
Details of CSC proposal 2017

Title: The Molecular Basis of Endospore Heterogeneity

Supervisor details

Name: Prof. dr. Stanley Brul (h-index 41)
Gender: ⊗ Male O Female
Date of birth: 1-12-1964
Supervised PhD students: 16
Director of the Biomedical Sciences program at UvA
E-mail: S.Brul@uva.nl

Name: Prof. dr. C.G. de Koster (h-index 44)
Tel: +31 (0)20 525 5457
Email: C.G.deKoster@uva.nl

Institute Departments: Molecular Biology & Microbial Food Safety, Mass Spectrometry of Biomacromolecules
Research School: Swammerdam Institute for Life Sciences, University of Amsterdam, Netherlands.
Science Park 904, 1098 XH, Amsterdam, NL

Project for: PhD student

Composition of the research group

Name and title	Specialization	Institution	Involvement
Prof. dr. Stanley Brul	microbiology	SILS	project leader/ supervisor
Prof. dr. Chris G. de Koster	biochemistry	SILS	co-supervisor
Dr. Gertien. J. Smits	cell biology	SILS	advisor
Prof. dr. Sander Woutersen	spectroscopy	HIMS	advisor
Dr. Leo de Koning	mass spectrometry	SILS	daily guidance
Dr. Erik Manders	advanced micr.	SILS	advisor
MSc Richard de Boer	molecular biology	SILS	technician
MSc Winfried Roseboom	mass spectrometry	SILS	technician
MSc Henk Dekker	mass spectrometry	SILS	technician
MSc Belinda Koenders	molecular biology	SILS	technician

8 PhD students and 1 post-doctoral fellow

Top 5 publications of the applicant and research group related to the proposed research

1.[@] Abhyankar, W.R., Kamphorst, K., Swarge, B.N., van Veen, H., van der Wel, N.N., **Brul, S.** , **de Koster, C.G.**, and de Koning, L.J. 2016. The influence of sporulation conditions on the spore coat protein composition of *Bacillus subtilis* spores. Front. Microbiol., 7, 1636.

2.[$] Zheng, L.; Abhyankar, W.; Ouwerling, N.; Dekker, H.L.; van Veen, H; van der Wel, N.N.; Roseboom, W.; de Koning, L.J.; **Brul, S.**[*]; **de Koster, C.G.**[*] 2016. *Bacillus subtilis* Spore Inner Membrane Proteome. J. Proteome Res. 15, 585-594.

3.[$] Pandey R., Vischer, N.O.E., Smelt, J.P.P.M., van Beilen, J.W.A., ter Beek, A., De Vos[*], W.H., **Brul**[*#]**, S.** and Manders[*], E.M.M. 2016. Intracellular pH response to weak acid stress in individual vegetative *Bacillus subtilis* cells. Appl. Env. Microbiol. 82, 6463-6471.

4.[@] Abhyankar, W.R., Pandey, R., Ter Beek, A., **Brul**, S., de Koning, L.J. and **de Koster, C.G.** 2015. Reinforcement of *Bacillus subtilis* spores by cross-linking of outer coat proteins during maturation. Food Microbiology *45* , 54-62.

5.[@] Abhyankar, W.R., Houssain, A., Djajasaputra, A., Permpoonpattana, P., Ter Beek, A., Dekker, H., Cutting, S., **Brul, S.**, de Koning, L.J. and **de Koster, C.G.** 2013. In Pursuit of Protein Targets: Proteomic characterization of Bacterial Spore Outer Layers. J. Proteome Research 12, 4507-4521.

Recent review: Abhyankar, W., Stelder, S., de Koning, L., de Koster, C. and **Brul, S[#]**. 2016. 'Omics' for microbial food stability: proteomics for the development of predictive models for bacterial spore stress survival and outgrowth. Int. J. Food Microbiol. pii:S0168-1605 (16)30217-3

[#]Corresponding author; [*]Equal contribution; All journals rank amongst the top in their respective categories. Food Microbiol. and Int. J. Food Microbiol. (see review above) are the top two journals of food microbiology.
[@]Papers all using our protocol for spore coat proteomics (Abhyankar, W.R. et al. 2011. Gel-free proteomic identification of the *Bacillus subtilis* insoluble spore coat fraction. Proteomics. 11, 4541-4550).
[$]Papers focussing on spore inner membrane (germination)protein identification and germination/outgrowth heterogeneity assessment using our live imaging system (Pandey et al. 2013. Live cell imaging of germination and outgrowth of individual *Bacillus subtilis* spores…….. analyzed with SporeTracker. Plos One 8(3): e58972).

Scientific summary

Upon nutrient starvation, species of *Bacillus* and *Clostridium* form endospores that can withstand a variety of chemical and environmental assaults. The resistance properties are in part attributed to spore surface proteins. Thermal resistance and germination heterogeneity are key features of spores against which no effective solution has yet been found. This is evident from the diseases and spoilage caused by spore formers in foods. Recently it was found that spore maturation, characterized by changes in coat protein structure, is needed for the acquisition of full spore heat resistance. In our previous research we identified a group of spore coat proteins to be affected by the maturation process in their level of protein-protein crosslinking. This led to the hypothesis that the extent of cross-linking is linked with the variation in the heat resistance and germination speed/time of spores: the more the proteins are cross-linked, the higher is spore thermal resistance and the slower and more heterogeneous is their germination. In the proposed research we will test this hypothesis, thus gathering novel insight in spore biology essential to address phenotypic spore heterogeneity. To this end a quantitative time lapse proteomics study will be performed using reference spores metabolically labelled with (^{15}N) and test un-labelled (^{14}N) spores sampled at different times of their maturation state. For the analysis we will use Fourier Transform Ion Cyclotron Resonance mass spectrometry. Additionally thermal resistance and the germination pattern of spores will be assessed with live-imaging and correlated with the protein digestion efficiency for the spore-coat proteins.

Summary for the broad scientific committee

Food preservation necessary in food manufacturing needs to strike a balance between required food safety and stability as well as optimal food quality. The thermal process generally applied at some point during the preservation regime is usually dictated by the presence of bacterial spore forming bacteria. The spores that these bacteria make are highly heat resistant as well as able to withstand many more or less harsh preservation treatments using antimicrobial compounds. The food preservation equilibrium has long been one dictated by being extra fail safe due to the observed heterogeneity in the behavior of spores from Bacilli and Clostridia under thermal and other preservation stress. The latter is expressed in significant heterogeneity in stress survival and thus leads to large fluctuations in calculated spoilage or food safety risks. To be on the (fail) safe side over-processing of a significant part of the produced foods is a significant issue to deal with. Such over treatment of manufactured foods is both from a purely economic perspective (heat input costs) as well as from a food quality perspective highly unwanted. Better insight in the mechanisms of heat resistance as well as detailed statistical analysis of (mechanisms of) heterogeneity in spore germination and outgrowth should allow for the generation of robust predictive models that can facilitate tailor made processing of food products where required microbial food safety and food quality can be in an optimal equilibrium.

Description of the proposed research

Objectives

The soil bacterium *Bacillus subtilis*, anaerobic *Clostridium* spp. and related organisms, in response to nutrient starvation, form endospores - small, metabolically dormant cellular structures that are remarkably resistant to heat, desiccation, radiation and chemical insult[1]. This state of dormancy achieves continuation of life and synchronization with the environments or periods to which the spore formers are adapted. Sporulation starts as a response to a single stress but the spores are resistant to a variety of stress conditions whether they are present in the environment, in human bodies or in food being produced in a food company. **Spore inactivation can be effectively achieved by wet-heat treatment but spores are generally resistant to higher temperatures when compared to vegetative cells. Such resistant behavior of**

spores is a cause of concern for the food industries as spores may eventually be responsible for food spoilage or even food becoming toxic.

Remarkably, in the absence of the stress or the limiting conditions the spores can germinate. Spore germination is an irreversible process by which the dormant and stress resistant spore wakes up and initiates vegetative cell growth[2]. All of the components necessary to facilitate germination are pre-assembled into the spore during sporulation. One striking feature of spore germination is the **heterogeneity** observed in the germinating spore populations. In a single population, while a great majority of spores germinate rapidly, a small percentage of spores ('super dormant' spores) germinate very slowly. **Due to the heterogeneity in germination[3,4] eradication of spores with minimal efforts cannot be achieved successfully, which poses challenges for the food industries.** Since spore formers like *B. anthracis* (causing the infectious disease anthrax), *C. difficile* (causing diarrhea and worse, often related to antibiotic treatments) are clinically important[5,6] there is also great medical importance for being able to effectively kill spores.

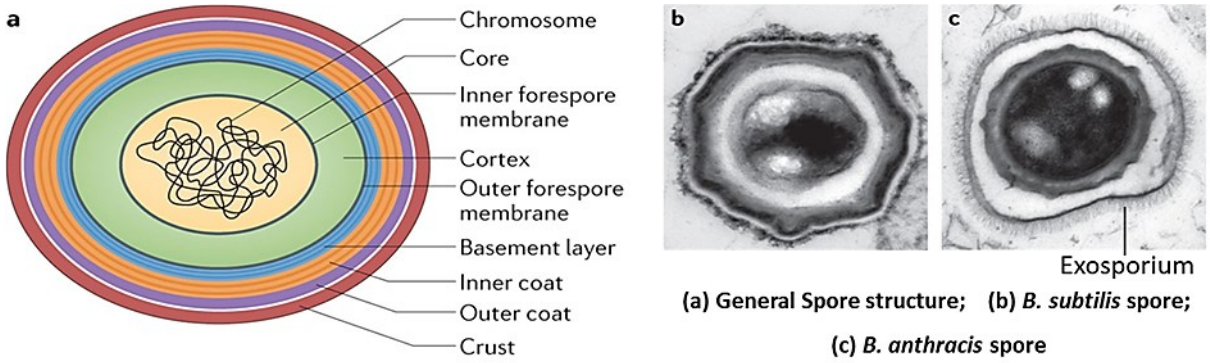

Figure 1: Structure of bacterial endospores. See text for details[1,7].

The resistance properties of spores are the result of its well assembled multi-layered structure (**Figure 1**). The outer layers called exosporium (absent in *B. subtilis*), crust (external glycoproteinaceous layer in *B. subtilis* spores; not an exosporium) and coat are mainly responsible for resistance and transmission of spores. Particularly, the heavily cross-linked proteinaceous coat surrounding the spore provides much of the chemical and enzymatic resistance[1,7]. Beneath the coat resides the basement layer and outer forespore membrane followed by a very thick layer of peptidoglycan called the cortex. Proper cortex formation is needed for dehydration of the spore core, which aids in resistance to high temperature[8]. The inner membrane, underneath the cortex, is a major permeability barrier against several potentially damaging chemicals. The centre of the endospore, the core, exists in a very dehydrated state and houses the cell's DNA, ribosomes and large amounts of dipicolinic acid (DPA). DPA can comprise up to 10% of the spore's dry weight and appears to play a role in maintaining spore dormancy. Small acid-soluble proteins (SASPs) are also uniquely to spores. They tightly bind and condense the DNA, and are in part responsible for resistance to UV light and DNA-damaging chemicals[8].

Three types of cross-links, namely, the dityrosine links, the ε-(ɣ)-glutamyl-lysine isopeptide linkages and the disulfide bonds are predicted to render 30% of the coat protein fraction insoluble. Although the spore coat protein composition in the released spores is presumably constant, there can be differences in the level of cross-linking amongst the proteins. The role of spore coat proteins as well as the inter-protein cross-linking in the spore's longevity has been a point of interest to the medical and food sectors. Thermal resistance has previously been attributed to the spore core water content, core mineralization and α/β-type SASPs[8]. A recent study concluded that spore maturation, after the spore's release, is an important factor in acquiring wet-heat resistance in spores[9]. On the same lines, we have identified the spore outer coat and crust layer proteins CotG, YurS, CotU, CotI, CotZ, CotY, CotB and CotC to be critical for the process of spore maturation and affected most likely in the extent of their cross-linking[10]. Thus we hypothesize that the difference in the extent of cross-linking amongst the spore coat proteins is also linked to the thermal resistance and the heterogeneity seen in spores.

This project will address the question of the remarkable thermal resistance of spores with a focus on spore coat protein cross-linking, which is not yet studied in detail. In order to achieve an answer, FT-ICR-MS/MS based proteomics using $^{14}N/^{15}N$ metabolic labelling, assessment of thermal heat resistance, analysis of spore DPA and water content and live cell imaging will be performed to get insights into (1) progression of protein cross-linking with time after spore's release, (2) cross-linked residue positions of proteins, (3) progression of thermal resistance with time after spore release, (4) role of cross-linking in thermal resistance and (5) germination

behaviour of young and mature spores. Our capability to probe for the germination proteins in the inner membrane and study comprehensively spore germination and outgrowth in detail at single spore level is key.

Innovative aspects

We will establish a quantitative relation between progression of protein cross-linking (amongst the spore coat proteins) and the built up of heat resistance, by tracking the efficiencies of tryptic digestion of the individual coat proteins during spore maturation. This will be accomplished by determining the change in levels of the released tryptic peptides for each coat protein relative to the corresponding ^{15}N metabolically labelled coat proteins from fully matured reference spores. Further, detailed MS/MS analyses of the digests of the coat proteins may reveal cross-linked tryptic peptides which expose the molecular nature of the cross-linking. To monitor the extent of di-tyrosine crosslinks, we will follow their formation using Raman spectroscopy for young and mature spores, in relation to their heat resistance, germination rate and germination heterogeneity of the population of spores under study. The comprehensive spore germination and outgrowth analysis at single spore level as well as our capability to interrogate the inner membrane germination proteins will uniquely position us to identify a firm molecular basis for the well-known phenotypic heterogeneity of spores with respect to extreme thermal stress.
In conclusion, this research will provide evidence that after the release of *Bacillus* spores (by the mother cell); the spore coat protein cross-linking contributes to the thermal resistance and heterogeneity of germination behaviour of a (genetically homogenous) bacterial spore population. As such the data and project are highly innovative and new. Some of the preliminary work has been presented at scientific meetings and it drew the attention of a large audience, including the key people in the field such at the American Scientist and 'father of spore biology' Prof. Peter Setlow from the University of Connecticut. The further understanding of the spore proteome building blocks will aid us in assessing the physicochemical properties of the spore cortex and coat and thus also in understanding the basis for the thermal stability of spore coat and cortex lytic enzymes.

Scientific approach

We will take, as a starting point, the group of spore coat and crust proteins identified by us to be affected by the maturation process[10].

Preparation, growth and sporulation of *B. subtilis* wild-type & mutant strains

The main strain used in this study will be *B. subtilis* PY79. For PY79 spores the thermal resistance was seen to increase as the time progressed. Therefore, the first step in our research will be to prepare *B. subtilis* deletion mutants[11] as mentioned in **Table 1** below. Protein CotG[12], Cot C and Cot U have been identified previously[13] to be involved in protein cross-linking presumably through di-tyrosine formation. Thus site-directed mutagenesis[14] will also be utilized for proteins CotG (amino acid residues 36-154), Cot C (amino acid residues 45-66) and CotU (amino acid residues 40-86). Since the SodA (superoxide dismutase[12]) and Tgl (transglutaminase[15]) proteins have been found to be crucial for cross-linking, *sodA* and *tgl* deletion mutants will also be prepared and used. The growth and sporulation behaviour of these mutants will be studied and optimized for the further experimentation as described in the literature[16]. To eliminate the effect of di-sulphide links, we will treat the intact spores with reducing agents such as β-mercaptoethanol (β-ME) or Dithiothreitol (DTT) and then test the thermal resistance of spores. Additionally the cysteine-rich crust proteins CotY & CotZ have already been listed

Mutant	***Wild-type protein characteristics***
B. subtilis PY79 (*ΔcotG*)*	*Outer coat putatively heavily cross-linked*
B. subtilis PY79 (*ΔyurS*)	*Outer coat / crust putatively cross-linked*
B. subtilis PY79 (*ΔcotU*)*	*Outer coat putatively dityrosine cross-linked*
B. subtilis PY79 (*ΔcotI*)	*Outer coat / crust putatively cross-linked*
B. subtilis PY79 (*ΔcotZ*)	*Spore crust formation cysteine cross links*
B. subtilis PY79 (*ΔcotY*)	*Spore crust component cysteine linked*
B. subtilis PY79 (*ΔcotYZ)*$^{+}$	*Crust components involved in cross-linking*
B. subtilis PY79 (*ΔcotB*)	*Outer coat putatively highly cross-linked*
B. subtilis PY79 (*ΔcotC*)*	*Outer coat putatively tyrosine cross-linked*
B. subtilis PY79 (*ΔsodA*)	*Outer coat generating dityrosine linkages*
B. subtilis PY79 (*Δtgl*)	*Generating γ-glutamyl lysine linkages*
B. subtilis PY79 (*ΔsodA Δtgl*)$^{+}$	*Dityrosine and γ-glutamyl lysine formation*

Table 1 Deletion mutant strains planned for use

* The genes *cotG*, *cotC* and *cotU* (*ynzH*) will also be used for site directed mutagenesis.

$^{+}$ The double mutants (*ΔcotYZ)* and (*ΔsodA Δtgl*) will also be studied.

for the mutants to be studied. The double mutant (*ΔcotYZ*) will also be tested. To eliminate the effect of the glutamyl-lysine crosslinks catalysed by transglutaminase, we will study the resistance pattern of *Δtgl* strain. There are known glutamine-rich proteins in the coat such as GerQ, CotM. Specific glutamines in these proteins will be replaced using site directed mutagenesis. For practice oriented validation analyses, we will generate mutants in coat protein cross-linking in true wild-type heat-resistance food isolate *B. subtilis* A163 (Unilever). The genome of strain A163 is available and the strain is genetically accessible (TNO). These experiments are planned towards the end of the project. The data to be obtained will form a basis for follow-up studies. All strains to be studied will be cultured as described previously[10] and spores will be generated accordingly. Typical spore characteristics such as basal thermal resistance and germination efficiencies will be documented as is standard practice in the laboratory (see reference 10 and references therein).

Time-resolved LC-FT-ICR MS/MS analyses of the digestion efficiency of coat proteins

The digestion efficiencies of the coat proteins are monitored relative to metabolically labelled fully maturated reference spores cultured using $^{15}NH_4Cl$ as the sole nitrogen source. For all the wild-type and mutants mentioned above the test spores will be prepared in ^{14}N-containing medium and will be harvested at several time-points (e.g. day 2, 4, 6 and 8). The labelled and unlabelled spores will be mixed in 1:1 ratio based on the optical density, the spore coats will be isolated and their proteins digested with trypsin. The $^{14}N/^{15}N$ ratios for the peptides and proteins will be determined using LC-FT-ICR-MS/MS analysis. Raw FT-MS/MS data will be processed with the MASCOT Distiller program. The processed data will be searched with the MASCOT server program against a complete *B. subtilis* ORF translation database and the isotopic ratios for all tryptic peptides are calculated using the Mascot Distiller quantification module. A decrease of the isotopic ratio over the spore maturation time indicates a decrease in digestion efficiency for individual coat proteins, which can be connected to the degree of crosslinking.

Study of thermal resistance of spore coat protein mutants

Thermal resistance of spores to wet heat will be assessed using the well-established screw-cap tube method[17]. In this way the % of mild heat stress survival of spores will be determined for each spore sample at a different stage of maturity (day 2, 4, 6 or 8). The significance of the data will be assessed statistically similar to the data processing reported by our group previously[18].

Estimation of di-tyrosine formation by Raman spectroscopy, spore DPA and water levels

In order to study the effect of di-tyrosine cross-links on spore properties we will follow dityrosine formation in the different aged spores of strains such as *ΔcotC*, *ΔcotU* mutants using mass spectrometry based methods[19]. We will pursue a Raman spectroscopy based method[20] for dityrosine determination as this allows more rapid kinetics measurement routines.

For each time point the DPA content for both ^{14}N and ^{15}N spores will be assessed using an established colorimetric method[21]. The spore water content will be measured using established spectroscopic methods[22,23] again in collaboration with Prof. Woutersen.

Live imaging and single spore analysis for the mutants using Spore Tracker followed by data analysis

A special microscopy technique using a closed air containing chamber, that we developed previously[18], will be used for phase contrast image acquisition. Spore germination times will be analyzed using a semiautomatic image analysis macro called Spore Tracker[18], a plugin for ObjectJ (http://simon.bio.uva.nl/objectj), which runs under ImageJ (http://rsb.info.nih.gov/ij). Spore Tracker allows single-spore live imaging from germination through outgrowth to vegetative outgrowth, as well as the automated quantification of each step. Frequency distribution plots of the germination times of individual spores from day 2 till 8 will be generated. We will use Spore Tracker in the proposed study to determine single-spore germination and outgrowth kinetics through measurements and analysis of growth curves and correlate them with progress in protein crosslinking. The data analysis will help in concluding if the extent of cross-linking and the digestion efficiency of spore coat proteins have a link with the heterogeneity observed in spore germination. As intra-spore physiological parameter of interest the pH will be monitored in quiescent, triggered, outgrowing spores and in resulting vegetative cells[24]. Hence we will be able to address the open question of why more heat resistant spores are the more difficult to germinate and germinate heterogeneously[25]. This is a key question in food microbiology linked to key open questions in fundamental developmental biology on the mechanisms of bacterial spore germination[26]. For practice oriented validation analyses, selected experiments and mutants will be tested in wild-type heat-resistance isolate *B. subtilis* A163 (Unilever), the spores of which are highly heat resistant. The genome of that strain is available (TNO).

Spore cortex peptidoglycan and inner membrane germination protein analyses

To study any likely role of the cortex peptidoglycan structure in thermal resistance of spores, cortex peptidoglycan analyses will be done[28] for the wild type as well as the mutant strains. This will involve HPLC and MALDI-TOF based analysis. This will also help to check any effects of mutations on the structure of the spore cortex with respect to peptidoglycan cross-linking. Inner membrane germination protein presence will be probed using the recently published approach[29].

Literature references

1 Driks, A & Eichenberger, P. The Spore Coat. *Microbiology Spectrum* **4,** 2, TBS-0023-2016 (2016).

2 Setlow, P. Germination of spores of *Bacillus* species: what we know and do not know. *Journal of bacteriology* **196**, 1297-1305, (2014).

3 Abee, T. *et al.* Germination and outgrowth of spores of *Bacillus cereus* group members: diversity and role of germinant receptors. *Food microbiology* **28**, 199-208, (2011).

4 Zhang, J. Q., Griffiths, K. K., Cowan, A., Setlow, P. & Yu, J. Expression level of *Bacillus subtilis* germinant receptors determines the average rate but not the heterogeneity of spore germination. *Journal of bacteriology* **195**, 1735-1740, (2013).

5 Alqurashi, A. M. Anthrax threat: a review of clinical and diagnostic measures. *Journal of the Egyptian Society of Parasitology* **43**, 147-166 (2013).

6 Pitt, T. L., McClure, J., Parker, M., Amezquita, A. & McClure, P. *Bacillus cereus* in personal care products: risk to consumers. *International journal of cosmetic science*, **37**, 165-174, (2014).

7 McKenney, P. T., Driks, A. & Eichenberger, P. The *Bacillus subtilis* endospore: assembly and functions of the multilayered coat. *Nature reviews. Microbiology* **11**, 33-44, (2013).

8 Setlow, P. Spores of *Bacillus subtilis*: their resistance to and killing by radiation, heat and chemicals. *Journal of Applied Microbiology* **101**, 514-525, (2006).

9 Sanchez-Salas, J. L., Setlow, B., Zhang, P., Li, Y. Q. & Setlow, P. Maturation of released spores is necessary for acquisition of full spore heat resistance during *Bacillus subtilis* sporulation. *Applied and environmental microbiology* **77**, 6746-6754, (2011).

10 Abhyankar, W. *et al.* Reinforcement of *Bacillus subtilis* spores by cross-linking of outer coat proteins during maturation. *Food microbiology* **45 Pt A**, 54-62, (2015).

11 Kim, H. *et al.* The *Bacillus subtilis* spore coat protein interaction network. *Molecular Microbiology* **59**, 487-502, (2006).

12 Henriques, A. O., Melsen, L. R. & Moran, C. P., Jr. Involvement of superoxide dismutase in spore coat assembly in *Bacillus subtilis*. *Journal of bacteriology* **180**, 2285-2291 (1998).

13 Isticato, R., Pelosi, A., De Felice, M. & Ricca, E. CotE Binds to CotC and CotU and Mediates Their Interaction during Spore Coat Formation in *Bacillus subtilis*. *Journal of bacteriology* **192**, 949-954, (2010).

14 Ramamurthi, K. S., Clapham, K. R. & Losick, R. Peptide anchoring spore coat assembly to the outer forespore membrane in *Bacillus subtilis*. *Molecular Microbiology* **62**, 1547-1557, (2006).

15 Ragkousi, K. & Setlow, P. Transglutaminase-mediated cross-linking of GerQ in the coats of *Bacillus subtilis* spores. *Journal of bacteriology* **186**, 5567-5575, (2004).

16 Abhyankar, W. *et al.* Gel-free proteomic identification of the *Bacillus subtilis* insoluble spore coat protein fraction. *Proteomics* **11**, 4541-4550, (2011).

17 Kort, R. *et al.* Assessment of heat resistance of bacterial spores from food product isolates by fluorescence monitoring of dipicolinic acid release. *Applied and environmental microbiology* **71**, 3556-3564, (2005).

18 Pandey, R. *et al.* Live cell imaging of germination and outgrowth of individual *Bacillus subtilis* spores; the effect of heat stress quantitatively analyzed with SporeTracker. *PloS one* **8**, e58972, (2013).

19 DiMarco, T. & Giulivi, C. Current analytical methods for the detection of dityrosine, a biomarker of oxidative stress, in biological samples. *Mass spectrometry reviews* **26**, 108-120, (2007).

20 Jarvis, R. M. & Goodacre, R. Discrimination of bacteria using surface-enhanced Raman spectroscopy. *Analytical chemistry* **76**, 40-47, (2004).

21 Janssen, F. W., Lund, A. J. & Anderson, L. E. Colorimetric assay for dipicolinic acid in bacterial spores. *Science* **127**, 26-27 (1958).

22 Groot, C. C. & Bakker, H. J. Hydration dynamics of aqueous glucose probed with polarization-resolved fs-IR spectroscopy. *The Journal of chemical physics* **140**, 234503, (2014).

23 Piatkowski, L., de Heij, J. & Bakker, H. J. Probing the distribution of water molecules hydrating lipid membranes with ultrafast Forster vibrational energy transfer. *The journal of physical chemistry. B* **117**, 1367-1377, (2013).

24 Pandey R., *et al.* Intracellular pH response to weak acid stress in individual vegetative *Bacillus subtilis* cells. *Appl. Env. Microbiol.* **82**, 6463-6471 (2016).

25 Ghosh, S., Zhang, P. F., Li, Y. Q. & Setlow, P. Superdormant spores of *Bacillus* species have elevated wet-heat resistance and temperature requirements for heat activation. *J. Bacteriol*. **191**, 5584–5591(2009).

26 Setlow, P. Germination of spores of *Bacillus* species: what we know and do not know. *J. Bacteriol.* **196**, 1297-1305 (2014).
27 Vizcaino J.A., Cote R.G., Csordas A., et al. The PRoteomics IDEntifications (PRIDE) database and associated tools:status in 2013. Nucleic acids research. **41**(Database issue):D1063-1069 (2013).
28 Meador-Parton, J., Popham, D.L. Structural Analysis of *Bacillus subtilis* Spore Peptidoglycan during Sporulation. *J. Bacteriol.* **182**, 4491–4499 (2000).
29 Zheng, L., Abhyankar, W., Ouwerling, N. et al. *Bacillus subtilis* Spore Inner Membrane Proteome. J. Proteome Res. 15, 585-594 (2016).

Timetable of the project

	Year 1				Year 2				Year 3				Year 4			
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Tasks																
1 Growth, sporulation and characterization of spores from *B. subtilis*	■	■	■													
2 Time-resolved analyses of $^{14}N/^{15}N$ spores for the digestion efficiency of coat proteins and peptides.			■	■	■	■	■	■	■							
3 Study of thermal resistance of spore coat protein mutants + Analysis of cortex cross-linking + Analysis of spore inner membrane proteins as well as DPA and water content.					■	■	■	■	■							
4 Analysis of di-tyrosine formation and its effect on thermal resistance and germination characteristics of spores.						■	■	■	■							
5 Live imaging and single spore analysis for the mutants using Spore Tracker followed by data analysis.							■	■	■	■	■					
6 Validation of selected findings in strain A163 a high thermal stress resistant spores forming food spoilage isolate.											■	■	■	■	■	■
Deliverables	**Year 1**				**Year 2**				**Year 3**				**Year 4**			
1 Produced mutant strains and their spores in stock available for further analysis.				X												
2 ^{15}N labelled reference (fully matured) spores for all control and mutant strains indicated put in stock for analysis.						X										
3 Trypsin digestibility determined of samples at various days of maturation compared to the ^{15}N labelled matured spores.										X						
4 Heat resistance (knock-out mutants), cortex analyses, DPA and water content of spores are documented.								X								
5 Di-tyrosine levels in relevant mutant spore coats documented.								X								
6 Thermal stress resistance and trypsin digestibility of reference and site-directed mutagenized strains reported.												X				
7a Germination and outgrowth characteristics of knock-out mutants documented.												X				
7b Germination and outgrowth characteristics of site-directed mutagenized strains documented.														X		
8 Thesis including validation experiments in wild-type heat resistant isolate A163.																X

Milestones	**Year 1**				**Year 2**				**Year 3**				**Year 4**			
1 Mutant strain construction and sporulation done.				X												
2 Control and mutant fully matured spores generated in the presence of ^{15}N ammonium.							X									
3 Trypsin digestion of samples at various days of maturation and ^{15}N labelled matured spores done.									X							
4 Heat resistance (knock-out mutants), cortex cross-linking, DPA and water content of spores analyzed.								X								
5 Di-tyrosine levels in relevant mutant spore coats analyzed.								X								
6 Thermal stress resistance and trypsin digestibility analyzed of reference and specific site-directed mutagenized strains.											X					
7a Germination and outgrowth characteristics of knock-out mutants established.												X				
7b Germination and outgrowth characteristics of site-directed mutagenized strains done.														X		
8 Validation experiments in wild-type heat resistant isolate A163 done & thesis draft finished																X

Scientific embedding of the proposed research

The dept. of Molecular Biology and Microbial Food Safety (MBMFS) headed by prof. Stanley Brul (including an endowed chair in Microbial Food Safety funded by the Dutch Food Safety Authority), is part of the Swammerdam Institute of Life Sciences (SILS) within the Faculty of Science of the University of Amsterdam. The group includes generally ~8-10 PhD students (often on projects with other SILS groups), post-docs and hosts many bachelor and master traineeships. SILS as a whole accommodates more than 200 researchers active in biology, (bio) chemistry, data analysis and systems biology. The MBMFS laboratory has state of the art facilities, shared with the laboratory for Microbiology (prof. Leendert Hamoen, expert of vegetative growth and cell division in *Bacillus*. Prof. Hamoen recently succeeded prof. Klaas Hellingwerf). The facilities include a top notch microbiological infrastructure ranging from well controlled fermenters to full fletched chemostats. We operate in the field of bacterial spore formers as one group together with the dept. of Mass Spectrometry of Bio-macromolecules (MSB) of Prof. dr. Chris de Koster. Jointly we focus on spore stress resistance, its dependency on sporulation conditions and on the molecular structure and function of the spore germinosome (protein complex with germination receptor proteins). Our research specifically aims at: (1) the role of spore layer protein-protein interactions in the development of spore heat resistance; (2) identification of the germinosome proteins and their interactions, (3) functional characterisation of the germinosome through quantitative proteomics and (4) heterogeneity in spore germination and outgrowth. We address major 'applied' challenges in food microbiology regarding spore thermal resistance, antimicrobial resistance and the identification of (targets for) novel antimicrobial compounds. The Brul and de Koster groups are highly regarded in the international spore community not in the least exemplified by their presentations at various international meetings. Most recently their results were presented at the 7th European Spore Conference (April 2016, London) and by prof. Brul as invited speaker at the European Symposium of the IAFP (June 2016, Athens). Prof. Brul has been invited to organise the session Food Microbiology & Safety at the 2017 6th FEMS European Congress of Microbiology. His 1999 review on the mode of action of preservatives has over 800 citations. Within SILS collaborations exist with Dr.Erik Manders (Van Leeuwenhoek Centre for Advanced Microscopy) facilitating the visualisation and time lapse microscopy analysis of spore germination. For chemical analyses using Raman spectroscopy we team up with prof. dr. Sander Woutersen of the Molecular Photonics group (Van't Hoff Institute for Molecular Sciences). For the statistical data analysis, we collaborate with Dr. Huub Hoefsloot from SILS Biosystems Data Analysis. Recently a (closer) collaboration has been established with prof. Peter Setlow (University of Connecticut, USA) regarding the analysis of the germinosome using the SILS state of the art microscopy facilities. With prof. Mike Peck (Institute for Food Research, UK) and Florence Postollec (ADRIA, University of Brest, France) established relations exist in studying applied aspects of spore germination and outgrowth. Prof. **Brul** co-edited a special issue of the Int. J. Food Microbiol. with papers from the 9th International Conference on Predictive Modelling in Food (Rio de Janeiro

sept. 2015). He also, recently, edited a Current Opinion in Food Science issue on Food Omics Technologies. **Brul, S.** (2016) The rise of the 'omics' technologies and their relevance to food. Curr. Opin. Food Sci. (in press).

Knowledge utilisation

Bacterial spores are a major problem in the food industry as well as in hospitals. Their high resistance allows them to escape the treatments designed to inactivate bacteria. In the final food products and in patients in hospitals, the spores can germinate, outgrow and multiply, leading to food-spoilage and intoxication. To reduce spore-related problems, substantial amounts are invested in food quality control and in special patient care in hospitals. Consumers nowadays prefer less processed food thereby allowing easy survival of spores. In addition, if food is treated with extreme measures to kill the spores then the nutritious properties as well as the organoleptic characteristics of food items are lost. Thus thorough understanding of the resistance mechanisms of spores will enable the industries to design processes that will more efficiently eliminate spores. This will lead to an extended shelf-life of food products, a reduction of spoilage and poisoning events, cheaper processes and better product quality as well as restricted spore-mediated outbreaks.

Beneficiaries identified:

Bacilli are well known to be a major source of Food spoilage and food borne disease. Some of the strains generate extremely heat resistant spores causing food producers to have to adopt harsh preservation strategies. Obviously such preservation approaches are not optimal for food quality! Hence consumers, major food companies like Unilever (Netherlands/UK), and the microbiology research departments of applied research organisations like, NIZO Food Research (Netherlands), TNO (Netherlands), as well as ADRIA (France) and IFR (UK) benefit from both scientific interaction with our groups and the results of this project. The research group of prof. Kuipers in Groningen, who has been heavily involved together with NIZO Food Research, in a Top Institute for Food & Nutrition project on high heat resistance in bacterial spores, is also an obvious user. The data of our project also provide valuable input, albeit obviously NOT directly in any joint project, for our collaboration with the Netherlands Food and Consumer Product Safety Authority (NVWA) that funds a collaborative strategic research program at the MBMFS group.

Education:

The PhD student will receive training in research and will also be trained to supervise master students as well as 3rd year bachelor students. Furthermore it is foreseen that the PhD student can develop him / herself through courses as appropriate. The candidate will be embedded in an academic research environment. Through participation in work discussions, biweekly spore meeting and scientific conferences the student will be exposed to microbial proteomics and molecular physiology. In the frame work of employability our institute offers training of personal skills to help the student to pursue an adequate position for a career in or outside academia.

Data management:

Upon completion of the project data are filed in an international proteomic data repository (PRIDE) as is our policy for all our proteomics projects. We will store protein and peptide identifications and quantitative values, the analysed mass spectra and the related technical and biological metadata including single-cell germination kinetics time–profiles. In the prepublication phase data are designated as private and will be accessible for the research team and the stake holders. Post-publication the data will be available for the public. Proteomics and germination and outgrowth data will be added in detailed supplementary materials to our publications.

Data distribution or integration:

Proteomics data and germination / outgrowth physiological meta-data will be stored in the international proteomic data repository PRIDE[27]. PRIDE is a centralized, standards compliant, public database for mass spectrometry proteomics data, including protein and peptide identifications (and the corresponding expression values, if available), post-translational modifications and supporting mass spectra evidence (both as raw data and peak list files) at the European Bioinformatics Institute. PRIDE is a core member in the ProteomeXchange (PX) consortium, which provides a standardised way for submitting mass spectrometry based proteomics data to public-domain repositories. Datasets are submitted to ProteomeXchange via PRIDE and are handled by expert biocurators. All PRIDE public datasets have their unique ID by which those datasets can be searched in ProteomeCentral, the portal for all ProteomeXchange datasets.

Name: Prof. dr. Stanley Brul
Place: Amsterdam Date: 07/11/2016