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Faculty of Veterinary Medicine and Biosciences
Department of Food Safety and Infection Biology

Genome-wide transcriptional profiling of Clostridium species during sporulation

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Preface

This thesis was performed at the department of Food Safety and Infection Biology, Oslo, and the department of Chemistry, Biotechnology and Food Science, Ås.

The most sincere thanks go to all who helped me throughout the project.

Special thanks for the patience go to my supervisors Marina Elisabeth Aspholm, Toril Lindbäck, Kristin O'Sullivan, Knut Rudi and Ekaterina Avershina. Everybody has been really supportive, even on rainy days.

Also wish to thank the researchers at Adamstua and Ås for being terrific company, especially Kristina Borch-Pedersen and Anne Kijewski, for the shortcuts and not-to-do's.

Also sincere thanks go to Antje Hofgaard for teaching, and allowing me to freely use the electron microscope at UiO.

It has been a pleasant adventure meeting everyone.

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Abstract

Dormancy strategies utilized by bacteria contribute to their survival under adverse conditions, as well as persistence and transmission between hosts. The formation of endospores produces some of the most resilient forms of life by members of the *Firmicutes* phylum. Genome studies of the human gut bacteria have shown that sporulation genes are widespread among commensal *Clostridia,* which play an essential role in maintaining gut homeostasis, but sporulation of non-pathogenic bacteria has been poorly studied. Defining the sporulation conditions and the genetic makeup behind cell differentiation into endospores may allow for practical applications in the treatment of gut disease and to promote well-being.

Select commensal *Clostridia* strains were cultivated under conditions that promote sporulation in *Bacillus* and *Clostridium* species. Spore-like cells were observed by phase-contrast and electron microscopy, but could not be cultured. Gene expression studies and 16s rRNA sequencing revealed that the strains belonged to non-sporulating species of *Actinotignum schaalii* and *Staphylococcus epidermidis*, which have close family members capable of exospore formation and entry into a viable but non culturable state. Differentially regulated genes during *S. epidermidis* entry into a VBNC state were analyzed using RNA sequencing. The upregulated expression of membrane proteins, cell transport, stress response, and a shift in metabolism towards protein and carbohydrate catabolism were similar to gene expression patterns in other bacteria previously reported to enter the VBNC state. The subject of bacterial dormancy has been gaining momentum through techniques such as RNA sequencing, which allows the discovery of genetic factors previously unassociated with cell differentiation.

Sammendrag

Hvile-stadier hos bakterier bidrar til deres overlevelse ved ugunstige forhold, i tillegg til å fremme standhaftighet og utspredning mellom verter. Formering av endosporer produserer noen av de mest resistente livsformer på jorda hos medlemmer i rekken *Firmicutes.* Genom studier av menneskets tarmbakterier har vist at sporulerings gener er utbredte blant kommensale *Clostridia*, som spiller en viktig rolle i å opprettholde homeostase i tarmen, men sporulering hos ikke-patogene arter har blitt lite studert. Å definere sporulerings forhold og den genetiske grunnlaget for celle differensiering kan bidra til behandling av tarmsykdommer og gi en økning i generell velferd.

Utvalgte *Clostridia* stammer ble kultiverte ved forhold som hadde vært tidligere vist til å fremme sporulering hos *Bacillus* og *Clostridium* arter. Spore-liknende celler ble observerte med fase-kontrast mikroskopi og elektron mikroskopi, men kunne ikke kultiveres. Genuttryks studier og 16s rRNA sekvensering viste at stammene tilhørte til ikke-sporulerende *Actinotignum schaalii* og *Staphylococcus epidermidis* arter, men som har nære familie slektninger med evne til å danne exosporer og til gå in en VBNC hvile-stadium

Differensialt uttrykte gener i løpet *S. epidermidis* overgang til en VBNC stadiet ble analyserte ved hjelp av RNA sekvensering. De oppregulerte gener som tilhørte membran proteiner, celle transport, stress respons, og en overgang til katabolisme av proteiner og karbohydrater, var i likhet til genuttrykk fra tidligere studier hos andre arter i VBNC stadiet. Hvile-stadier hos baketerier har fått en økning i oppmerksomhet og fremgang ved hjelp av nyere metoder som RNA sekvensering, som tilltater oppdagelse av nye genetiske faktorer som tidligere var ikke assosierte med celle differensiering.

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Abbreviations

BHI	Brain-heart infusion media
BLAST	Basic local alignment search tool
CCUG	Culture Collection, University of Gothenburg, Sweden
DPA	Dipicolinic acid
DTT	Dithiothreitol
FPKM	Fragments per kilobase of transcript per million mapped reads
FTM	Fluid-thioglycolate media
NCBI	National Center for Biotechnology Information
OD	Optical density
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
RIN	RNA integrity number
Rpf	Resuscitation promoting factor
SASP	Small acid-soluble spore protein
SDS	Sodium dodecyl sulfate
SEM	Scanning electron microscope
VBNC	Viable but non-culturable

1. Introduction

1.1 Commensal *Clostridia*

The human gut's performance and homeostasis is constantly modulated by the microbiota that inhabits it. Pathogenic bacteria belonging to the obligate anaerobe class *Clostridia* are often implicated in gut disease and dysbiosis, but 10-40% of the normal gut microbiota consists of commensal *Clostridia* that ferment complex carbohydrates, synthesize compounds that are essential to the host and other commensals, and form a vital barrier against invading bacteria [1]. Understanding the mechanisms for transmission of bacteria between hosts, and how the bacteria colonize the gut and persist there over time, are necessary to treat gut-related disease, modulate the after effects of antibiotic treatment, and to promote well-being. Pathogenic gut bacteria have been studied extensively, such as the enteropathogen *Clostridium difficile*, and one mode of persistence that causes recurring infections in the gut is the formation of endospores [2]. Sporulation genes are widespread in the genomes of commensal human gut bacteria as well, compared to environmental microbiomes [3], and it is predicted that as much as 50% of the genera in gut bacteria are capable of endospore formation [4].

1.2 Sporulation programs

Endospores are dormant cell structures capable of surviving extreme environmental, and even extraterrestrial, conditions [5]. Cell differentiation into endospores is primarily a stress response and a survival mechanism, unlike spore production in *Actinobacteria* and *Fungi* which is a means of reproduction [6], because the formation of endospores is a costly and irreversible process involving more than 500 differentially regulated genes [7], where one mother-cell gives rise to a single endospore. The process of sporulation at the molecular level is best studied in *Bacillus subtilis* [8] and a few *Clostridium* [9] model organisms, such as *Clostridium perfringens* and *Clostridium acetobutylicum*. Many of the core sporulation genes are conserved among endospore formers [10], although there are major differences in how the sporulation cascade is initiated and regulated in *Bacillus* and *Clostridia*, as well as the proteins involved in the endospore structure.

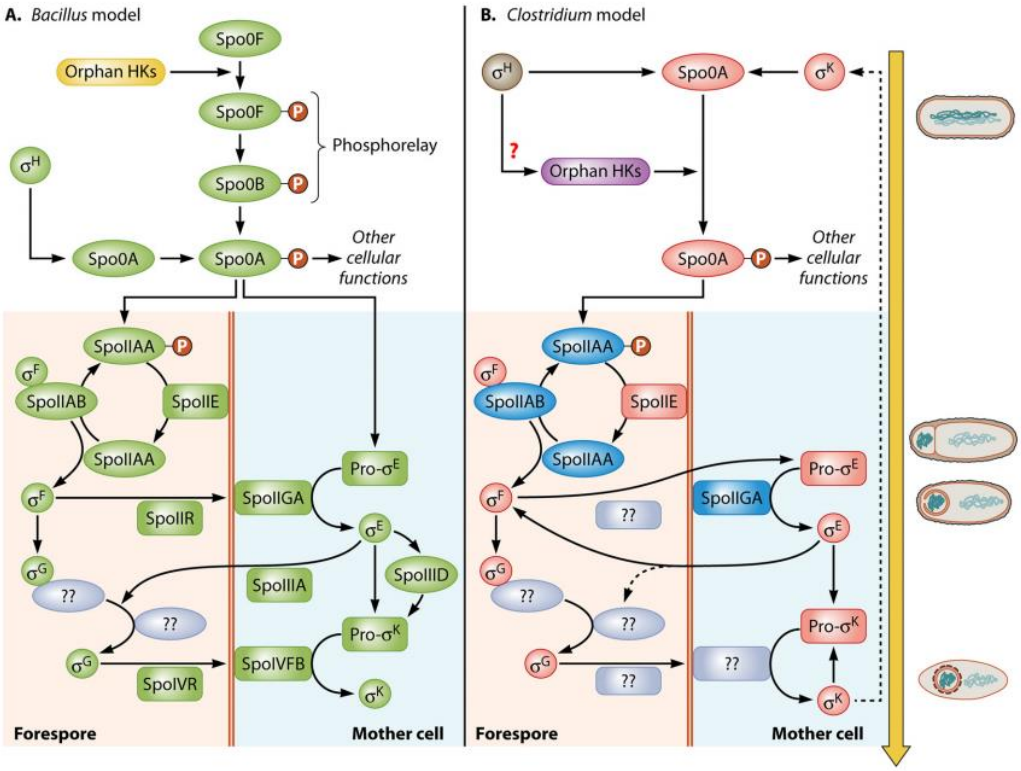

Figure 1. A general comparative model for regulation of sporulation in *Bacillus* and *Clostridia* [9]. Sequence of regulation events and the proteins involved varies between both *Bacillus* and *Clostridium* species.

1.2.1 Initiation of sporulation

Entry into sporulation is controlled by the master transcriptional regulator Spo0A, which is activated by phosphorylation and initiates the expression of the sporulation cascade. At low levels of phosphorylated Spo0A proteins in *B. subtilis*, behavior such as competence, biofilm formation and cannibalism is regulated instead, but once the concentration of activated proteins surpasses a threshold, Spo0A initiates the transcription of sporulation specific sigma factors, through a switch-like mechanism of positive feedback loops, and sporulation becomes irreversible (Figure 1) [8]. Phosphorylation of Spo0A is controlled by several histidine kinases that auto-phosphorylate in response to environmental signals, and in *B. subtilis* a phosphorelay system mediates the transfer of the phosphate group from the kinases to their response regulators and Spo0A [8]. Clostridial endospore formers lack a phosphorelay system, the Spo0A transcriptional regulator is phosphorylated directly by orphan histidine kinases

resulting in passively high *spo0A* expression levels, but the amount of phosphorylated proteins is maintained by phosphatase-like enzymes [9].

1.2.2 Regulation of sporulation

Following DNA replication in the normal cell cycle, the cells that have committed to sporulate will form a septum, which unevenly divides the cell into a smaller forespore compartment that contains one copy of the DNA, and a mother cell compartment which is separately regulated, and where a portion of the developing endospore proteins will be synthesized [11]. Further differentiation into endospores is facilitated by prespore and mother cell specific sigma factors, which are regulated at transcription, and post-translation, to coordinate and time the development (Figure 1). The first sigma factor activated by Spo0A~P is sigma F, which is transcribed together with an anti-sigma factor SpoIIAB and an anti-anti-sigma factor SpoIIAA belonging to the same operon [9]. After translation, sigma F is inactivated by the repressor SpoIIAB, until a membrane bound phosphatase SpoIIE dephosphorylates SpoIIAA which allows it to bind SpoIIAB, thus releasing and activating sigma F [9]. Similarly, sigma F induces a positive feedback loop of its own expression, and transcribes a prespore specific sigma factor G and a mother cell specific sigma factor E [9]. After translation, the sigma factor G is repressed by an unknown protein, until it is activated again by SpoIIIA and SpoIIIJ and induces the transcription of prespore specific proteins such as small acid-soluble proteins (SASPs) [8, 9]. The mother cell sigma factor E is initially transcribed in its inactive form, and requires cleavage by the SpoIIGA protease to activate the transcription of downstream genes responsible for granulose formation, an amylopectin storage material not found in *Bacillus* species, and the swollen cigar shape of sporulating *Clostridium* cells [9]. The last mother cell specific sigma factor K, which is also transcribed in an inactive form and is necessary for the release of the forespore from the mother cell, has been shown to also be essential for the early transcription of *spo0A* in *C. acetobutylicum*, but not in other *Clostridium* species [9]. Many of the specific molecular mechanisms involved in the regulation of sporulation in *Clostridia* have not yet been identified, but differences in the sequence that the sporulation events are initiated and the transcriptional regulators involved, show great variation between *Clostridium* species.

1.2.3 Endospore structure

Endospores attain their extreme resistance through a complex and multilayered structure which provides protection from environmental stress such as desiccation, heat, radiation, oxidation and bactericides (Figure 2).

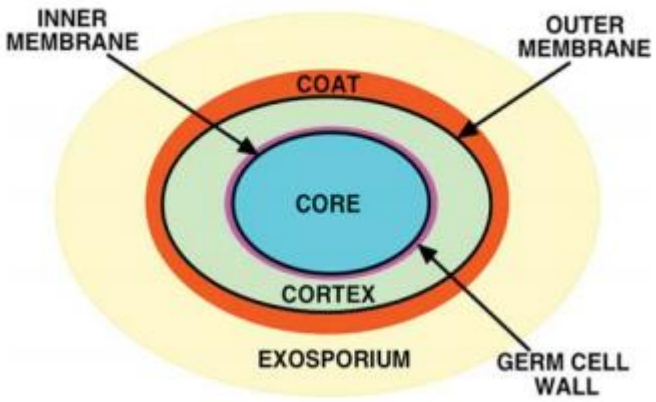

Figure 2. Schematic representation of the various endospore layers [12].

The endospore core contains DNA and essential proteins necessary to resume growth. The core has very low water content, and instead contains a large amount of dipicolinic acid (DPA) that is chelated by divalent cations such as Ca^{2+}, as well as several SASPs that bind to and help stabilize DNA, greatly increasing the endospore resistance to heat and DNA damage [12]. Transport of DPA into the core, and the synthesis of SASPs, is initiated in the forespore by the sigma factor G [9], which leads to increased density in the forespore and can be observed with phase contrast microscopy as a phase-bright body. The endospore's permeability barrier starts with an inner membrane surrounding the spore core, containing rigid and viscous lipids that are highly impassible even for water molecules [12]. Surrounding the inner membrane lays a thin layer of peptidoglycan, followed by a thicker layer of rigid, highly crosslinked and modified peptidoglycan termed the spore cortex [13]. The spore cortex is covered by and outer membrane, and although it is essential during forespore formation, the outer membrane does not appear to play a significant role in spore resistance, besides containing a few pigments that may contribute to radiation resistance [12]. Outside the outer membrane is the spore coat, containing numerous spore proteins that act as a permeability barrier, and a mechanical sieve for larger molecules such as lysozyme, that may attack the spore cortex. In addition, various antioxidative enzymes may be present in the spore coat

contributing to the spores chemical resistance, as well as pigments that absorb in the UV range and shield DNA from damage [12]. Although not present in all endospores, an additional outer exosporium layer may reside above the spore coat, which reduces the permeability of larger molecules such as antibodies [12]. The importance of various endospore structures is generally understood, but the precise mechanisms involved in e.g. spore killing by wet-heat remain unclear.

1.2.4 Germination of endospores

In order for endospores to resume active growth and cell division, they need to rehydrate the spore core and shed the protective cortex and coat layers. Germination is achieved through a set of sensory kinases termed germinant receptors, which sense favorable conditions by responding to nutrient stimulus such as amino acids and sugars [14]. The germination machinery is assembled in the endospore during sporulation and initiates an automated response to germination signals [9]. The Ca-DPA content in the spore core is released through a dedicated channel in the spore inner membrane, followed by the uptake of water, which dissociates SASPs from DNA and initiates their breakdown as well as allowing the cell to resume transcription and metabolism. Release of the spore core ion content is followed by the degradation of the cortex with cortex-lytic-enzymes, which allows further hydration, swelling and elongation of the germinating cell [14]. The specific nutrient combinations that induce germination vary between species, but non-nutrient germinants, such exogenous Ca-DPA and lysozyme, have been also shown to induce germination [15].

1.3 RNA sequencing

Gene expression studies using qPCR and microarray assays are limited to beforehand knowledge of the genes involved in the test condition, and their sequences, for probe design. RNA sequencing allows identifying all expressed transcripts through reverse transcription of mRNA to cDNA, and to make a relative comparison of expression levels between samples. This is useful when working with novel or poorly characterized phenomenon. Previously unassociated genes may become apparent after RNA sequencing and allow for individual studies using conventional PCR methods. There is a great variety of tools for sequence data processing, depending on experimental conditions such as organism

type, sequencing platform and read length.

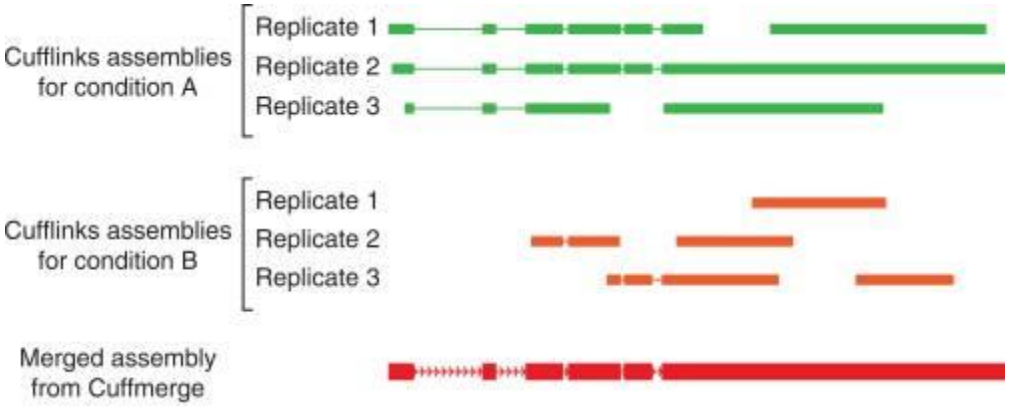

Figure 3. Schematic of read assembly into transcripts with Cufflinks [16].

The Cufflinks [16] package aims to assemble short read fragments into whole gene transcripts by doing a basic local alignment with a reference genome (Figure 3). Read counts per transcript are normalized in a convention expressed as Fragments Per Kilobase of transcript per Million mapped reads (FPKM), to avoid the bias where longer genes are assigned higher short fragment counts, not reflecting an actual increase in transcript expression levels. Once assembled, the samples are merged and normalized again, according to their FPKM values. The change in expression levels between conditions is the calculated as a fold-change, and a statistical t-test is performed to see if the replicate variance is not above the change in expression between conditions. The final output is a list of significant, differentially regulated genes between the input conditions.

2. Materials & Methods

2.1 Strains and growth media

Freeze-dried cultures of 9990 *Blautia producta*, 26784 *Eubacterium fissicatena* and 58757 *Lachnoanaerobaculum umeaense* were ordered from CCUG (Culture Collection, University of Gothenburg, Sweden). The freeze-dried bacteria were resuspended in cryogenaic vials with BHI/glycerol media and frozen at -80 °C. For culturing, the three strains were inoculated from frozen stocks into Brucella (BD Difco, New Jersey, United States), brain-heart infusion (BHI)(BD Difco), fluid-thioglycolate (FTM)(BD Difco) and Robertson broth (BD Difco), as well as onto blood (BD BBL) and Brucella (BD Difco) agar plates. The cultures were incubated overnight at 37 °C in anaerobic jars.

2.2 Induction of sporulation

Endospore formation was followed by phase contrast microscopy (Olympus Bx51, 10 x 100 magnification) using a digital mounted camera (ColorView II). During spore formation, the bacterial cells turn from phase-dark to phase-bright [17].

A protocol for *Clostridium perfringens* sporulation [18, 19] was used to induce sporulation in the tested strains. This was done by transferring a colony from overnight blood agar to 10 ml fresh Robertson media and incubating anaerobically at 37 °C for 7 days. The culture was then heated for 20 min at 77 °C in a water-bath, and 1 ml of the heat treated culture was used to inoculate 10 ml of FTM media followed by incubation for 16 hours. A volume of 1ml of the 16-hour culture was transferred to 100 ml of Duncan Strong broth (VWR, Radnor, United States), prepared and autoclaved the day before, and incubated anaerobically overnight. The growth in Duncan Strong broth was followed for three days.

Other strategies tested to induce sporulation in Brucella media were:
-Heat-shock [20] of an overnight culture at 50-70 °C in a water bath for 0-15 min followed by transfer to fresh media (1% v/v concentration).
-Simulation of stationary growth phase conditions by increasing the cell density [21]. This was done by centrifuging an overnight culture, removing 1/4 of the supernatant volume and resuspending the cells for further incubation.

-Supplementing an overnight culture with 1% Na-acetate, a fermentation end-product [22, 23].
-Addition of either 0.5-1% $CaCl_2$, KCl, MgCl, $FeSO_4$ or $MnSO_4$ [24] or a combination of these as well as thiamine [25] and glucose [26] into the growth medium.

2.3 Preparation of spores

Various methods for isolation of spores from vegetative cells and debris were tested such as digestion with lysozyme [20, 27], daily water washes [28], sonication [20], washing with detergents [27], a two-phase extraction system [29], differential centrifugation and density gradient centrifugation. The most effective procedure was to pellet liquid cultures, resuspend them in 0.05% SDS by vortexing, centrifuge at 13 000 rpm for 5min and wash the pellets three times with water. A Nycodenz density gradient was then prepared by adding 4 ml of a cold 40% wt/wt solution at the bottom of a 15 ml falcon tube, which was then overlaid by 4 ml of a cold 30% solution. Then, the washed spore pellet was resuspended in 4 ml of 20% Nycodenz and added on top of the gradient, followed by centrifugation at 4 500 rpm (Allegra X-22, Beckman) for 1h at 4 °C. The supernatant was then removed and the resulting pellet was washed three times with water. The spore preparations were stored at 4 °C until use.

2.4 Germination of spores

To screen for potential germinants, 25 µl of spore preparations were incubated at 37 °C for 1-24 hours with various combinations of nutrients and non-nutrient compounds in a total volume of 100 µl, with and without heat shocking at 60°C for 10 min prior to the incubation. Both aerobic and anaerobic conditions were tested.

Table 1. Germinants tested to resuscitate the spores.

Amino acids	Saccharides	Co-germinants	Non-nutrient
40 mM	1% w/v	50 mM	45 mM
L-alanine, L-methionine, L-serine, L-cysteine	D-glucose, D-fructose, D-fucose, D-(+)-mannose	Inosine, lactate, bicarbonate	Ca-DPA
0.2 g/ml		0.2 g/ml	
Casein hydrolysate		Bile salts	

Spore germination was assessed by phase-contrast microscopy, looking for darkened and swollen spores, or colony formation on blood agar for up to three days. ImageJ software was used for automatic cell counting from microscopy images [30]. Cells were detected and separated from the image background by setting a threshold for color, particle size and roundness. The separated cells were then counted and reported by the software. Advantage over manual counting is that once the filter settings are set, any number of images can be counted from the same sample.

Additional methods were tested for germinating spores including chemical denaturation of the protein coat and/or pretreating with proteases, followed by washing and plating on BHI agar containing 1 µg/ml lysozyme (Sigma-Aldrich, Missouri, United States). The principle is to make the spore protein coat permeable enough for lysozyme to access the spore cortex, which it may then cleave and allow hydration of the spore core, initating germination, similar to how native spore cortex-lytic enzymes work. For chemical removal of spore protein coat, the spore suspensions were incubated in 50 mM tris-HCl (pH 8), 8 M urea, 1% SDS and 50 mM DTT for 90 min at 37 °C [31, 32]. An alternative method for removal of the protein coat was to incubate the spore suspensions in 0.1 M sodium borate (pH 10) and 2% 2-mercaptoethanol for 60 min at 37 °C [33]. In the third method, the spore suspensions were pretreated with 50 mM DTT in tris-HCl (pH 9.3) for 3 h, followed by digestion with 100 µg/ml proteinase K (Sigma-Aldrich) for 3 h at 37 °C [32].

2.5 RNA isolation

Total RNA for expression studies was isolated using the RNeasy mini kit (Qiagen, Düsseldorf, Germany). Cell cultures of *A. schaalii* were centrifuged at 4 500 rpm (Allegra X-22, Beckman) for 20 min at 4 °C and the pellets were then resuspended in 495µl SET buffer. A volume of 50 µl 10 mg/ml lysozyme (Sigma Aldrich) was added to the samples, followed by vortexing and incubation at 37 °C for 30min. Following lysozyme treatment, 50 µl of 10% SDS and 5 µl of 25 mg/ml proteinase K was added and the whole cell solution was incubated in a 55 °C water bath for 30 min to lyse the cells. After proteinase treatment, 350 µl of buffer RLT was added to the samples, vortexed and centrifuged at 14 000 rpm for 2 min. The supernatants were

carefully removed, and 250 µl of 100 % ethanol was added to the samples as described in the kit manual for bacteria. The samples were then immediately loaded onto RNeasy spin columns. Total RNA was eluted in 30 µl of RNase free water according to the kit protocol, and the concentration and purity of the RNA preparations was determined using Nanodrop spectrophotometer (NanoDrop 1000, Thermo Scientific). Nucleic acids have their maximum absorbance at 260 nm, proteins at 280 nm and other contaminants such as alcohols absorb at 230 nm (NanoDrop 1000 Manual). The absorbance intensity at 260 nm determines RNA quantity, while a 260/280 nm absorbance ratio is used to determine RNA purity, where a ratio of 2.0 or above is considered pure for RNA. The samples were then immediately treated with DNase using Turbo DNase (Invitrogen, California, United States). After DNase treatment, the RNA concentration was measured on a Nanodrop spectrophotometer and the samples were stored at -80 °C until use. For isolation of total RNA from *B. producta,* the same protocol was modified by extending incubation time with lysozyme to 60 min followed by a bead-beating step (Mini-Beadbeater-24, Biospec). After adding lysis buffer RLT, the samples were transferred to a FastPrep lysing matrix B tube (0.1mm silica beads, MP-Bio), vortexed and bead beat for 1 min. The tubes were then centrifuged for 2 min at 14 000 rpm, the supernatants transferred to new tubes and centrifuged at 14 000 rpm for 1 min to ensure that no solid material is carried over to the spin column.

2.6 16 rRNA sequencing

To confirm the identity of *E. fissicatena* its 16s rRNA gene was amplified by PCR and sequenced. A single colony from a blood agar plate was resuspended in 200 µl of H_2O and microwaved for 4 minutes. The sample was centrifuged at 13 000 rpm for 5 min and 2 µl of the supernatant was used as a template in the following PCR reaction. DreamTaq PCR reagent kit (Thermo Scientific) was used for amplification following kit protocol. A universal 16s rRNA forward primer 15F (5’ - ACGGGAGGCAGCAG -3’) and the reverse primer 4R (5’ - ACGGGCGGTGTGTRC - 3’) were used to generate an amplicon of about 1 000 base pairs. A single PCR product was confirmed on an agarose gel before sequencing.

2.7 RNA sequencing

For the differential expression study of *B. producta,* samples were taken 6 hours after inoculation at an OD_{600} of 0.21 representing logarithmic growth phase, and after 27 hours at

OD_{600} of 0.733 representing the onset of spore-like formation at stationary phase. For sequencing of total RNA, the 'NEBNext Ultra RNA library preparation kit for Illumina' (New England Biolabs) was used following the manufacturer's instructions. To determine the incubation time for fragmenting the samples into ~200 nucleotide inserts, based on RNA integrity, the RNA preparations were examined using a Bioanalyzer (Agilent Technologies, California, United States) with RNA-nano 6000 chips. Based on RIN values of 8.3, a 15 min fragmentation time at 94 °C for intact RNA was chosen. To determine the input amount of RNA, the total RNA concentrations were measured using Qubit RNA HS Assay Kit (Life Technologies, California, United States). Final sequencing was performed on a MiSeq sequencer (Illumina, California, United States) using paired-end 300 base pair reads.

2.8 Differential-expression

Analysis of sequencing data was done in a Linux environment (Appendix A) because the tools have dependencies that are native to Linux systems. This can be setup on a Windows machine for temporary use through a VMware player (www.vmware.com, freeware). Quality assessment of sequenced reads was done with FastQC (Babraham Bioinformatics). To align sequenced reads to a reference genome the Bowtie2 [16] tool was used by indexing the genome and doing a paired-end alignment. Cufflinks [16] package was used to assemble reads into transcripts and measure the difference in expression levels between samples. Expression data was managed and visualized using the R package cummeRbund.

2.9 Comparative analysis

Comparative studies of homologous genes for sporulation and germination in *B.producta* and *A.schaalii* were done by aligning protein sequences of characterized proteins from Bacillus and Clostridia against their genomes. NCBI's BLAST search was done using the tblastn module with a protein query aligned against a translated nucleotide database of the target genomes. Standard settings were used with an expected E-value threshold of 10^{-6}. Protein sequences were obtained from the UniProtKB database, preferably Swiss-Prot reviewed sequences where available (Appendix B). All (18) available genomes of *Listeria spp.* from the NCBI genome database were used as controls for finding non-redundant genes that are specific to spore formers. For hits with high sequence identity to known germination

receptors, a reverse search was done by blasting nucleotide sequences of 1000 nucleotides in the flanking regions under the assumption that germination genes may be expressed in a clustered, e.g. tricistronic fashion such as in *B. subtilis*. This was done using blastx module for searching protein databases with a translated nucleotide query.

2.10 Electron microscopy

Scanning electrom microscopy was performed at the University of Oslo's EM-lab. Samples for SEM were prepared by washing spore suspensions and vegetative cells from an overnight culture three times with distilled water followed by fixation overnight at 4°C with 2.5% glutaraldehyde in Ca-codylate buffer (0.05 M). Dehydration of the samples was performed by personnel at the EM-lab by exposing the samples to an increasing ethanol concentration, starting at 30 % and finishing with 100 % anhydrous ethanol. To further dry the samples without destroying their structure due to surface tension, a critical point drying method with pressured $C0_2$ was used which replaces remaining liquid content in the samples with liquid $C0_2$. The specimens were then mounted onto metal stubs and sputter coated with conductive palladium material. Imaging of SEM samples was done on a Hitachi S-4800 microscope (Hitachi High Technologies, Schaumburg, United States).

3. Results

3.1 Cultivation of strains

Strains were resuscitated from the frozen state on blood agar plates. *B. producta* did not form visible colonies on blood agar from the stock frozen at -80 °C after three culturing attempts. However, subsequent spreading from the inoculated plates, with no visible growth, onto fresh blood agar plates showed the appearance of very small colonies after anaerobic incubation at 37°C overnight. *E. fissicatena*, *B. producta* and *L.umeaensis* strains showed turbid growth when inoculated from blood agar cultures to Brucella and BHI broths, as well as colony formation on Brucella and BHI agar plates. For all the three strains, slower growth was observed in Robertson and FTM broths.

During the study, one of the Clostridium strains was identified by 16s rRNA sequencing to be *Actinotignum schaalii* and not *E. fissicatena*, which was later confirmed by CCUG. *A. schaalii* is reported to be non-sporulating in the literature. The sporulation and germination tests were done before the identity of the strain was known. Interestingly, however, several close family members of *A. schaalii* are spore-formers.

Similarly, the bacterium which was recovered on blood agar, and thought to be *B. producta*, was during the project identified by RNA sequencing to be contamination with *Staphylococcus epidermidis*. Staphylococci are described to be non-sporulating in literature, although several strains including *S. epidermidis* have been recently reported to enter a VBNC state.

3.2 Induction of sporulation

As a starting point, the conditions that induce sporulation in clostridial endospore-forming model organism *C. perfringens,* described in section 2.2, were tested on *A. schaalii*, *S. epidermidis* and *L. umeaensis*. The strains were first incubated in Robertson broth for one week. Some turbidity and precipitate, indicating growth, was observed in *A. schaalii*, *S. epidermidis* and *L.umeaensis* cultures. Subsequent heat treatment and incubation in FTM media resulted in no observed growth, neither in the following Duncan Strong broth incubation.

As shown in Figure 4, signs of endospore formation such as swollen and phase-bright cells were observed under phase-contrast microscope in Brucella, FTM and Robertson cultures incubated for one week. However, heat treatment of the cultures, at 77 °C for 20 min, followed by anaerobic incubation at 37 °C on blood agar plates, to test for growth of spores, showed no colony formation for any of the strains.

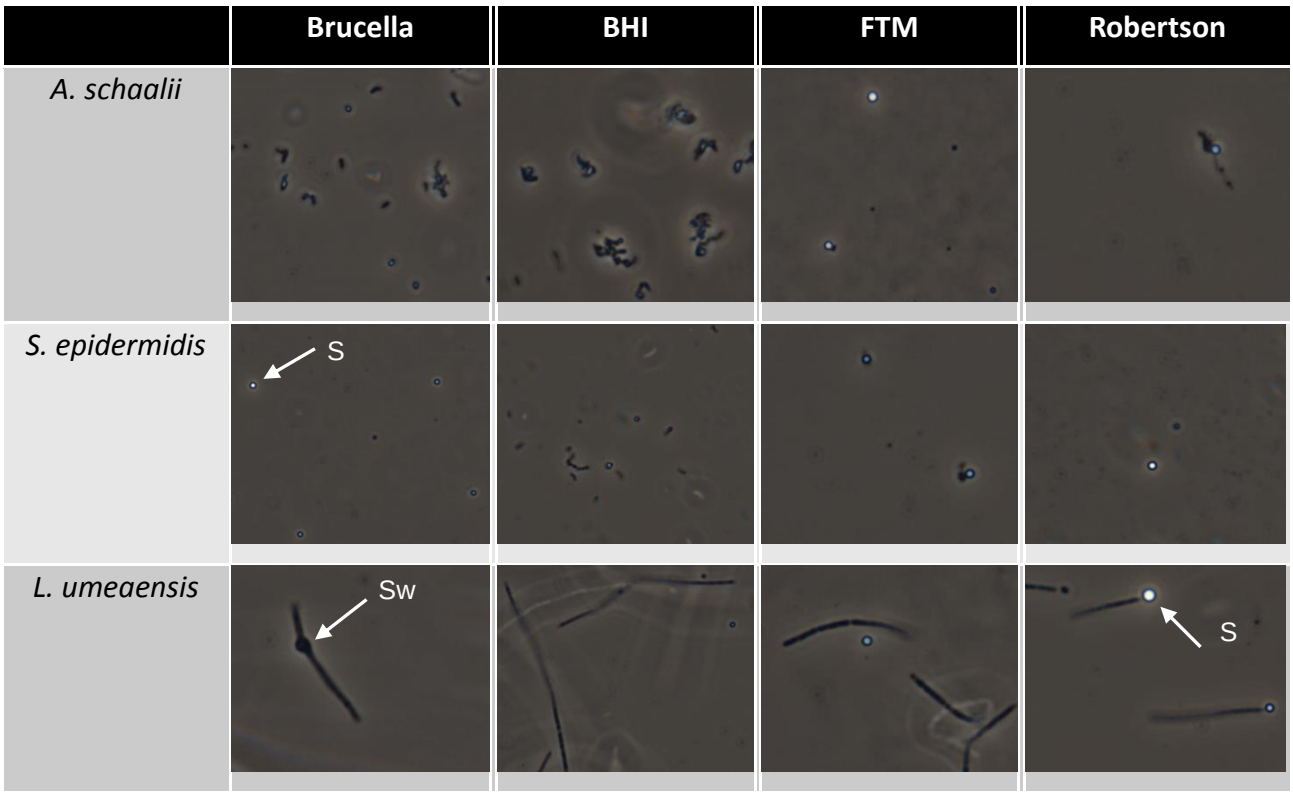

Figure 4. Cultures of *A. schaalii*, *S. epidermidis* and *L. umeaensis* incubated in different media for one week as seen with phase-contrast microscopy. The white arrows indicate S: Spores. Sw: Swollen cells.

3.3 Purification of crude spore suspensions of *A. schaalii* and *S. epidermidis*

Initial attempts to isolate and concentrate spores by centrifugation or washing (Procedures described in Materials and Methods section 2.3) were inefficient. Centrifugation at various speeds and times did not enrich for spore-like cells in either the pellet or supernatant as the same proportion of cells to spores was observed. Repetitive washing and centrifugation also resulted in an extensive loss of sample material. Equal proportions of spores and vegetative cells were observed in the hydrophobic PEG phase in the two-phase system as well, which took extensive washing to remove PEG/phosphate residues. Lysozyme digestion appeared to

only partly degrade the cells without fragmenting them, and required several washes to remove the debris, making it highly inefficient.

3.4 Sporulation

3.4.1 *A. schaalii*

Brucella media was chosen for further culturing and optimization of sporulation, as spore-like cells were observed for the three strains when grown in this media. Increasing the amount of spore-like cells was first attempted by simulating stationary culture conditions by increasing the cell density. Increased cell density, however, did not induce any increased concentrations of phase bright spore-like cell types. Addition of 1% Na-acetate appeared to swell the cells and reduced clumping, but no spore-like structures could be observed.

Divalent cations such as calcium have previously been shown to promote spore formation [24]. When supplementing the growth media with 0.5% w/v $CaCl_2$ followed by incubation overnight, phase bright spore-like cell types that composed less than 1% of total cells appeared (Figure 5). Increasing the concentration of $CaCl_2$ did not increase the yield of phase bright cells. Other cations tested did not appear to have any effect, while SO_4^{2-} anions inhibited growth.

Brucella broth cultures containing 0.5% w/v $CaCl_2$ were further supplemented with thiamine or glucose in an attempt to increase the fraction of spore-like cell types. The presence of thiamine has previously been reported to promote sporulation in *Bacillus* and *Clostridium* species [25, 26]. Addition of 1 ppm thiamine increased phase-bright cell forms to about 10% of total cells. Varying thiamine concentrations ten-fold had no noticeable effect. The addition of small amounts of glucose [26] to sporulation medium has also been shown to increase total spore yields for *B. subtilis*. Supplementing growth medium with glucose increased cell density, but fewer phase-bright cell forms were observed.

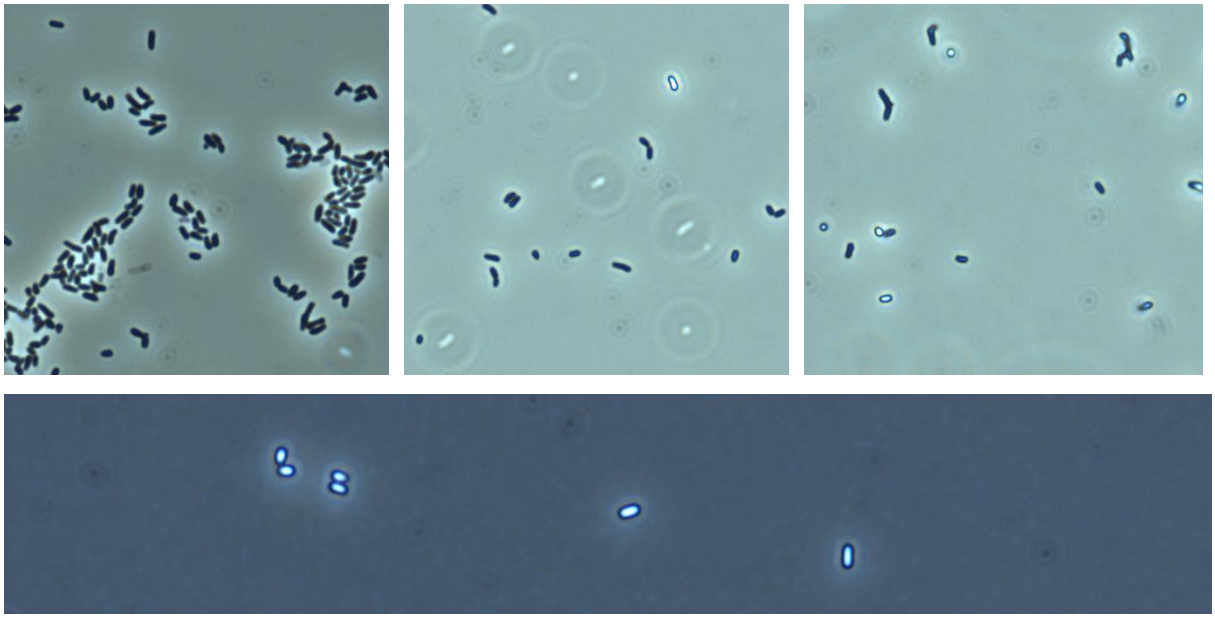

Figure 5. Appearance of phase-bright cell forms in *A.schaalii* cultures incubated for two days. Left: Brucella medium. Middle: Brucella medium supplemented with 0.5% $CaCl_2$. Right: Brucella medium supplemented with 0.5% $CaCl_2$ and 1 ppm thiamine. **Bottom**: For comparison, *B. subtilis* endospores.

Heat-shocking cultures, at sub-lethal temperatures, increased the yield of spore-like cell types. Incubation at 60°C for 5 min resulted in a maximum increase in phase bright *A. schaalii* cells. Notably, the positive effect of heat-shock, on the formation of spore-like cells, was greatly diminished when heat-treated cultures (60 °C for 5 min) were immediately cooled down in a water-bath at room temperature prior to incubation at 37 °C (Figure 6).

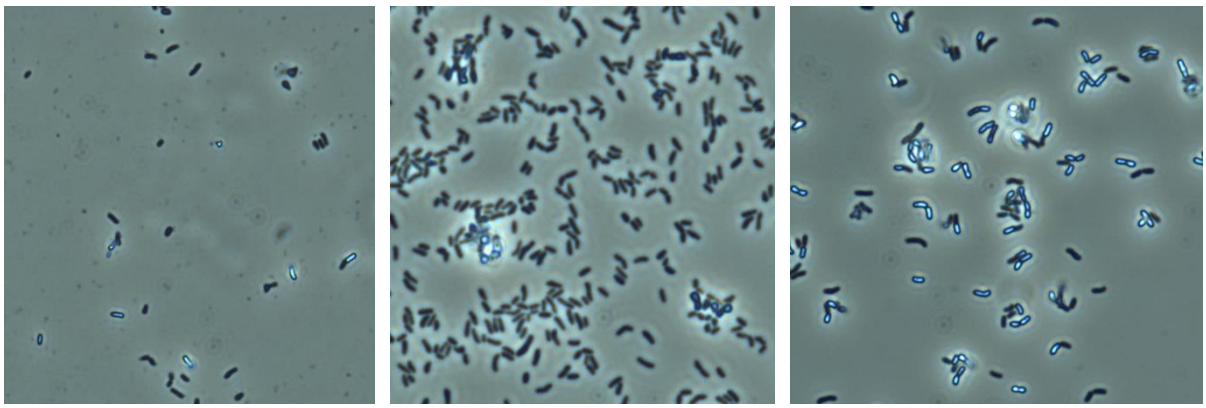

Figure 6. Effect of heat-shock at 60°C for 5 minutes on *A. schaalii* cultures incubated overnight. Left: Brucella medium. Middle: Brucella medium with $CaCl_2$ and thiamine cooled before inoculation. Right: Brucella medium with $CaCl_2$ and thiamine.

3.4.2 *S. epidermidis*

Phase-bright cell forms were observed in *S. epidermidis* cultures grown in Brucella broth for more than 24 h (Figure 7). At the end of the exponential growth phase, the cultures underwent major auto-lysis where the vegetative cell count decreased while the proportion of phase bright cells increased. After five days of incubation in Brucella broth, only the phase-bright cells could be observed as well as a large amount of cell debris.

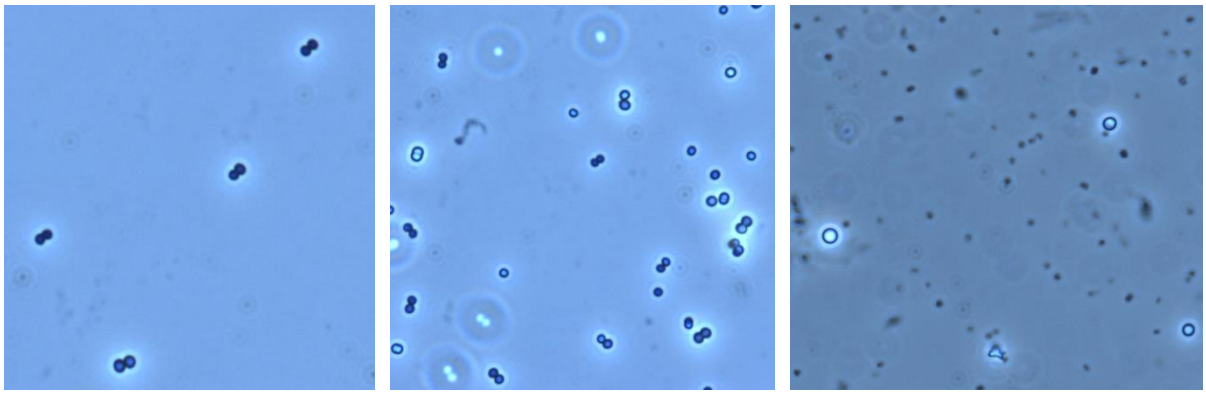

Figure 7. Appearance of phase-bright cells in aging *S. epidermidis* cultures. Left: Cells after 8 hours of culturing. Middle: Cells after 27 hours of culturing. Right: Cells after 6 days of culturing.

3.4.3 *L. umeaensis*

Incubation in Brucella media for more than three days resulted in a swollen cell morphology (Figure 8), but no further development of spore-like structures were observed with prolonged incubation. Supplementing the growth medium with $CaCl_2$ and thiamine increased the degree of cell swelling and some bright elliptical cell forms could be observed by phase-contrast microscopy after incubating the cultures for at least 5 days in anaerobic conditions at 37°C.

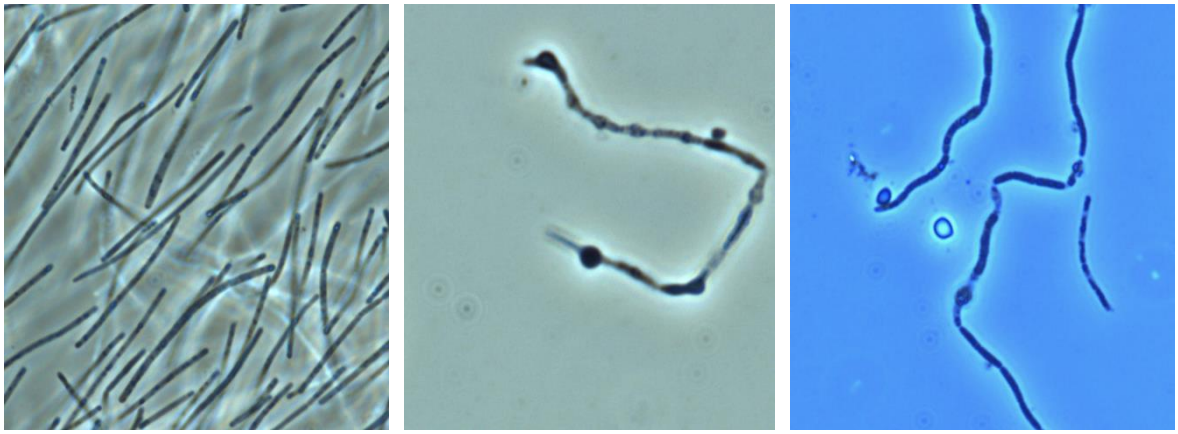

Figure 8. Swelling and phase-bright cells in aging *L. umeaensis* Brucella broth cultures incubated under anaerobic conditions at 37°C. Left: Overnight culture. Middle: Culture incubated for three days. Right: Culture incubated for one week in media supplemented with $CaCl_2$ and thiamine.

3.5 Density gradient purification of *A. schaalii* and *S. epidermidis* spores

Gradient separation of cultures containing a high degree of spore-like cells resulted in a low-density band containing vegetative cells and a higher density band dominated by spore-like structures (Figure 9). Plating the purified cell suspensions on blood agar showed only a few contaminant colonies. After storing purified spores suspended in water at 4°C for a week no viable colony forming units were observed.

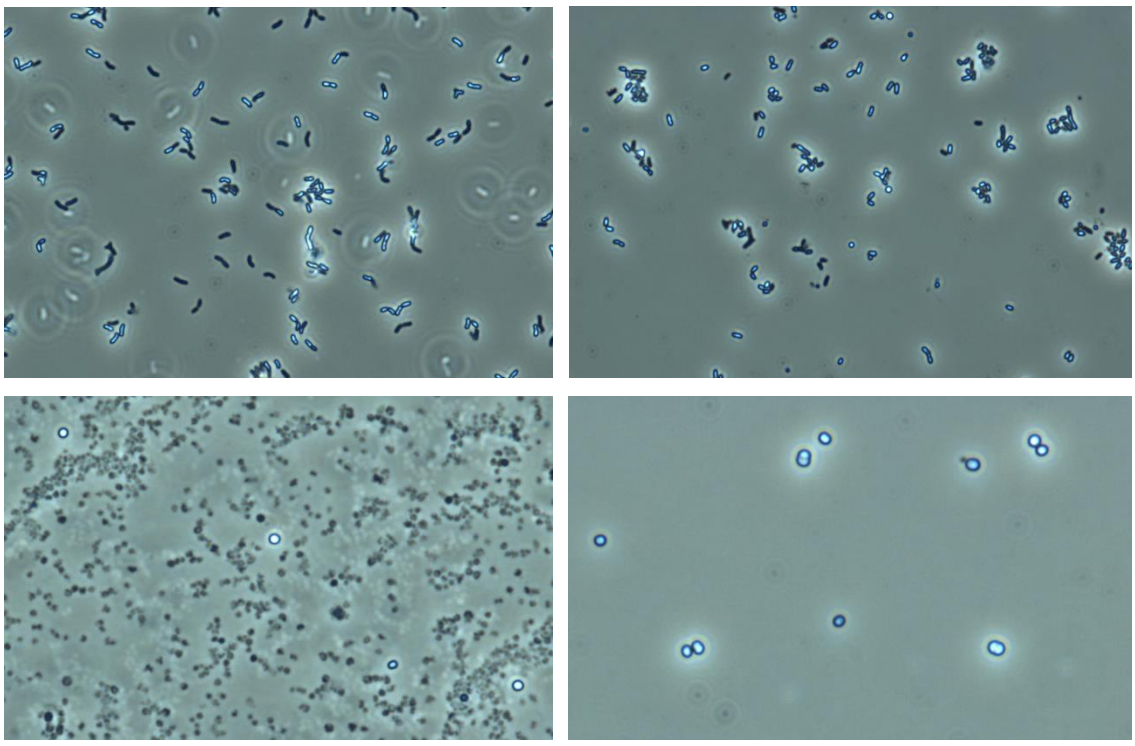

Figure 9. Purification of *A. schaalii* (top) and *S. epidermidis* (bottom) spores using Nycodenz density gradient. Left: Sporulating culture. Right: After density gradient centrifugation.

3.6 Resuscitation of *A. schaalii* and *S. epidermidis* spores

The spore like structures of *A. schaalii* and *S. epidermidis* could not be resuscitated by spreading on either blood agar or Brucella agar, nor by attempts to culture them in Brucella or BHI broth media. Exposure to various nutrient combinations often induced partial darkening of the cells, when observed by phase contrast microscopy, but no colony forming units could be recovered following inoculation into growth media. It has been shown in previous studies that endospores of *Bacillus* and *Clostridium* species can be germinated, by permeabilizing their protein coat, and exposing the spore cortex to lysozyme [32, 33], regardless of endospore germinat receptors. Chemical removal of the protein coat was performed, but while the spore-like structures remained intact and phase bright after treatment, phase darkening was not induced by the following exposure to lysozyme.

3.7 Scanning electron microscopy of *A. schaalii* and *S. epidermidis*

A. schaalii and *S. epidermidis* vegetative cells and spore suspensions were observed by SEM to image their outer structure. Vegetative *A. schaalii* cells appeared as short rods with a rough, wrinkled surface (Figure 10, Top). Indented cell division rings were seen on the surface. An additional extruded ring was present on a small portion of the cells. In contrast to vegetative cells, the spore-like cell types appeared to have a smooth surface and most of them were greatly elongated. Several protrusions were observed, resembling budding (Figure 10, Bottom).

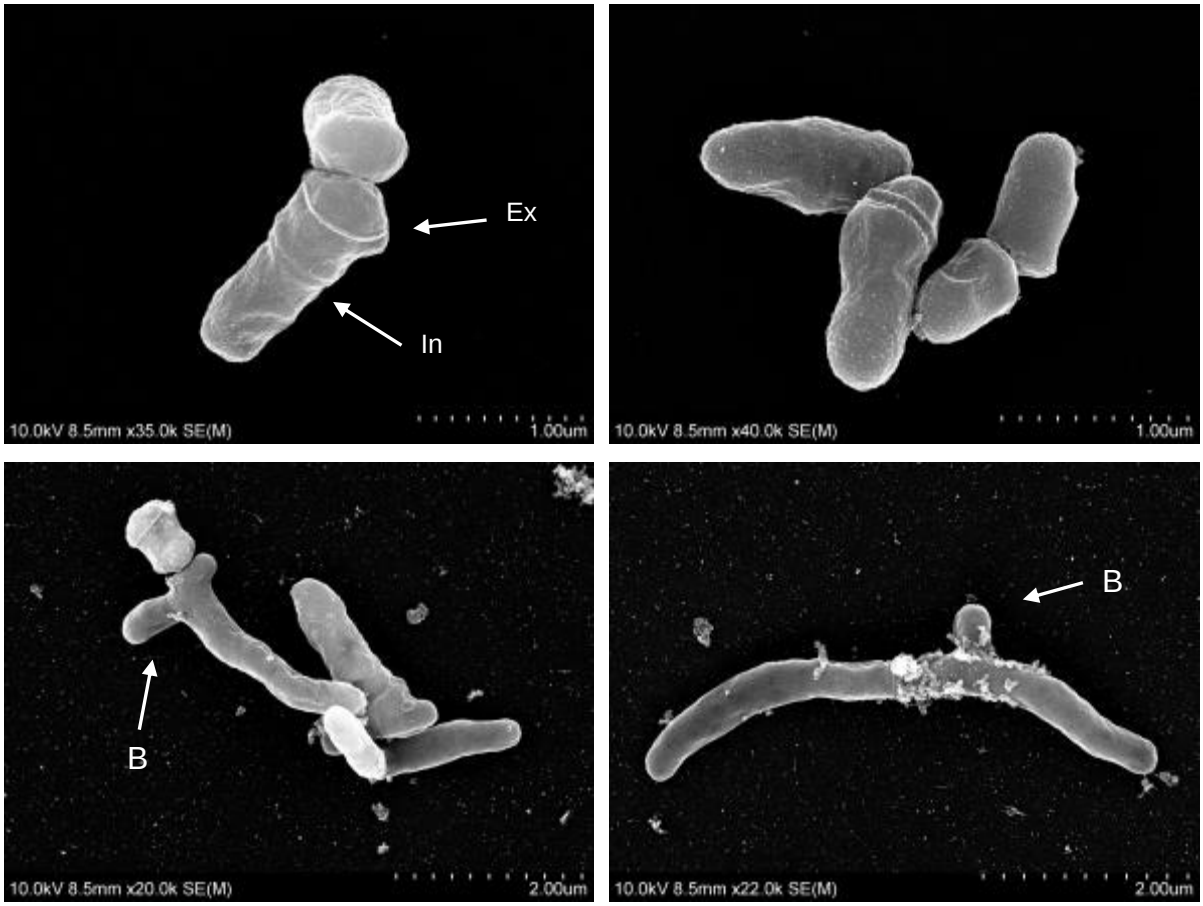

Figure 10. SEM imaging of *A.schaalii* vegetative cells (Top) and spore-like cell forms (Bottom). The white arrows indicate Ex: extruded rings. In: indented rings. B: buds.

The vegetative cells of *S. epidermidis* were uniform in size with a grainy surface. Indented cell division rings were present. In comparison, most of the spore-like forms had wrinkled, folded surfaces with protrusions and were slightly smaller in size (Figure 11). Varying amounts of appendages were seen attached to the microscope stage surface and other cells

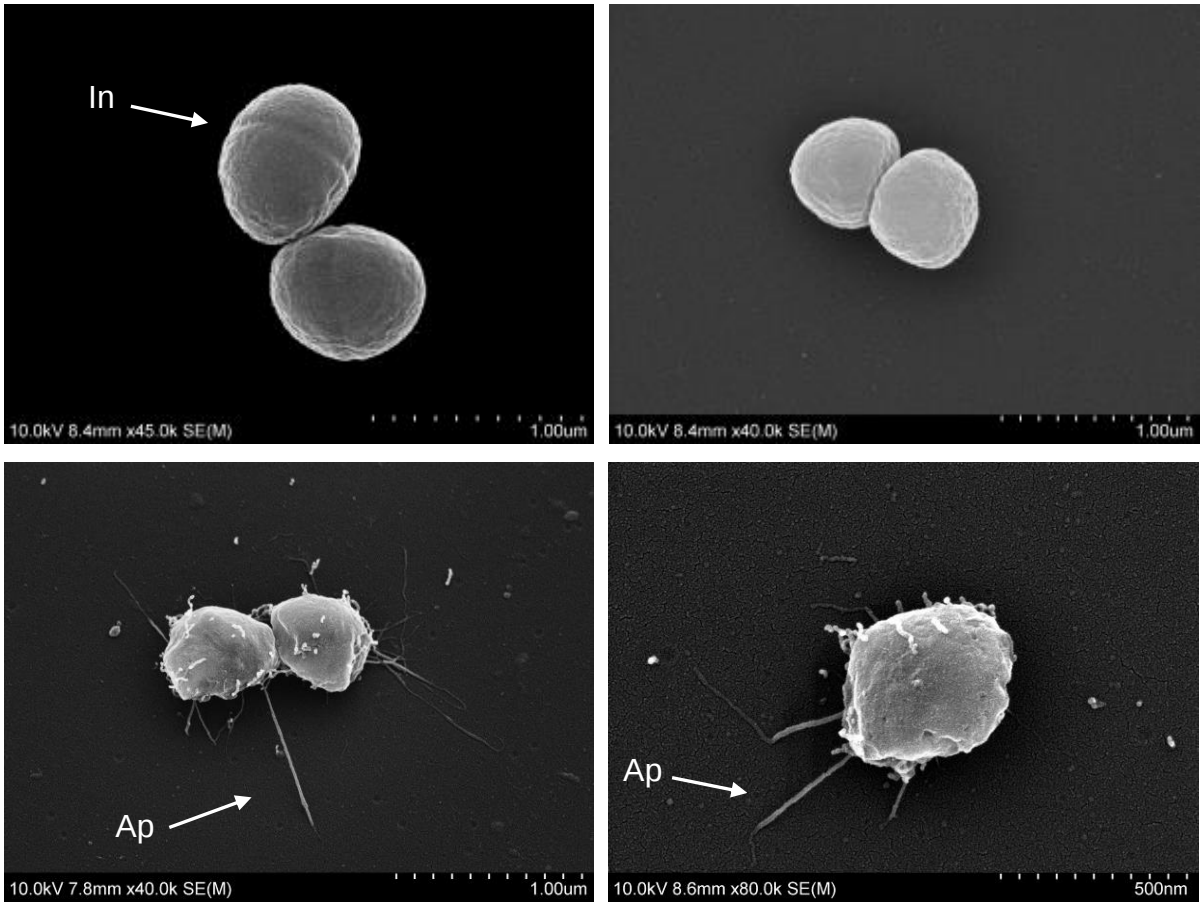

Figure 11. SEM imaging of *S. epidermidis* vegetative cells (top) and spore-like structures (bottom). The white arrows indicate In: Indented ring. Ap: appendages.

3.8 Comparative study of sporulation genes

To assess the presence of known sporulation, germination and spore structure genes from *Bacilli* and *Clostridia*, a comparative homology approach was used. Comparison to the *Listeria* genus was done to assess which genes may not be unique identifiers of sporulation (Table 3).

Table 3. Know sporulation genes present in *B. producta* and *A. schaalii*. Genes that appear present in all sporulating bacilli and clostridia are shown in bold [10]. Homologous genes also found in non-sporulating bacteria are shown underlined.

	A. schaalii	*B. producta*	*Listeria spp.*
Stage 0	***spo0A*, *spo0J*, *obgE***	***spo0A*, *sigH*, *spo0J*, *obgE***	***spo0A, sigH, spo0J, obgE***
Stage II	***sigF*, *sigE***	***spoIIAA*, *spoIIAB*, *sigF*, *spoIID*, *spoIIE*, *spoIIGA*, *sigE*, *spoIIR***	***spoIIAA, spoIIAB, sigF, spoIIE, sigE***
Stage III	***dapA*, *dapB*, *spoIIIE*, *spoIIIJ*, *sigG*, *jag***	***cwlD*, *dacB*, *dapA*, *dapB*, *dpaA, dpaB, spmA, spmB, spoIIIAA, spoIIIAD, spoIIIAE, spoIIID*, *spoIIIE*, *spoIIIJ*, *sigG*, *jag***	***cwlD, dacB, dapA, dapB, spoIIIE, spoIIIJ, sigG, jag***
Stage IV	***sigK***	***spoIVA, spoIVB, sigK***	***sigK***
Stage V	***spoVC*, *spoVD*, *yncD***	***spoVAC, spoVAD, spoVAEB, spoVB*, *spoVC*, *spoVD*, *spoVG*, spoVK, *spoVT*, *stoA*, *ylbJ*, *yqfD*, *ytvI*, *yyaC*, *yncD***	***spoVB, spoVC, spoVD, spoVG, spoVT, ytvI, yncD***
SASPs		*ssp1-3, ssp4*	
Coat	*yckK*, *yhaX*	*yhdD*, *yisY*, *yjqC*	*lipC, yckK, yhaX, yhbA, yhbB, yisY*
Coat maturation	*spsI*, *spsJ*, *spsK*	*dacB*, *spsC*, *spsD*, *spsI*, *spsJ*, *spsK*	*dacB, spsA, spsC, spsE, spsI, spsJ, spsK*
Cortex		*cwlH*, *cwlC*, *cwlD*, *sleB, ylbJ, yqfD, sleC, sleL, lytH, sleM*	*cwlA, cwlH, cwlC, cwlD*
Germination	***igt***	*gerAA/BA/KA*, ***igt***, ***gpr***, *yndD, yfkQ, cspC, cspBA*	***igt***

The majority of genes essential for sporulation in model spore-formers are present in *B. producta,* but many of the early-stage sporulation genes also have homologs in the non-sporulating *Listeria* genus. Endospore-associated genes, expressed in later stages of sporulation, are present in the *B. producta* genome. Among these genes are homologs for dipicolinic acid synthesis genes *dpaA*/*dpaB*, clostridial SASPs *ssp1-3* and *ssp4*, cortex-lytic enzymes *sleB*/*sleC*/*sleL*/*sleM* and various germination associated genes.

All homologs to sporulation genes, found in *A. schaalii,* could also be found in the *Listeria* genus, which makes it unlikely that the species is capable of endospore formation.

No homologs were found in *B. producta* for several conserved genes, essential for sporulation in *B. subtilis*, such as *spoIIM/spoIIP* anchor protein genes and genes encoding *spoIIIAB-H* gated-channel components required for forespore engulfment [10, 34] (Table 4).

Table 4. Missing genes from *B. producta* genome that appear to be conserved in sporulating bacilli/clostridia [10].

	B. producta
Stage II	*spoIIM, spoIIP*
Stage III	*spoIIIAB, spoIIIAC, spoIIIAF, spoIIIAG, spoIIIAH*
Stage V	*spoVS, yabP, yabQ, ylmC, yqfC*

3.9 Gene expression studies

3.9.1 RNA isolation

To study gene expression in exponentially growing and spore-like cells of *A. schaali* and *S. epidermidis,* total RNA was isolated. The RNA quality was determined as shown in Figure 12 and 13.

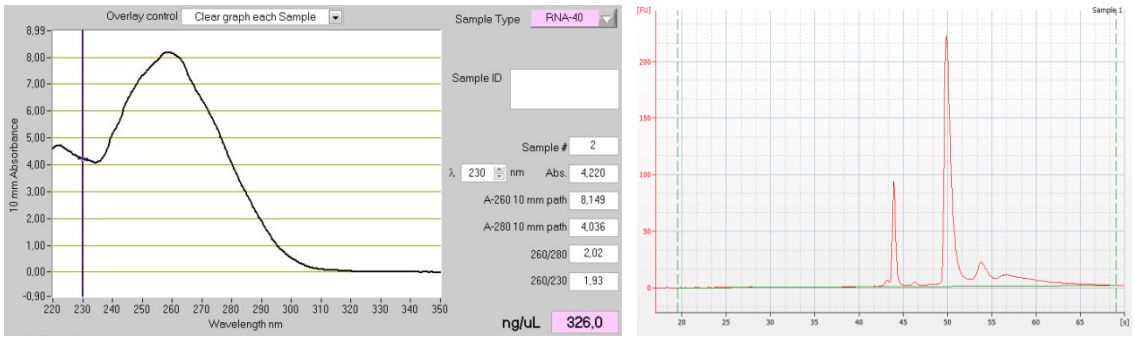

Figure 12. Total RNA isolated from *A. schaalii* as quantified on NanoDrop and Bioanalyzer after DNase treatment.

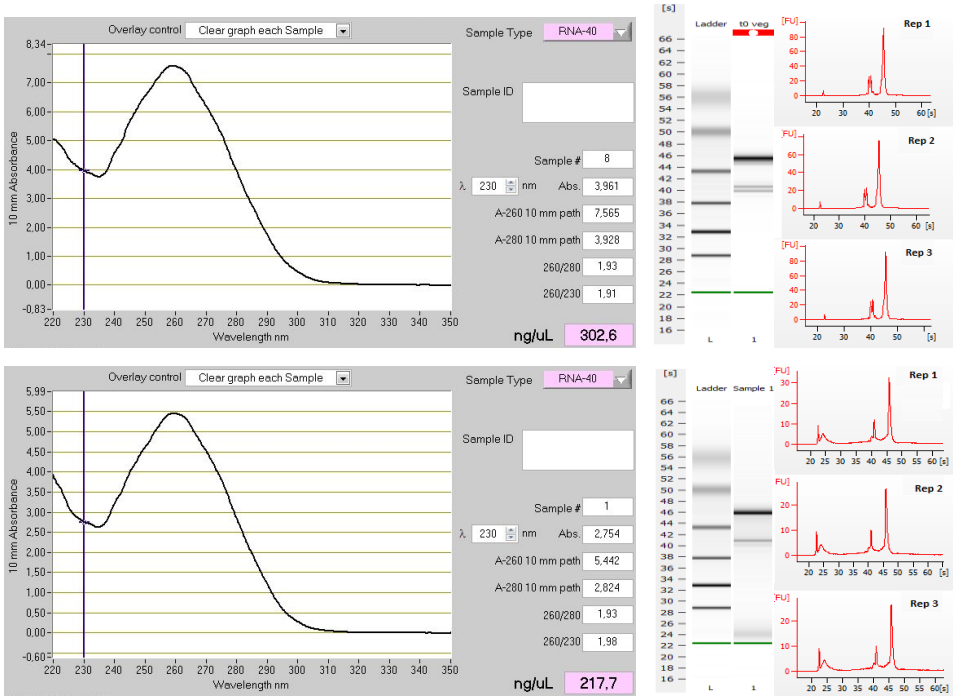

Figure 13. Total RNA isolated from *S. epidermidis* as quantified on NanoDrop and Bioanalyzer after DNase treatmenr. Upper: RNA isolated after 6 hours. Lower: RNA isolated after 27 hours.

Relatively pure RNA was isolated from *A. schaalii* and *S. epidermidis* cultures with a 260/280 nm absorbance ratio of 1.9-2.0, and a low absorbance peak at 230 nm. When running Bioanalyzer, uneven 16s to 23s rRNA ratios were measured between samples taken at 6h and 27h for *S. epidermidis* (Figure 13).

3.9.2 16S rRNA sequencing

Initially, it was planned to perform qPCR experiments to see if sporulation genes were expressed during the appearance of the spore-like cells. Attempts to amplify select sporulation genes, using primers complementary for *E. fissicatena,* were unsuccessful, and after extensive troubleshooting of the PCR setup and annealing temperatures, it was decided to perform 16s rRNA sequencing of *E. fissicatena*. Sequencing of the 16s rRNA gene identified the bacteria to be *A. schaalii* strain CCUG 27420 and not *E. fissicatena*. Due to time limitations, RNA sequencing was then performed directly for *B. producta.* Aligning sequenced reads to *B. producta* genome resulted in only 4% alignment rate. The longest sequenced reads of rRNA with highest frequency were used to blast the NCBI database resulting in a 100% match to *S. epidermidis*. Running the alignment of sequenced reads to *S. epidermidis* genome resulted in a 95% alignment rate.

3.9.3 Differential expression

To see which genes were up or downregulated during the transition of *S. epidermidis* vegetative cells to the spore-like forms, RNA sequencing data was analyzed using Cufflinks. The distribution of assembled transcripts was assessed using a volcano plot (Figure 14-A). Outliers seen beyond 2^{20}-fold change are genes either completely downregulated or newly expressed after 27 hours compared to samples taken after 6 hours. Out of 2729 annotated genes in the *S. epidermidis* genome, 1763 were detected as transcripts. Among the differentially expressed genes, 54 were significantly upregulated and 119 were significantly downregulated. Variance between technical replicates for significant differentially expressed genes was visualized in a heat-map (Figure 14-B). The rRNA/tRNA genes were manually removed from the dataset. They were identified in total RNA samples as outliers having FPKM values 100-fold above average transcript levels.

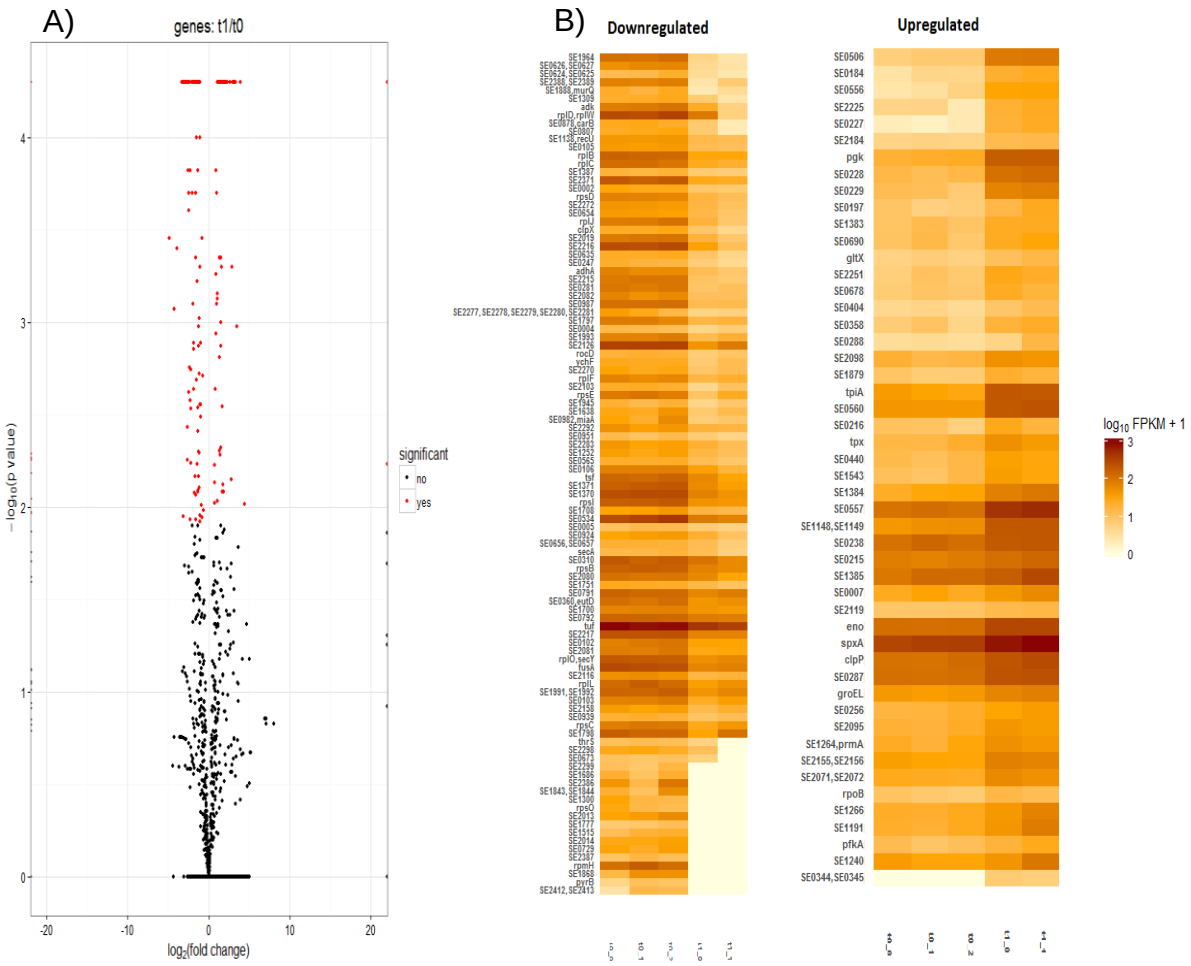

Figure 14. A) Volcano plot of all assembled transcripts. B) Heat-map of transcript levels between replicates and samples in significantly up or down-regulated genes.

3.9.4 Upregulated genes

Significantly upregulated genes were extracted from the dataset and sorted by fold-change in expression levels together with their relative transcript values in FPKM (Figure 15). The newly expressed genes SE0344 (AMP-binding domain), SE0345, and the most upregulated gene SE0227 (membrane transport domain), as well as the highly upregulated genes SE1148 (metallopeptidase domain) and SE2251 have no known function. The majority of top upregulated genes are involved in metabolism and substrate degradation, especially sugars, followed by stress response proteins and transcription factors. Other notable upregulated genes were proteases, such as SE0184 which encodes for an extracellular cysteine protease that may be involved in colonization of tissues [35, 36].

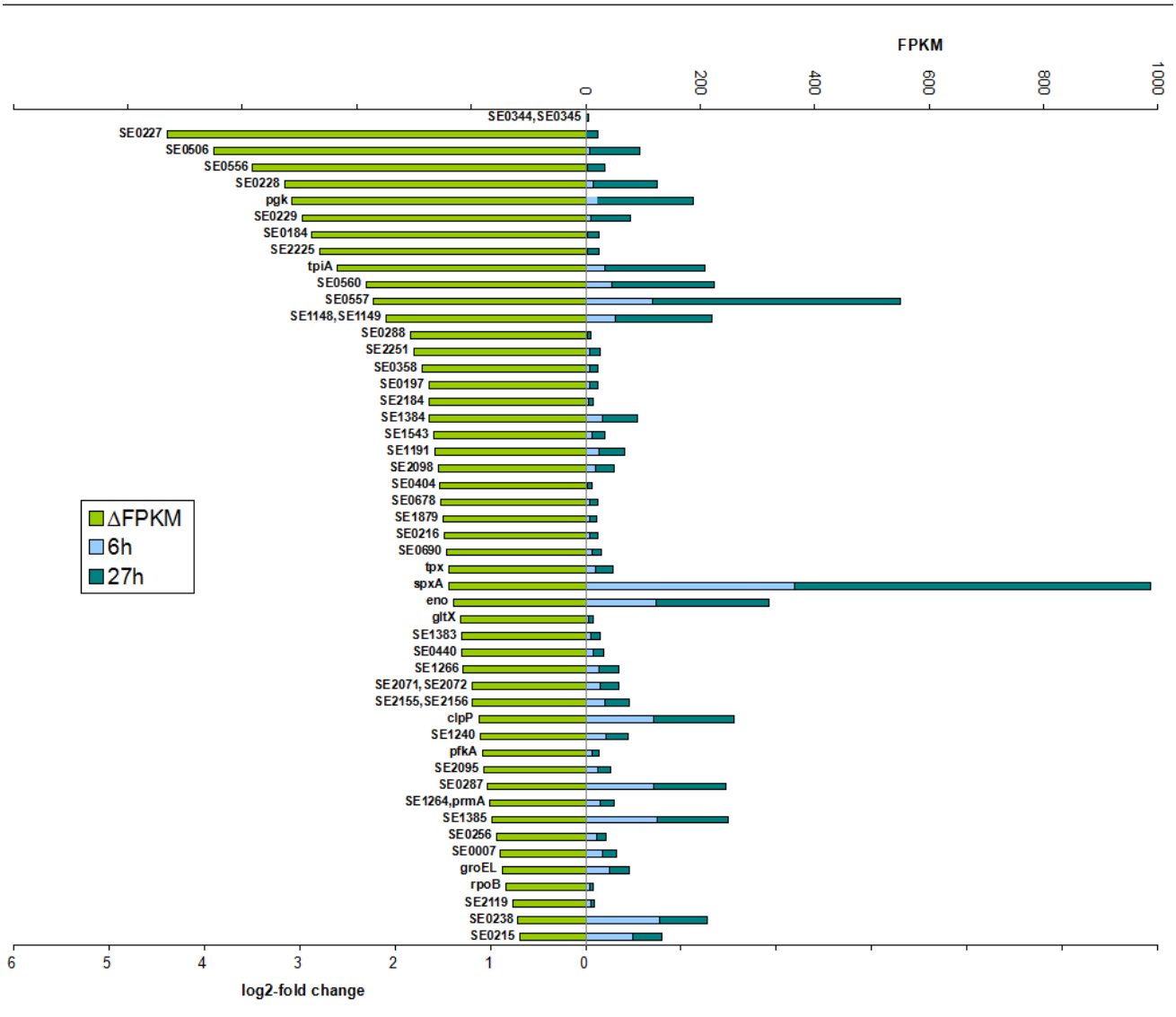

Figure 15. Relative abundance and fold-change of significantly upregulated genes. Transcripts

expressed as Fragments Per Kilobase of transcript per Million mapped reads. Significance threshold set at p value < 0.05.

Significantly upregulated genes were grouped into functional categories (Table 5). The largest functional category of upregulated genes (44.4%) is involved in metabolic processes such as degradation of sugar or protein substrates or in lipid biosynthesis. A fraction of 14.8% of the upregulated genes was involved in stress response performing DNA repair, antioxidation and protein folding. Remaining upregulated genes were grouped with functions in cell transport (5.6%), transcription (5.6%) and various regulatory and extracellular proteins (13%). Of all significantly upregulated genes 16.7% had no predicted function.

Table 5. Functional categories of significantly upregulated genes in the spore-like cell forms of *S. epidermidis*. Many of the gene functions are predicted from conserved domains and grouped by their most likely function.

Gene symbol	Function	Gene name/locus	Function
[3/54]	**Cell transport 5,6 %**	**[7/54]**	**Other 13 %**
SE0404	ion transporter-like	*prmA*	Ribosomal protein L11 methyltransferase
SE0440	oxygen transporter-like	SE0184	Extracellular cysteine protease
SE2119	Copper-exporting P-type ATPase A	SE0358	Chlorite dismutase
[24/54]	**Metabolism 44,4 %**	SE0690	thimet oligopeptidase-like
SE2184	Dihydrolipoamide dehydrogenase	SE1264	Methyltransferase E
clpP	ATP-dependent Clp protease proteolytic subunit	SE1383	X-Pro aminopeptidase
eno	Enolase	SE1543	Glutamyl endopeptidase
gltX	Glutamyl-tRNA synthetase	**[8/54]**	**Stress response 14,8 %**
pfkA	6-phosphofructokinase	*groEL*	60 kDa chaperonin
pgk	Phosphoglycerate kinase	SE0288	DNA repair protein radA
SE0007	Seryl-tRNA synthetase	SE1240	Superoxide dismutase [Mn/Fe]
SE0197	Acetoin(diacetyl) reductase	SE1266	Chaperone protein dnaJ
SE0215	Pyruvate formate-lyase-activating enzyme	SE1385	Putative universal stress protein
SE0216	Probable succinyl-diaminopimelate desuccinylase	SE1879	Putative 2-hydroxyacid dehydrogenase
SE0228	Carbamate kinase 1	SE2071	Putative aldehyde dehydrogenase aldA
SE0229	Ornithine carbamoyltransferase	*tpx*	Probable thiol peroxidase
SE0256	2-oxoacid dehydrogenase family	**[3/54]**	**Transcription 5,6 %**
SE0287	ATP-dependent Clp protease ATP-binding subunit clpC	*rpoB*	DNA-directed RNA polymerase subunit beta
SE0344	Putative long chain fatty acid-CoA ligase vraA	SE0556	sugar-binding transcription factor
SE0557	Glyceraldehyde-3-phosphate dehydrogenase 1	*spxA*	Regulatory protein spx
SE0560	2,3-bisphosphoglycerate-independent phosphoglycerate mutase	**[9/54]**	**Unknown 16,7 %**
SE0678	Fatty acid biosynthesis	SE0227	Hypothetical membrane protein
SE1191	alpha-glucosidase	SE0238	Hypothetical membrane transport protein
SE1384	Alanine dehydrogenase	SE0345	unknown
SE2098	alcohol dehydrogenase	SE1148	unknown
SE2156	Fructose-bisphosphate aldolase class 1	SE1149	unknown
SE2225	short-chain dehydrogenase	SE2072	unknown

tpiA	Triosephosphate isomerase	SE2095	unknown
		SE2155	unknown
		SE2251	unknown

3.9.5 Downregulated genes

Several genes were completely downregulated in the spore-like cell forms of *S. epidermidis* including four hypothetical transcription factors SE1868, SE1686, SE2299 and SE2386, two genes similar to extracellular murein hydrolase-inhibitors (SE2013 and SE2014), as well as metabolic and uncharacterized genes (Figure 16). The majority of downregulated genes may be associated with exponential growth such as rapid nutrient turnover, protein synthesis, DNA replication and cell division (Table 6). A fraction of 40.3% of all significantly downregulated genes were involved in metabolism. In particular, many synthases were downregulated. Genes involved in transport of nutrients and energy consumption were significantly downregulated as well.

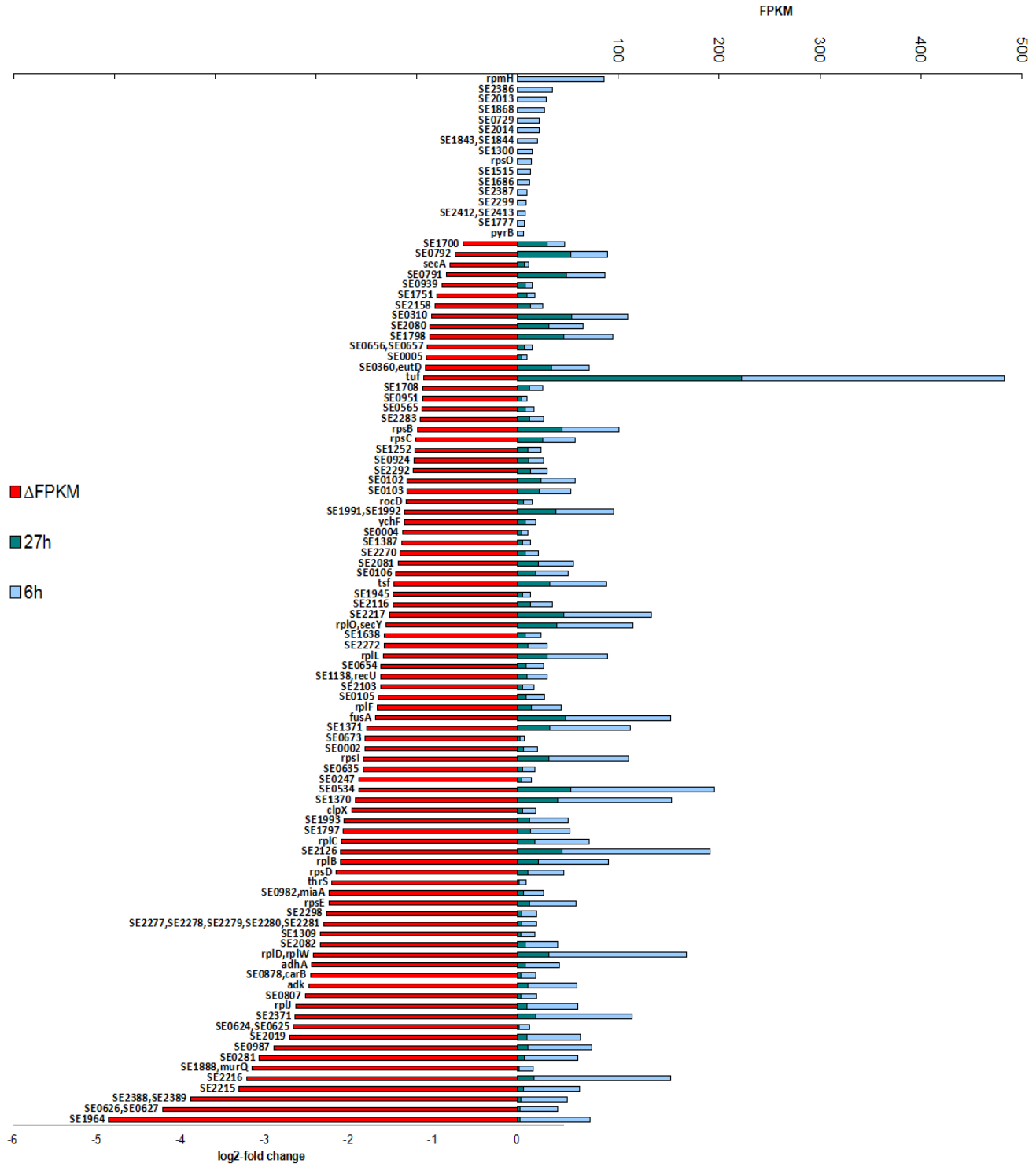

Figure 16. Relative abundance and fold-change of significantly downregulated genes in the spore-like cell forms of *S. epidermidis*. Transcripts expressed as Fragments Per Kilobase of transcript per Million mapped reads. Significance threshold set at p value < 0.05.

Table 6. Functional categories of significantly downregulated genes in the spore-like cell forms of *S. epidermidis*. Many of the gene functions are predicted from conserved domains and grouped by their most likely function.

Gene symbol	Function	Gene name/locus	Function
[5/119]	**Cell signaling 4,2 %**	**[5/119]**	**Energy processing 4,2 %**
SE2013	Antiholin-like protein lrgA	*clpX*	ATP-dependent Clp protease ATP-binding subunit clpX
SE2014	Antiholin-like protein lrgB	SE0635	NADH dehydrogenase-like protein
SE2126	Probable transglycosylase isaA	SE1309	ATP-binding
SE2298	probable signal peptidase II YaaT	SE1700	ATP synthase subunit beta
secA	Protein translocase subunit secA 1	SE2388	Energy production and conversion
[7/119]	**Cell structure 5,9 %**	**[48/119]**	**Metabolism 40,3 %**
SE0625	Membrane bound O-acyl transferase	*adhA*	Alcohol dehydrogenase
SE0627	Cell envelope biogenesis, outer membrane	*adk*	Adenylate kinase
SE0673	Acyltransferase	*carB*	Carbamoyl-phosphate synthase large chain
SE1138	peptidoglycan synthesis	*eutD*	Phosphate acetyltransferase
SE1992	transport systems inner membrane component	*miaA*	tRNA Delta(2)-isopentenylpyrophosphate transferase
SE1993	lysine-arginine-ornithine-binding protein	*murQ*	N-acetylmuramic acid 6-phosphate etherase
SE2272	cell division protein FtsH	*pyrB*	Aspartate carbamoyltransferase
[6/119]	**Cell transport 5,0 %**	*rocD*	Ornithine aminotransferase
SE0105	amino acid transporter	SE0102	Carbamate kinase 2
SE0247	transmembrane sugar transporter	SE0103	Ornithine carbamoyltransferase 1, catabolic
SE1945	L-lactate permease	SE0106	Arginine deiminase 1
SE1964	dicarboxylic acid transport	SE0281	Lipase
SE1991	ABC transporter-like	SE0360	Biotin/lipoate A/B protein ligase
SE2215	amino acid transporter	SE0624	D-alanine--poly(phosphoribitol) ligase subunit 1
[5/119]	**DNA processing 4,2 %**	SE0626	D-alanine--poly(phosphoribitol) ligase subunit 2
recU	Holliday junction resolvase recU	SE0654	Amino acid transport and metabolism
SE2280	Transcription-repair-coupling factor	SE0656	Argininosuccinate lyase
SE2292	Mg-dependent DNase	SE0657	Argininosuccinate synthase
SE2299	DNA binding	SE0791	Pyruvate dehydrogenase E1 component subunit alpha
SE2386	DNA binding	SE0792	Pyruvate dehydrogenase E1 component subunit beta
[4/119]	**DNA replication 3,4 %**	SE0807	protein syntheis
SE0002	DNA polymerase III subunit beta	SE0878	Carbamoyl-phosphate synthase small chain
SE0004	DNA gyrase subunit B	SE0924	Succinyl-CoA ligase [ADP-forming] subunit alpha
SE0005	DNA gyrase subunit A	SE0939	Prolyl-tRNA synthetase
SE2371	Single-stranded DNA-binding protein	SE0951	Polyribonucleotide nucleotidyltransferase
[17/119]	**Ribosomal 14,3 %**	SE0987	Glutamine synthetase
rplB	50S ribosomal protein L2	SE1252	Glycyl-tRNA synthetase
rplC	50S ribosomal protein L3	SE1370	Isocitrate dehydrogenase [NADP]
rplD	50S ribosomal protein L4	SE1371	citrate synthase
rplF	50S ribosomal protein L6	SE1387	Acetate kinase

rplJ	50S ribosomal protein L10	SE1708	UDP-N-acetylglucosamine 2-epimerase
rplL	50S ribosomal protein L7/L12	SE1751	Glucosamine--fructose-6-phosphate aminotransferase [isomerizing]
rplO	50S ribosomal protein L15	SE1777	Zinc-type alcohol dehydrogenase-like protein SE_1777
rplW	50S ribosomal protein L23	SE1843	molybdopterin biosynthesis
rpmH	50S ribosomal protein L34	SE1844	Molybdopterin-converting factor subunit 2
rpsB	30S ribosomal protein S2	SE2019	alcohol dehydrogenase
rpsC	30S ribosomal protein S3	SE2080	Amino acid transport and metabolism
rpsD	30S ribosomal protein S4	SE2081	Glycine, serine and threonine metabolism
rpsE	30S ribosomal protein S5	SE2103	Pyruvate metabolism
rpsI	30S ribosomal protein S9	SE2116	1-pyrroline-5-carboxylate dehydrogenase
rpsO	30S ribosomal protein S15	SE2158	Probable malate:quinone oxidoreductase 4
SE0310	30S ribosomal protein S7	SE2216	Ornithine carbamoyltransferase 2, catabolic
SE1798	30S ribosomal protein S11	SE2217	Arginine deiminase 2
[3/119]	**Other 2,5 %**	SE2270	Cysteine synthase
SE0565	RNA binding, nuclease activity	SE2278	tetrapyrrole methylase
SE2277	RNA binding	SE2279	Polysaccharide metabolism
SE1515	Cadmium resistance transporter	SE2283	Ribose-phosphate pyrophosphokinase
[5/119]	**Transcription 4,2 %**	*thrS*	Threonyl-tRNA synthetase
SE0982	tanscription factor	**[9/119]**	**Unknown 7,6 %**
SE1638	Accessory gene regulator protein A	SE0534	unknown
SE1686	hypothetical metal-sensitive transcription repressor	SE0729	unknown
SE1797	DNA-directed RNA polymerase subunit alpha	SE1300	unknown
SE1868	hypothetical transcription factor	SE1888	unknown
[5/119]	**Translation 4,2 %**	SE2082	unknown
fusA	Elongation factor Ts	SE2387	unknown
SE2281	Peptidyl-tRNA hydrolase	SE2389	unknown
tsf	Elongation factor Ts	SE2412	unknown
tuf	Elongation factor Tu	SE2413	unknown
ychF	Ribosome-binding ATPase		

4. Discussion

4.1 Cultivation

Strains of *E. fissicatena*, *B. producta* and *L. umeaensis* were previously reported to grow on blood-agar media [23, 37, 38] which was chosen for resuscitation from frozen stock. When no growth was observed for *B. producta* it was assumed the strain had reduced viability due to freeze-storage. Attempting to revive and enrich viable cells (Section 3.1) resulted in colony formation of coccoid cells matching the description of the cellular morphology of *B. producta* [37]. The contamination with *S. epidermidis* occurred either during the inoculation or it was enriched from the stock cultures.

4.2 Induction of sporulation

There is great variation in sporulation conditions between spore-forming species in terms of which nutrients, minerals, and stress factors [20, 39, 40] that trigger spore formation. In several *Bacillus* and *Clostridium* species it has been shown that during the transition to stationary growth phase the adaptive process of spore formation is initiated [41, 42]. It was therefore expected that culturing past the stationary phase will have at least a small fraction of cells developing into spores in one of the media tested in section 2.1. Typical signs of sporulation were observed, such as cell swelling in *L. umeaensis,* and transition from phase dark to phase-bright cells in *S. epidermidis* (Figure 4). They were distinguished from artifacts by being reproducible and dependent on the growth phase. Since no colonies were recovered following heat treatment, it was assumed that the spores might be more heat sensitive than most previously studied bacterial endospores, or have specific germination requirements related to their niche. While endospores are thermally stable structures [12], studies have shown that their heat-resistance is dependent on the mineral content of the sporulation medium [13]. Instead of being killed by heat treatment by damage to the spore core proteins [12], endospores may be inactivated due to damage to the germination machinery such as germinant receptor proteins or cortex-lytic enzymes. It has also been shown that alkali [15] or heat injured [43] spores may still be recovered with high efficiency upon exposure to

lysozyme. In a study of guinea pig gut bacterium *Metabacterium polyspora*, the endospores could not be revived in standard laboratory media, but germinated in the presence of cecum homogenate [44]. Several studies have shown that the endospores of enteropathogen *C. difficile* recognize bile salts, particularly taurocholate, as a co-germinant [14], and it has been recently shown that taurocholoate effectively triggers germination in enteric spores of commensal bacteria as well [4]. It was necessary to increase the fraction of sporulating cells and to isolate them, to determine their heat-resistance and germination requirements.

4.3 Spore formation and isolation

Addition of Ca^{2+} ions to the growth medium induced the formation of spore-like structures in *A. schaalii* cells, with a phase-bright appearance highly similar to *B. subtilis* endospores (Figure 5). Supplementation of the growth media with divalent cations such as Ca^{2+}, Mg^{2+} and Mn^{2+} have been shown to promote endospore formation in bacilli [26, 39] and clostridia [24] as they play an important role in various spore structures and chelation of DPA in the spore core. Similarly, Ca^{2+} ions promote the formation of arthrospores in Streptomyces species [45] and are found at a high concentration in their spore core.

To produce pure spore suspensions, differences in cell properties such as density, hydrophobicity [46] or heat-resistance may be exploited. The majority of methods used in section 2.3 are based on such differences and are previously reported to be effective for endospore purification in *Bacillus* and *Clostridium* species [20, 28, 47]. The vegetative cells and the spore-like structures appeared to have similar hydrophobicity properties and therefore the method of separation in aqueous polymer two-phase system [29] turned out to be highly inefficient for isolation of spore-like structures of *A. schaalii* and *S. epidermidis*. However differences in cell density made it possible to separate the spore-like structures of *A. schaalii* and *S. epidermidis* from vegetative cells by density-gradient centrifugation (Figure 9).

4.4 *A. schaalii*

The phylum *Actinobacteria* contains many members capable of spore formation, as well as entering a VBNC state. The *A. schaalii* cells were similar to spores, when observed by phase-contrast microscopy, although they did not form internally of a mother cell as seen in Bacillus and Clostridium endospores [9, 11] or fragmenting hyphae of exospores [48] (Figure 5). Heat-shocking and calcium supplements that enhance endospore-formation in *B. subtilis* [26] and many *Clostridium* species [20, 24] also appear to induce cell differentiation in *A. schaalii.* The spore-like structures of *A. schaalii* had an increased cell density as seen by phase-contrast microscopy and density-gradient centrifugation. The family *Mycobacteriacea*, and previously *Thermoactinomyces*, in the *Actinobacteria* phylum have previously been reported to be endospore formers, based on ultrastructure, heat resistance, sporulation related orthologous genes and the presence of dipicolinic acid [49, 50]. However, in the case of *Mycobacterium* [51], the sporulation related orthologous genes, that were found, are also present in non-sporulating bacteria [52]. This is similar to the results presented in Table 3. The genus of *Thermoactinomyces*, which forms spores on hyphae, has been moved from the class *Actinobacteria* to the class of Bacilli in the *Firmicutes* phylum [53]. There is an overall lack of genetic studies on sporulation in *Actinobacteria*, but it is currently suggested that the sporulation machinery required for endospore formation evolved once in a common ancestor of the *Bacilli* and *Clostridia* [54] and therefore, it is expected that endospore formers are restricted to the *Firmicutes* phylum. The apparent absence of highly conserved genes necessary for endospore formation, make it unlikely that the spore-like cells of *A. schaalii* are endospores. The elongated cell shape and protrusions of *A. schaalii* spore-like forms seen in Figure 10 more closely resemble the monosporous *Actinomycetes* [48] belonging to the same family.

The observed cellular changes may be a result of reinforcement in the cell wall layers or a buildup of material in the cytoplasm, and not spore formation. Changes in the cell wall composition have previously been reported for bacteria entering a VBNC state [55, 56]. A group of enzymes, with lysozyme-like muralytic activity, termed resuscitation-promoting factors (Rpf) have been isolated from various *Actinobacteria*. These enzymes are essential for degradation of the cell wall in dormant cells to allow them to resume vegetative growth [57,

[58]. The concept of Rpfs has been extended to the *Firmicutes* [59], although various other factors such as growth medium composition during entry into dormancy, temperature shift and limited time windows for resuscitation have been reported [60] to influence cell recovery from the VBNC state. The mechanisms by which Rpfs induce germination in dormant cells appear similar to the function of cortex-lytic enzymes during germination of endospores. However, cells in the VBNC state do not appear to carry the enzymes inherently. Instead, they rely on enzymes secreted by actively growing cells [61], to escape from dormancy. This comprises a completely different strategy for initiation of germination compared to that utilized by endospores, and would explain why the spore-like cells of *A. schaalii* did not resume growth when exposed to various nutrient combinations and media tested in section 2.4.

4.5 *S. epidermidis*

The *Staphylococcus* genus is known to be non-sporulating, although several strains were reported to enter a VBNC state [62, 63]. Recent advances on how of *S. aureus* cells resuscitate from dormancy, with a Rpf-like protein secreted by vegetative cells, have made it less controversial whether the bacteria are simply dead or in the VBNC state [64]. *S. epidermidis* has been reported to enter the dormant state in biofilms which is an important risk factor for developing post-operative infections in orthopedic implants, prosthetic joints and catheters [65].

A fraction of *S. epidermidis* cells appeared to differentiate into spore-like structures (Figure 7), while the remaining vegetative cells were autolyzed upon reaching stationary culture phase. Release of extracellular DNA during autolysis has been associated with increased biofilm formation and increased adhesion capabilities for the remaining population [66]. The phase-bright appearance indicates increased cell density and may be a result of a thicker cell wall or the smaller cell size observed by SEM microscopy (Figure 11). In addition, cross-linking and acetylation of the cell wall, as previously described in bacteria entering a VBNC state, may increase the cell rigidity and provide protection from endogenous and extracellular autolysins [56, 67]. It is likely that the dormant cells of *S. epidermidis* can be

resuscitated by the same strategy as used for *S. aureus* cells, although the study reported a narrow time window between 13 and 19 days during which the treatment was effective [64]. This limited period of time when cells can be resuscitate in laboratory conditions has been reported as a "resuscitation window" for many bacteria capable of entering a VBNC state, varying between a few days to several years among species [60].

Given the broad range of conditions that promote entry into the VBNC state in different bacteria, and those required for resuscitation, as well as differences in VBNC resistance and lifetime, it appears that the VBNC state is a general term for various forms of dormancy in bacterial cells. There is currently a huge lack of knowledge on the VBNC state. Several studies on gene expression have been performed in VBNC bacteria and these show some common patterns such as a shift in metabolism, differentially regulated transcription, cell wall modification and changes associated with oxidative stress ressistance [68, 69]. Major differences in expression patterns were also found between strains of the same species which entered the VBNC state [70]. Gene expression data for *S. epidermidis* shows downregulation of amino-acid and protein synthesis and energy turnover, and this was also indicated by downregulation of ribosomal genes (Table 6). This was, however, expected in cultures entering the stationary phase. Upregulation of genes participating in carbohydrate and protein degradation, protein modification, stress response, transport, as well as uncharacterized membrane proteins are suggestive of entry into dormancy and has previously been described in VBNC bacteria [71]. Before RNA sequencing of *S. epidermidis* was performed, it was thought that the spore-like structures were endospores, for which RNA would have to be isolated before endospore formation was complete to see the expressed sporulation genes. If the bacteria are in a VBNC state and potentially actively transcribing, it would be possible to isolate RNA from only VBNC cells to see which genes are expressed during dormancy as well.

4.6 *L. umeaensis*

In the original report where isolation of *L. umeaensis* strain was first described, it was observed to form spores during prolonged incubation in Brucella medium [38]. Sings of spore formation, such as cell swelling, was also observed in this study (Figure 8), but further development of phase-bright endospores was not seen here. Supplementing the media with

Ca^{2+}-ions and thiamine, to enhance sporulation, produced some phase-bright elliptical cells similar to endospores, but they did not germinate in standard media. *L. umeaensis* was not prioritized in the project because it lacked a sequenced genome for comparative studies.

4.7 RNA sequencing

For RNA sequencing of bacterial samples, the choice for library preparation is currently between depleting rRNA using commercial beads or sequencing total RNA directly. The difference lies in price as the resulting expression data provides the same information. Initially, we planned to test an experimental method that involved hybridizing the total RNA from one sample to another sample using custom adapters and beads to get an unbound remainder of mRNA reflecting the differentially expressed genes. However, due to different ratios of 16s to 23s rRNA measured in the samples (section 3.9.1, Figure 13), which would hybridize unevenly it was decided to sequence total RNA directly. The initial approach was the reason for choosing paired-end 300 nucleotide reads for sequencing, but is otherwise of little advantage in prokaryote RNA sequencing where splicing does not occur. Total RNA requires a 10-fold increased sequencing depth to account for rRNA and get the same coverage. This is the more cost effective approach for a few samples compared to the rRNA depletion kits, but scaling up the number of samples will exponentially increase sequencing costs. Therefore, development of new methods for mRNA isolation could bring down the price of RNA sequencing considerably.

RNA sequencing is a relative comparison of expression levels between samples and a statistical threshold is necessary to determine if the expression levels are significantly altered. One of the replicates for the samples taken at 27 hours (section 2.7) failed to yield sequence data, either due to a technical error or indexing. This had an impact on the number of genes that were considered significantly altered in expression level. In the volcano plot (Figure 14A), several genes demonstrated large fold-changes in expression levels that were considered to be insignificant due to variance between replicates. Increasing the statistical power by having more replicates may have revealed additional genes that were differentially regulated. This is of interest since small changes in regulatory genes, such as transcription and sigma factors, can have large downstream effects in cell differentiation [72].

4.8 Future studies

It would be fascinating to study the most resilient form of dormancy, endospores, and their genetic makeup in commensal *Clostridium* genera such as *Eubacterium* and *Blautia*. Determining the sporulation conditions for *B. producta* would allow for gene expression studies, such as RNA sequencing, to detect previously unassociated sporulation factors in Clostridia. Follow up with molecular studies, such as gene knockout or protein labeling in endospore thin-sections, would be necessary to determine the gene function and protein location. Screening for germination requirements of commensal clostridial endospores would be important to understand the mechanisms of transmission, establishment and resilience of commensal bacteria in the human gut. These studies may also allow for commercial ventures in the treatment of disease and promotion of well-being. Also, since taurocholate has been shown to be a major germination factor for commensal endospores in humans [4], it would be interesting to see if commensal endospores in other species, such as insects which do not synthesize bile salts [73], have evolved similar niche adaptations in germination requirements.

The exospore form of dormancy in bacteria is lacking genetic studies on the genes involved in cell differentiation. *A. schaalii* is reported to be a non-sporulating, emerging pathogen and it would be interesting to see a comparative study with other spore formers in *Actinobacteria* to shed light on the observed spore-forms. One approach could be to do a transcriptome sequencing of sporulating *A. schaalii* cells in parallel to a well-described *Actinomyces* spore former belonging to the same family, and then search for common orthologs in their upregulated genes.

Lastly, it would be of interest to try to resuscitate *S. epidermidis* cells from the VBNC state with Rpf-like proteins, described previously [64], and to assess the VBNC morphotype adhesion and resistance properties, as well as their lifespan and the regulatory factors leading to their entry into dormancy. Antibiotic resistance or resilience to clinical sterilization techniques would have implications in the medical environment, especially for VBNC bacteria of pathogenic strains.

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Appendix A.

Processing of RNA sequencing data with cufflinks

1. Download *S. epidermidis* genome (NCBI) in .fasta format:

>gi|27466918|ref|NC_004461.1| Staphylococcus epidermidis ATCC 12228 chromosome, complete genome

And gff annotation of the genome in .gff3 format, then open and compare the headers, make sure that both sequence identifiers are the same or edit manually as in:

Genome.fasta: >NC_004461.1
Genome.gff: NC_004461.1

Important to make sure identifiers are identical before indexing/aligning or it will be difficult to retrieve DE gene annotation/sequences.

2. In the linux command shell, run bowtie alignment with following commands:

Create an index for bowtie:
bowtie2-build genome.fasta yourindexname

Paired-end read alignment:
bowtie2-align-s -x yourindexname -S outputname.sam -1 filePathToFastaQfile1stEnd.fastq -2 filePathToFastaQfile2ndEnd.fastq

3. Convert .sam to sorted .bam files:

samtools view -bS samfilepath.sam | samtools sort - outputName

4. Running cufflinks:

cufflinks -g genomeannotationFilePath.gff -o output sortedBAMfilepath.bam

5. Running cuffmerge:

Make a .txt file with a list of file-paths, each on a new line, of the cufflinks output folders for all the samples, then run:

cuffmerge -o outputName -g genomeannotation.gff -s genome.fasta trans_list.txt

6. Finally run cuffdiff:

cuffdiff -L sampleName1,sampleName2 -o outputName cuffmergeOutput.gtf sample1replicate1.bam, sample1replicate2.bam, sample1replicate3.bam sample2replicate1.bam, sample2replicate2.bam, sample2replicate3.bam

Transcript values, fold-changes in expression and gene-ids can be found in the output gene_exp.diff

Appendix B.

List of genes and their UniProt ID`s used in the comparative study

	A.schaalii		B.producta		Listeria ssp.		UniProt
Stage 0	Hit	E val	Hit	E val	Hit	E val	
spo0A	+	5e-15	+	2e-87	+	4e-14	P06534
spo0H (sigH)	-		+	4e-43	+	1e-55	P17869
spo0J	+	3e-55	+	9e-60	+	6e-99	P26497
obgE	+	2e-84	+	9e-99	+	6e-153	P20964
Stage II							
spoIIAA	-		+	1e-15	+	9e-13	P10727
spoIIAB	-		+	8e-38	+	2e-09	P10728
spoIIAC (sigF)	+	9e-27	+	2e-68	+	1e-36	P07860
spoIID	-		+	4e-24	-		P07372
spoIIE	-		+	3e-38	*	2e-08	P37475
spoIIGA	-		+	7e-13	-		P13801
spoIIGB (sigE)	*	1e-10	+	3e-82	+	3e-14	P06222
spoIIM	-		-		-		P37873
spoIIP	-		-		-		P37968
spoIIR	-		+	4e-34	-		P39151
Stage III							
cwlD	-		+	3e-25	+	3e-16	P50864
dacB	-		+	2e-44	+	2e-24	P35150
dapA	+	3e-50	+	9e-67	+	8e-96	Q04796
dapB	+	4e-40	+	4e-21	+	6e-94	P42976
dpaA	-		+	4e-27	-		Q04809
dpaB	-		+	7e-62	-		Q04810
spmA	-		+	1e-40	-		P35157
spmB	-		+	6e-31	-		P35158
spoIIIAA	-		+	8e-77	-		Q01367
spoIIIAB	-		-		-		Q01368
spoIIIAC	-		-		-		P49780
spoIIIAD	-		+	1e-19	-		P49781
spoIIIAE	-		+	1e-13	-		P49782
spoIIIAF	-		-		-		P49783
spoIIIAG	-		-		-		P49784
spoIIIAH	-		-		-		P49785
spoIIID	-		+	3e-23	-		P15281

spoIIIE	+	2e-137	+	5e-152	+	1e-154	P21458
spoIIIJ	+	4e-25	*	7e-11	+	4e-80	Q01625
spoIIIG (sigG)	+	9e-24	+	2e-110	+	3e-29	P19940
jag	+	7e-17	+	2e-24	+	4e-53	Q01620
Stage IV							
spoIVA	-		+	1e-126	-		P35149
spoIVB	-		+	3e-58	-		P17896
SigK	+	4e-23	+	5e-40	+	2e-21	P12254
Stage V							
spoVAC	-		+	1e-22	-		P40868
spoVAD	-		+	1e-76	-		P40869
spoVAEB	-		+	3e-16	-		C0H450
spoVB	-		+	5e-40	+	6e-18	Q00758
spoVC	+	4e-29	+	7e-57	+	9e-77	P37470
spoVD	+	9e-46	+	6e-101	+	7e-96	Q03524
spoVG	-		+	4e-31	+	6e-45	P28015
spoVK	-		*	6e-06	-		P27643
spoVS	-		-		-		P45693
spoVT	-		+	2e-53	*	4e-14	P37554
stoA	-		*	3e-06	-		O31687
yabP	-		-		-		P37558
yabQ	-		-		-		P37559
ylbJ	-		+	5e-30	-		O34765
ylmC	-		-		-		Q04811
yqfC	-		-		-		P54468
yqfD	-		+	3e-25	-		P54469
ytvI	-		+	4e-23	+	7e-19	O34991
yyaC	-		+	4e-43	-		P37521
yncD	+	2e-45	+	2e-79	+	3e-81	P94494
SASPs							
sspA-P	-		-		-		P04831
ssp1-3	-		+	2e-18	-		P41371
ssp4	-		+	4e-14	-		Q17ZW6
tlp	-		-		-		Q45060
csgA	-		-		-		P54379
Spore Coat							
cotA-Z	-		JC-JB		A		P07788
yncD	+	2e-45	+	5e-81	+	3e-81	P94494
gerQ							P39620
lipC					+	1e-14	P42969
oxdD							O34767
tgl							P40746

yabG							P37548
yckK	+	2e-45			+	6e-92	P42199
ydhD			+	1e-23			O05495
yeeK							O31510
yhaX	+	1e-10			+	1e-24	O07539
yhbA					+	2e-150	P97030
yhbB					+	1e-13	O31589
yhcN							P54598
yhcQ							P54601
yheC							O07544
yheD							O07545
yhjR							O07572
yisY			+	1e-70	+	3e-17	O06734
yjqC			+	5e-16			O34423
yjzB							O34891
yknT							O31700
ylbD							O34880
ymaG							O31793
yodI							O34654
ypeP							P54164
yppG							P50835
ypzA							O32007
yraD							O06010
yraE							O06011
yraF							O07949
yraG							O07919
ysxE							P37964
ytxO							P46916
yutH							O32123
yuzC							O32089
ywqH							P96720
yxeE							P54944
yxeF							P54945
yybI							P37495
ysnD							P94563
bclA							Q81JD7
bxpA							Q81R91
bxpB							Q81TN4
bxpC							Q81QT5
eag							P94217
exsB							Q81RJ9
exsC							Q81PB7
exsD							Q81Q22
exsH							Q81QH5

exsK							Q81Q79
rihA	+	1e-30	+	4e-19			Q81PC3
Spore cortex							
coxA	-		-		-		P94446
cwlA	-		-		+	7e-32	P24808
cwlH	-		+	1e-19	+	3e-13	P54450
cwlC	-		+	1e-31	+	3e-39	Q06320
cwlD	-		+	4e-26	+	3e-16	P50864
cwlJ	-		-		-		P42249
sleB	-		+	2e-11	-		P50739
stoA	-		-		-		O31687
ylbJ	-		+	2e-30	-		O34765
yabP	-		-		-		P37558
yabQ	-		-		-		P37559
yqfC	-		-		-		P54468
yqfD	-		+	2e-25	-		P54469
sleC	-		+	5e-71	-		Q97E72
sleL	-		+	1e-40	-		P37531
lytH	-		+	1e-24	-		O32130
ypeB	-		-		-		P38490
sleM	-		+	3e-09	-		O06496
Coat Maturation							
cgeA							P42089
cgeB							P42090
cgeC							P42091
cgeE							P42093
cgeD							P42092
dacB			+	1e-46	+	2e-24	P35150
spsA					+	7e-14	P39621
spsB							P39622
spsC			+	3e-46	+	2e-51	P39623
spsD			+	5e-07			P39624
spsE					+	3e-55	P39625
spsF							P39626
spsG							P39627
spsI	+	2e-52	+	1e-64	+	1e-61	P39629
spsJ	+	2e-81	+	7e-91	+	8e-91	P39630
spsK	+	3e-57	+	8e-60	+	6e-59	P39631
spsL							Q7WY56
Germination							
gerAA			+	7e-64			P07868
gerAB							P07869
gerAC							P07870

gerD							P16450
igt	+	2e-18	+	2e-27	+	9e-87	O34752
gerM							P39072
gpr			+	6e-67			P22322
yfkR							O35028
gerBA			+	9e-75			P39569
gerBB							P39570
gerBC							P39571
gerKA			+	2e-84			P49939
gerKB							P49940
gerKC							P49941
gerPA							O06721
gerPB							O06720
gerPC							O06719
gerPD							O06718
gerPE							O06717
gerPF							O06716
yndD			+	1e-71			O31808
yndE							O31809
yndF							O31810
yfkQ			+	8e-80			O34486
yfkT							O34573
gerXB							Q736Z6
cspC			+	4e-46			Q185C0
cspBA			+	2e-92			Q185B9

Uniprot: bacillus - blue, clostridium – purple, weak hits - orange