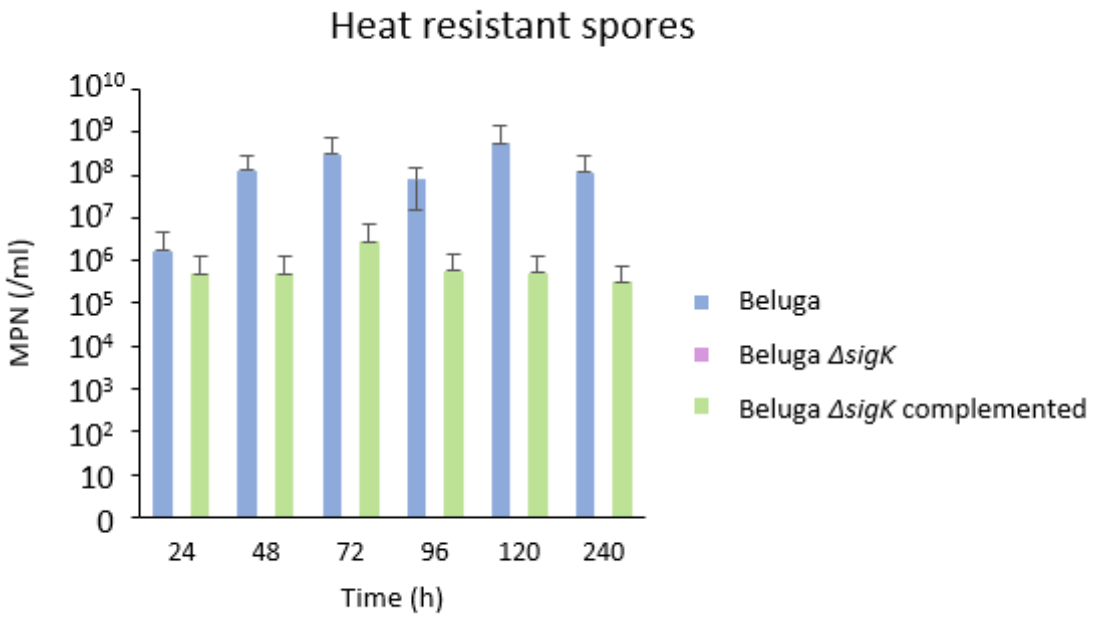


Figure 11. Most probable number of heat resistant spores per milliliter in each strain at the sampling points. Note that the scale is logarithmic. The error bars represent standard deviations.

7.4 ELISA Toxin Concentration Results

Because there were so many samples, the ELISAs were performed during three different dates, and for each day there was a different standard curve. The samples that were used as standards created a sigmoid curve. The samples that created the linear part of the sigmoid were used to create a linear regression. The ideal coefficient of determination, R^2-value, of a linear standard curve is as close to 1 as possible. Another issue that must be considered when making the standard curve is the toxin concentrations of the samples. The calculated toxin concentrations of the samples must fit to the standard curve's range. Otherwise the calculations are unreliable, and further testing is required. The standard curves can be seen in figure 12.

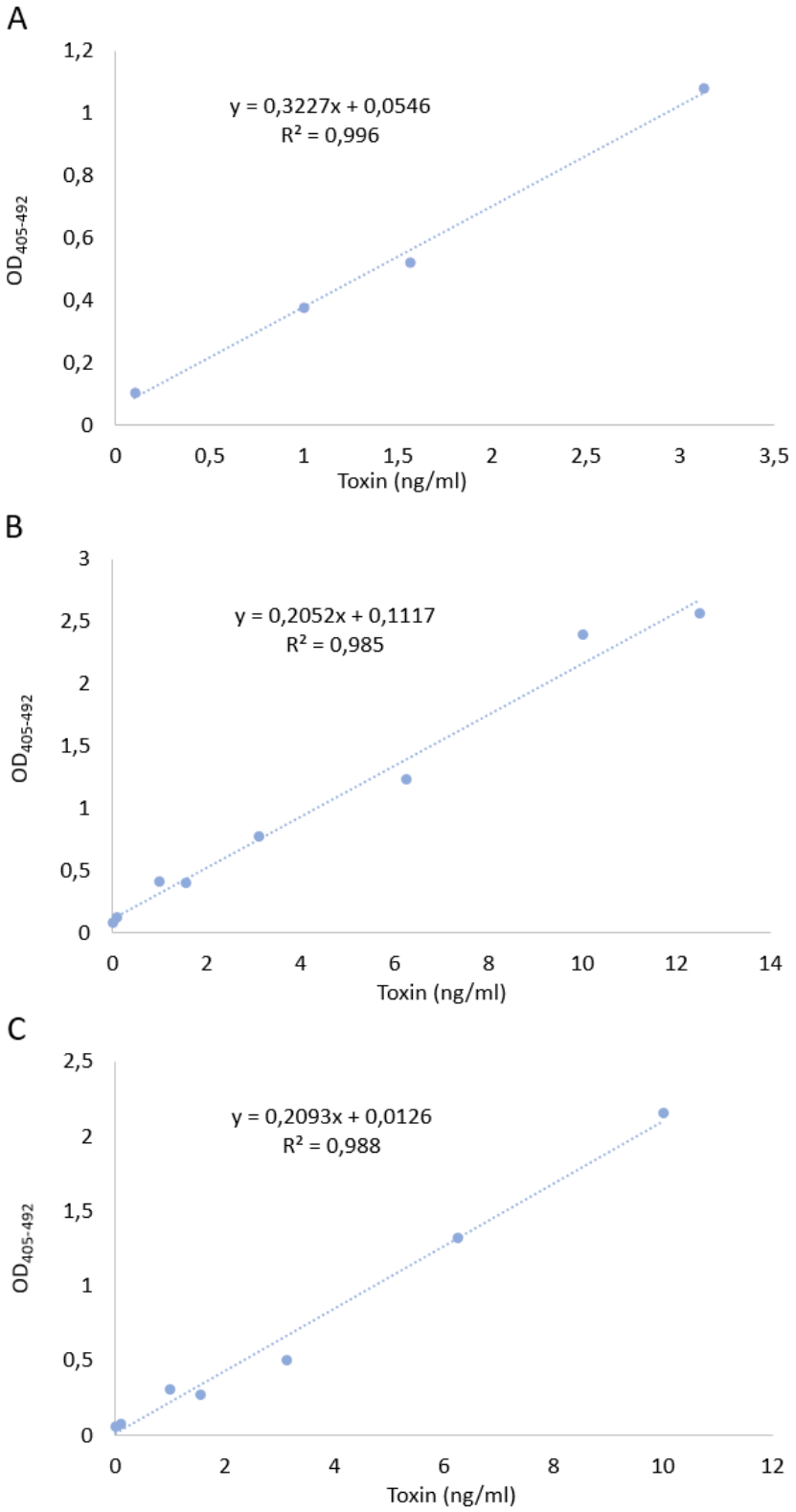


Figure 12. Standard curves of each ELISA test. Replicates 1–3 from each strain at time points 24 and 48 h were measured during the first day (A). During the second day samples from time points 72 and 96 h were measured (B), and during the third day samples from time points 120 and 240 h (C).

Looking at the standard curves in figure 12, the linearities of especially curves (B) and (C) are not ideal, but the R^2-value of each curve is higher than 0.95, which is acceptable. The linearity of day 1 is nearly ideal with an R^2-value of 0.996, but only four standard solutions are taken into account. Luckily only one sample, Beluga replicate 1 pellet at the 24-hour-time-point, fell off the standard curve's range and was unreliable because of this.

The average botulinum neurotoxin type E concentrations of each strain are shown in figure 13. The toxin in the pellet is inside the bacteria, and the toxin in the supernatant has been secreted out of the cell or released upon cell death. Both the Beluga *ΔsigK* (B) and Beluga *ΔsigK* complemented (C) have a similar toxin profile, where the toxin concentrations are the lowest at 240 hours and highest at 120 and 72 hours. The toxin concentrations are also highest in the pellet, except for the samples taken at 240 hours, where the toxin concentration is higher in the supernatant, and the Beluga *ΔsigK*'s samples taken at 96 hours, where the toxin is about evenly distributed to the pellet and the supernatant. The total toxin concentrations in the strain where *sigK* is removed (B) is higher than in the strain where it has been added in a plasmid (C). It could be because since the spores are not released and the mother cell is not lysing, the cells have more time to produce toxin before they die.

The toxin profile of the wild type Beluga (A) is very different from the other two strains. The highest toxin concentrations are at 96 and 24 hours and the lowest are at 240 and 120 hours. The toxin concentrations of the supernatant are also usually closer to the concentration of the pellet, or even higher, for example at 96 and 240 hours. It seems that removing the *sigK* gene (B) causes the bacteria to handle toxin differently than the wild type. There is slightly less toxin produced at the beginning, but then at around 72 hours the toxin concentrations more than double compared to the previous sample. The toxin concentration then falls, but at 120 hours it is even higher than at 72 hours. At this highest measured toxin concentration of the Beluga *ΔsigK* strain (B), the amount of toxin per 1 ml is 3040 ng higher than the highest amount measured from the wild type (A).

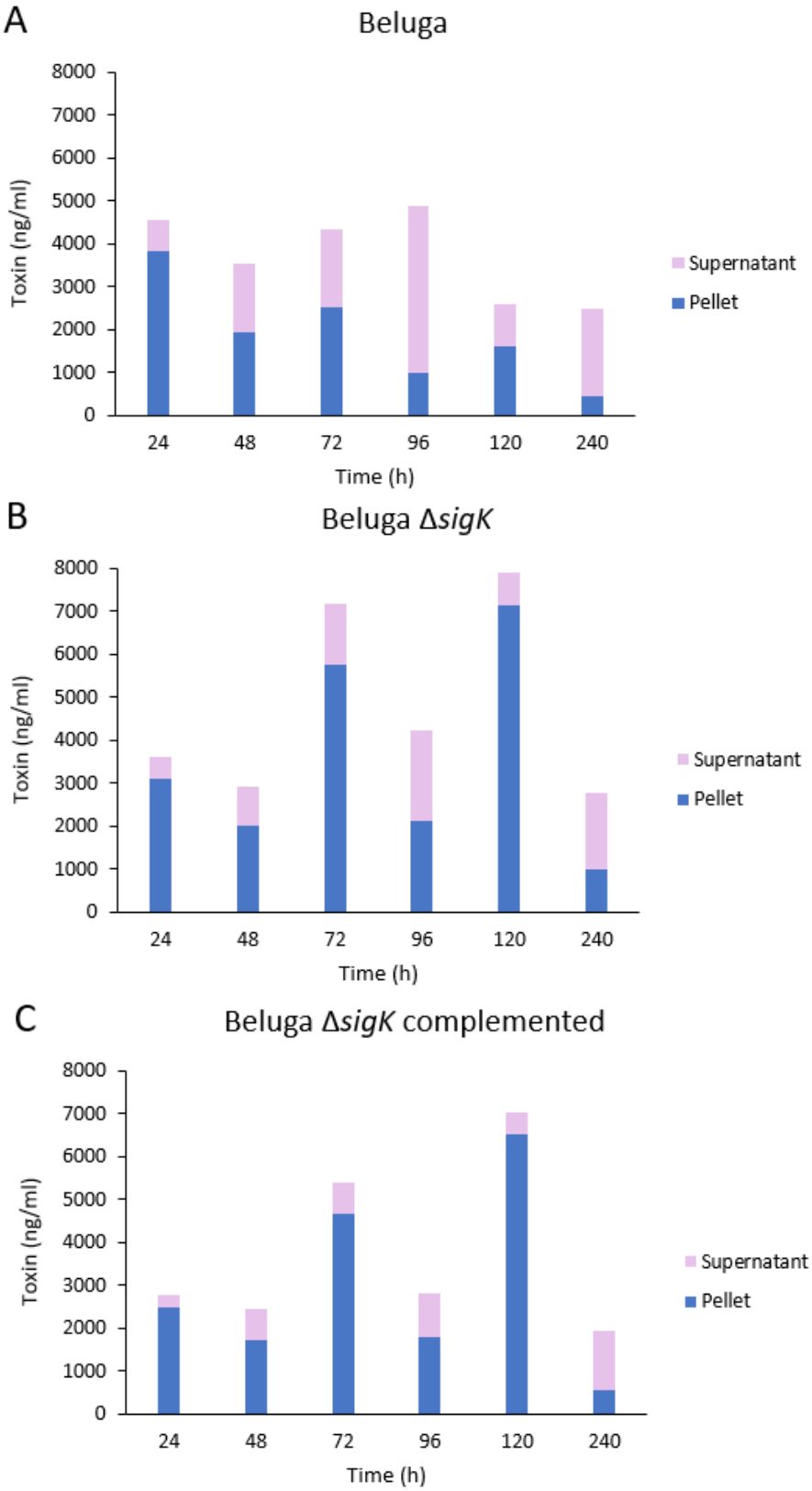


Figure 13. Average BoNT/E concentrations of the supernatant and pellet at different sampling points. Beluga is shown above (A), Beluga *ΔsigK* is below that (B) and Beluga *ΔsigK* complemented is at the bottom (C). The replicate 1 of Beluga (A) pellet at the 24-hour

sampling point is removed from the calculations here, because the sample did not fit in the standard curve's range.

7.5 Assessment of Reliability of the Results

Like in every study, there are some factors that impact the overall reliability of this study. Only three replicates of each strain were used and sometimes they showed drastically different results, making it difficult to estimate them. For example, the ELISA results were sometimes very different for each replicate. The linearity of the standard curves for ELISAs might have something to do with it, but also the fact that this was the first time I have performed ELISA tests. There were differences between the MPN results a well. The MPN results are affected by many factors, for example the age and homogeneity of the medium, the homogeneity of the samples and the quality of the pipettes and the pipette tips. The multi-channel pipettes used in this study did not always pipette equal volumes into each tip, which can affect the results.

During microscopy at the later sampling points, the cells started to have dots inside them in the pictures. The dots were a normal occurrence perceived by the laboratory researchers in their previous studies, but they still made determining of the sporulation stage more difficult and prone to errors. In addition, during the last few sampling points most of the cells were dead especially in the modified strains. This made it almost impossible to count a relevant number of cells.

8 Conclusions

In this study, the effects that the deletion of *sigK* has on the spore formation and release of *Clostridium botulinum* group II type E, were studied. Not much about the genetic mechanisms behind sporulation of the different strains of *C. botulinum* is known. The deletion of the gene had many effects on the bacteria. The spores got trapped inside the mother cell at the final stages of sporulation when the mother cells did not lyse. Only a small percentage of spores were released and germinated, but no growth was detected after heat treatment. Either the spores were not resistant to the heat treatment, or there was a very small amount of them that were able to germinate and replicate. The spores

appeared less bright on the microscope compared to the wild type, which could mean that their maturation was incomplete. Since the sporulation was halted and there were little to no spores to germinate, the population became gradually smaller over time. After ten days had passed from inoculation, the population was only 0.006 % of the wild type's population at that same time. Because the cells were not lysing, the individual bacteria died slower compared to the wild type. This probably allowed them to have more time to produce and secrete toxin, so there was overall more botulinum neurotoxin type E produced compared to the wild type. The toxin was also found more at different time points than in the wild type and the biggest peaks in toxin concentration were at 120 and 72 hours. Most of the toxin produced remained in the pellet, unlike in the wild type.

If the *C. botulinum* group II type E had no *sigK* in its genome, but gained it in a plasmid, the ability to sporulate was partially restored. Some similar problems described above still occurred. Spores were released, but the amounts were not as high as in the wild type. Just like in the strain where the *sigK* was absent, the population became gradually smaller over time and the cells died slower, but the effects were not as dramatic. The toxin production pattern was also similar, the biggest peaks in toxin concentration occurred at roughly the same times and most of the toxin was not secreted out of the cell. It seems that if the *sigK* is part of the bacterium's genome in a plasmid, it does not work as effectively as if it was in its normal location in the chromosome.

A previous study done on group I *C. botulinum* ATCC 3502 showed that *sigK* deletion leads to the bacteria being unable to enter sporulation at all and the process being stopped at stage 0 [22]. Our study showed that group II *C. botulinum* type E Beluga strain behaved in a very different way, when the gene was deleted. The bacteria entered sporulation and even produced spores but had problems releasing them. Based on these studies, it seems that *sigK* has a more restricted role in the sporulation of *C. botulinum* group II type E than in group I type A.

The results of this study could help shed some light on the relatively unknown mechanisms that drive the sporulation process of *C. botulinum*. These results have confirmed the fact that the many strains of *C. botulinum* are indeed very different from each other genetically. This study had only three replicates, which sometimes behaved in very

different ways, so further research should be done before drawing any absolute conclusions based on this study alone.

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Compositions of Growing Mediums and Agarose Gel

Agarose gel for mounting microscopy samples:

- Ingredients:
  - SeaKem® LE Agarose: 1.7 g
  - Water: 100 ml
- Microwaved until boiled
- Dispensed into 1.5 ml or 2 ml Eppendorf tubes
- Stored in RT.

Meat agar for culturing *C. botulinum* samples:

- Ingredients:
  - Cooked Meat Medium pellets: 7.5 g
  - 75 ml of liquid medium made of the following ingredients:
    - D(+)-Glucose anhydrous: 0.1125 g per one bottle made
    - Bacteriological agar: 1.8 g per one bottle made
    - Distilled water: 0.15 l per one bottle made.
- Dispensed into 250 ml glass bottles
- Sterilized by autoclaving
- Stored in 4 °C.

TPGY for MPN plates:

- Ingredients:
  - Bacto™ Tryptone: 50 g
  - Bacto™ Peptone: 5 g
  - Bacto™ Yeast Extract: 20 g
  - D(+)-Glucose anhydrous: 4 g
  - Sodium thioglycolate: 1 g
  - Distilled water: 1 l.

- Dispensed into 250 ml glass bottles
- pH adjusted to 7 with 6 M sodium hydroxide and 1 M hydrochloric acid
- Sterilized by autoclaving
- Stored in 4 °C.

Machines and Equipment

Centrifuge:

- Biofuge pico
- Heraeus instruments.

Cuvettes:

- Optical polystyrene
- Semi-micro
- 340–900 nm
- Disposable
- 634-0676
- VWR.

Microscope:

- Inverted model
- Leica Model TL LED
- Camera: Hamamatsu Digital Camera Orca Flash V2 LT
- FM4-64 filter: Custom, emission 726 nm
- DAPI filter: Filter cube DA/FI/TX, emission 462 nm
- MetaMorph® software: Molecular Devices.

Mini centrifuge:

- Sprout® Mini Centrifuge
- Heathrow Scientific.

Spectrophotometer:

- Genesys 20
- Thermo Spectronic.

Spectrophotometer for microwell plates:

- Multiskan Ascent
- Thermo Electron Corporation.

Water bath:

- Labnet International Inc.

Reagents

Agar, bacteriological:

- $(C_{12}H_{18}O_9)n$
- VWR
- J637.

Bacto™ Peptone, Enzymatic Digest of Protein:

- BD
- 211677.

Bacto™ Tryptone, Pancreatic Digest of Casein:

- BD
- 211705.

Bacto™ Yeast Extract, Extract of Autolysed Yeast Cells:

- BD
- 212750.

Cooked Meat Medium pellets:

- Formula:
  - Heart muscle 454 g/l
  - Peptone 10 g/l
  - 'Lab-Lemco' powder 10 g/l
  - Sodium chloride 5 g/l
  - Glucose 2 g/l.
- pH 7.2 ± 0.2 at 25°C
- Oxoid

- CM0081.

DAPI (4',6-Diamidino-2-Phenylindole, Dihydrochloride) 5 mg/ml:

- $C_{16}H_{17}Cl_2N_5$
- MW: 350.25 g/mol
- Thermo Fisher Scientific
- D1306.

D(+)-Glucose anhydrous:

- $C_6H_{12}O_6$
- MW: 180.16 g/mol
- VWR
- 101176K.

FM4-64 (N-(3-Triethylammoniopropyl)-4-(6-(4-(diethylamino)phenyl) hexatrienyl)pyridinium dibromide) 1 mg/ml:

- $C_{30}H_{45}Br_2N_3$
- MW: 607.5 g/mol.

Formalin solution 10 %:

- Sol.formaldehyd.cntr. 37 % and Aq.purif.
- 
- Yliopiston Apteekki
- 85102A.

Hydrochloric acid:

- HCl
- MW: 36.46 g/mol

- 
- 1 M.

PBS, Phosphate buffered saline powder:

- pH 7.4 at 25 °C
- Sigma-Aldrich
- P3813.

SeaKem® LE Agarose:

- Lonza
- 50005.

Sodium hydroxide:

- NaOH
- MW: 39.997 g/mol
- 
- 6 M.

Sodium thioglycolate for microbiology:

- $C_2H_3NaO_2S$
- MW: 114.1 g/mol
- 
- Merck KGaA
- 1.06691.0500.

ELISA Solutions

ABTS:

- Ingredients:
  - 1 tablet containing 50 mg of ABTS
  - 50 ml ABTS Substrate Solution.
- Stable for 3 months
- Stored in dark at 4 °C
- ROCHE.

Antigen-dilution buffer:

- 0.1 % BSA in PBS
- pH 7.2
- Filtered (0.2 µm).

Casein buffer:

- Formula:
  - 0.05 M Tris
  - 0.625 % (w/v) Casein
  - 0.005 % (w/v) 5-Bromo-5-nitro-1,3-dioxane
  - 0.0025 % (v/v) Tween20.
- Thermo Fisher Scientific.

PBS, Phosphate buffered saline:

- Commercialized powder in water
- pH 7.4 at 25 °C
- Filtered (0.2 µm)
- Sigma-Aldrich
- P3813.

PBS + Tween20:

- PBS, filtered (0.2 µm)
- 0.1 % Tween20.

MPN Numbers

Table 7. Translation of the number of positive tubes into MPN numbers [34].

No. of Tubes Positive in			MPN in the inoculum of the middle set of tubes	No. of Tubes Positive in			MPN in the inoculum of the middle set of tubes
first set	middle set	last set		first set	middle set	last set	
0	0	0	‹0.03	2	0	0	0.091
0	0	1	0.03	2	0	1	0.14
0	0	2	0.06	2	0	2	0.20
0	0	3	0.09	2	0	3	0.26
0	1	0	0.03	2	1	0	0.15
0	1	1	0.061	2	1	1	0.20
0	1	2	0.092	2	1	2	0.27
0	1	3	0.12	2	1	3	0.34
0	2	0	0.062	2	2	0	0.21
0	2	1	0.093	2	2	1	0.28
0	2	2	0.12	2	2	2	0.35
0	2	3	0.16	2	2	3	0.42
0	3	0	0.094	2	3	0	0.29
0	3	1	0.13	2	3	1	0.36
0	3	2	0.16	2	3	2	0.44
0	3	3	0.19	2	3	3	0.53
1	0	0	0.036	3	0	0	0.23
1	0	1	0.072	3	0	1	0.39
1	0	2	0.11	3	0	2	0.64
1	0	3	0.15	3	0	3	0.95
1	1	0	0.073	3	1	0	0.43
1	1	1	0.11	3	1	1	0.75
1	1	2	0.15	3	1	2	1.20
1	1	3	0.19	3	1	3	1.60
1	2	0	0.11	3	2	0	0.93
1	2	1	0.15	3	2	1	1.50
1	2	2	0.20	3	2	2	2.10
1	2	3	0.24	3	2	3	2.90
1	3	0	0.16	3	3	0	2.40
1	3	1	0.20	3	3	1	4.60
1	3	2	0.24	3	3	2	11
1	3	3	0.29	3	3	3	›24

Details of Results

Table 8. Average percentages of cells in different stages of sporulation.

Strain	Time point (h)	Cell types and percentages (%)					
		Vege-tative	Stage 2	Stage 3/4	Stage 5/6	Free spores	Germi-nating spores
Beluga	24	47,6	16,3	26,7	8,8	0,6	0,0
Beluga	48	28,0	5,9	18,8	9,6	28,2	9,4
Beluga	72	20,4	3,9	13,4	2,2	43,1	17,0
Beluga	96	11,2	1,6	9,7	1,3	56,1	20,0
Beluga	120	5,5	0,0	10,1	2,4	55,8	26,2
Beluga	240	1,0	0,0	0,4	0,8	59,2	38,6
Beluga *ΔsigK*	24	52,7	16,4	24,7	6,1	0,0	0,0
Beluga *ΔsigK*	48	38,8	12,3	23,6	25,3	0,0	0,0
Beluga *ΔsigK*	72	22,3	6,2	41,6	28,1	1,2	0,5
Beluga *ΔsigK*	96	18,1	3,0	45,6	28,9	3,0	1,4
Beluga *ΔsigK*	120	17,4	2,1	60,0	16,9	2,5	1,1
Beluga *ΔsigK*	240	29,2	2,1	53,2	7,7	3,6	4,2
Beluga *ΔsigK* complemented	24	59,1	11,4	24,2	5,3	0,0	0,0
Beluga *ΔsigK* complemented	48	36,8	10,6	23,8	28,6	0,1	0,1
Beluga *ΔsigK* complemented	72	26,0	5,0	31,8	35,3	0,2	1,6
Beluga *ΔsigK* complemented	96	14,8	2,4	31,8	34,8	13,4	2,8
Beluga *ΔsigK* complemented	120	14,7	1,4	39,4	34,8	7,0	2,7
Beluga *ΔsigK* complemented	240	21,0	1,5	17,7	22,3	20,3	17,3

Table 9. Average optical densities of the samples.

	Time points (h) and ODs (A_{600})					
Strain	24	48	72	96	120	240
Beluga	1.926	1.569	1.11	0.941	0.907	0.901
Beluga *ΔsigK*	1.908	1.664	1.332	1.142	1.026	0.801
Beluga *ΔsigK* complemented	1.92	1.73	1.301	1.113	0.984	0.748

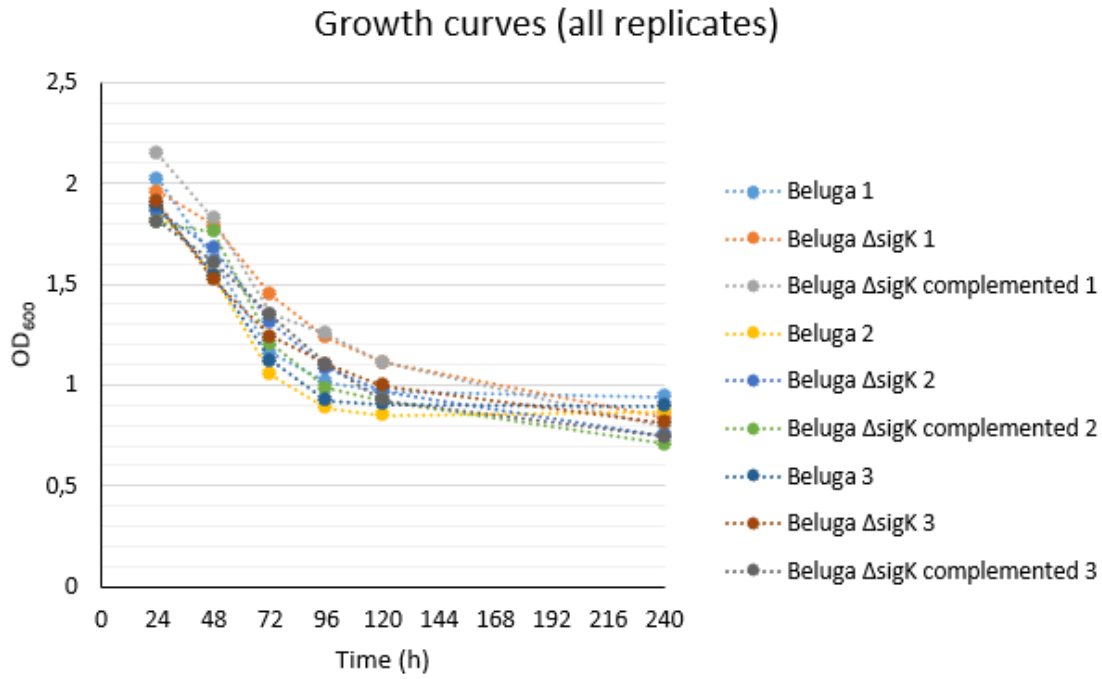

Figure 14. Growth curves of individual replicates.

Table 10. Average MPNs of viable cells.

Strain	Time points (h) and MPNs (MPN/ml)					
	24	48	72	96	120	240
Beluga	2.78×10^8	3.75×10^8	1.78×10^8	1.01×10^8	1.68×10^8	8.48×10^7
Beluga *ΔsigK*	2.69×10^8	4.34×10^7	3.70×10^6	150556	27400	4818
Beluga *ΔsigK* complemented	1.67×10^8	2.68×10^7	7.45×10^6	5.26×10^6	860256	494526

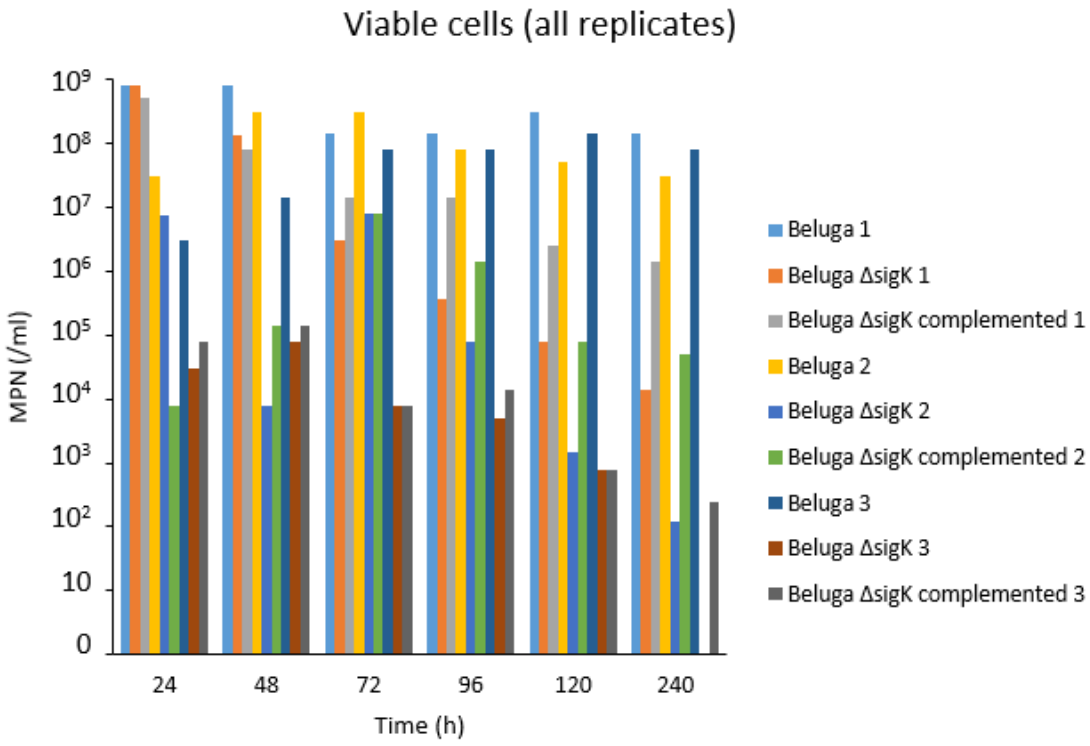

Figure 15. Viable cell MPNs of all replicates.

Table 11. Average MPNs of heat resistant spores.

	Time points (h) and MPNs (MPN/ml)					
Strain	24	48	72	96	120	240
Beluga	1.67×10^6	1.25×10^8	3.04×10^8	7.92×10^7	5.54×10^8	1.16×10^8
Beluga *ΔsigK*	0	0	0	0	0	0
Beluga *ΔsigK* complemented	477818	480434	2.75×10^6	581367	504611	314611

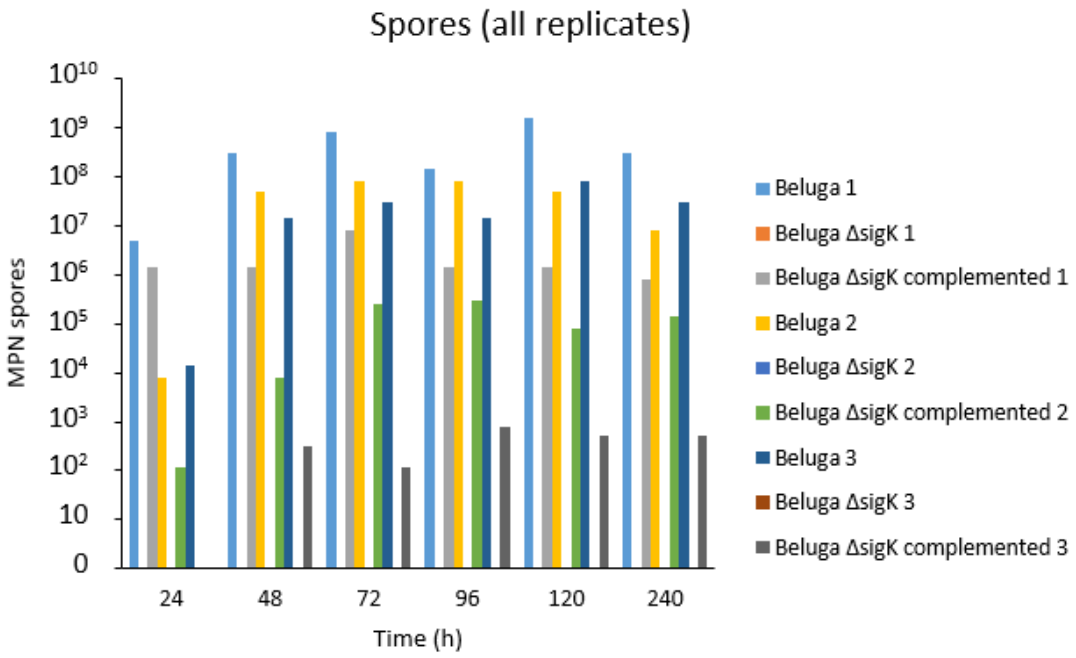

Figure 16. Spore MPNs of all replicates.

Table 12. ELISA results of all replicates' pellets.

Strain	Replicate	Time points (h) and toxin pellet (ng/ml)					
		24	48	72	96	120	240
Beluga	1	8338	1898	3091	1212	565	467
Beluga *ΔsigK*	1	3455	2446	8054	2043	9368	1047
Beluga *ΔsigK* complemented	1	2122	1427	5003	2291	9837	493
Beluga	2	3213	1876	2956	1027	3387	467
Beluga *ΔsigK*	2	2841	1901	3785	2062	4763	1047
Beluga *ΔsigK* complemented	2	2847	1842	2771	1570	3387	493
Beluga	3	4465	2062	1541	702	851	388
Beluga *ΔsigK*	3	3027	1659	5412	2233	7285	909
Beluga *ΔsigK* complemented	3	2494	1923	6163	1472	6358	639

Table 13. ELISA results of all replicates' supernatants.

Strain	Replicate	Time points (h) and toxin supernatant (ng/ml)					
		24	48	72	96	120	240
Beluga	1	1148	1217	1498	3580	906	2020
Beluga *ΔsigK*	1	754	821	1293	1660	756	2020
Beluga *ΔsigK* complemented	1	293	467	440	393	448	1112
Beluga	2	627	1478	1863	3570	1045	1991
Beluga *ΔsigK*	2	423	852	854	2586	751	1571
Beluga *ΔsigK* complemented	2	175	498	689	1806	522	1647
Beluga	3	355	2054	2014	4525	992	2106
Beluga *ΔsigK*	3	330	1130	2107	2089	808	1676
Beluga *ΔsigK* complemented	3	392	1199	1108	851	546	1466