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### Proteome dynamics during sporulation and heat resistance in *Bacillus subtilis* spores

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## Summary

Bacterial spores are one of the common concerns for the food industry. Sources of contamination by bacterial spores in the food chain may include, but are not limited to, water, soil, food ingredients and the processing chain itself. The extreme thermal resistance exhibited by the spores favors their survival of food processing and long-term persistence in foods. Beside the problems caused in the food chain, some species of *Bacillus* and *Clostridium* are causative agents of several serious human diseases. Therefore, the investigation of the sporulation (spore formation) characteristics at the molecular physiological level and thus, among others the spore proteome, are crucial to further our understanding of spore physiology and to ensure the most efficient control of the problems caused by spores.

**Chapter 1** concisely introduces the general knowledge of spore structure, spore germination and outgrowth, sporulation and sporulation regulatory systems, and sporulation heterogeneity.

**Chapter 2** describes how the artificial induction of *kinA* in *B. subtilis* can minimize the heterogeneity of sporulation initiation and therefore homogenize sporulation to result in 70% spore formation of the population within 8 hours. Moreover, spores from a cell population in which *kinA* is induced showed an elevated resistance to wet heat compared to the spores of the wildtype strain. The induced spores also showed a delayed germination behavior compared to wildtype spores. Transmission electron microscopy analysis indicates that the induced spores had a relative thicker cortex and coat layer. Proteome comparison of spores of the *kinA* induced cell population and the wildtype cell population reveals the upregulation of sets of proteins enriched in sporulation but also the downregulation of other sets of proteins enriched in sporulation, metabolism and coping with stress functionality. Thus, while *kinA* overexpression enhances synchronicity in sporulation initiation, it also has profound effects on the central equilibrium of spore formation, and spore resistance and germination, through modulation of the spore's molecular composition.

**Chapter 3** reports on the proteome changeover during the

homogeneous sporulation described in chapter 2. Four co-expressed modules, modules brown, green, blue, and yellow, were revealed thorough a protein co-expression analysis. Among them, modules brown and green are associated with sporulation. Module blue is associated with ribosomal and metabolic proteins. Module yellow is co-expressed with all other three modules. Remarkably, the levels of some of the coat proteins decreased late in sporulation. A logical assumption is that these coat proteins could have played roles in guiding or helping assembly of other coat proteins, after which they became “surplus” and were degraded. This study highlights the dynamics of protein expression during sporulation at high temporal resolution and illustrates its highly dynamic nature.

In **chapter 4**, the DPA level of spores and the proteome of high heat resistant spores of the food isolate strain *B. subtilis* A163 was investigated. Significantly higher amounts of DPA were observed in spores of *B. subtilis* A163 than in *B. subtilis* PY79, a low heat resistance strain. In addition, the release rate of spore DPA from *B. subtilis* A163 at 98°C was in our hands lower than that of *B. subtilis* PY79. In total, 2011 and 1901 proteins were identified in the spores and the cells of *B. subtilis* A163. Among them 108 proteins in spores and 93 proteins in cells were differentially present compared to *B. subtilis* PY79. In addition, morphogenetic protein SpoVM has no homologs found in *B. subtilis* A163. These findings form a basis for further mechanistic analysis of the high thermal resistance and the low DPA release rate of the *B. subtilis* A163 spores.

In **chapter 5**, the single-pot, solid phase-enhanced sample-preparation (SP3) was successfully applied to the proteome study of *B. subtilis* spores and cells. With SP3, more membrane proteins were qualitatively and quantitatively identified. In addition, SP3 could replace the “one-pot” method to process spores and cells of *B. subtilis* in proteomic analysis studies.

**Chapter 6** summarizes the results achieved in this thesis, and further discusses the mass spectrometry-based proteomic strategies used in this thesis, the implications of the data for spore thermal stress resistance and the future perspectives derived from this data presented in this thesis.

## Samenvatting

Bacteriële sporen zijn een van de problemen voor de voedingsindustrie. Bronnen van deze vervuiling met bacteriele sporen zijn onder andere water, grond, ingrediënten en de verwerkingsketen zelf. De resistentie van de sporen tegen extreme hitte helpt ze overleven tijdens het verwerkingsproces en bij de langdurige overleving in het voedsel. Naast de problemen in de voedingsindustrie, zijn enkele soorten van *Bacillus* en *Clostridium* ook de veroorzakers van serieuze humane ziektes. Onderzoek naar de sporulatie (sporevorming), de eigenschappen op het moleculaire fysiologische niveau en het proteoom van de sporen zijn daarom cruciaal in het onderzoek naar de fysiologie en efficiënte controle van de problemen die door deze sporen worden veroorzaakt.

**Hoofdstuk 1** introduceert kort de algemene kennis over de structuur van sporen, de sporenkieming en uitgroei, sporulatie en de regulatoire systemen en de heterogeniteit van het sporulatie proces.

**Hoofdstuk 2** beschrijft dat kunstmatige inductie van *kinA* in *B. subtilis* heterogeniteit in de initiatie van sporulatie kan minimaliseren. Hierdoor is het mogelijk om sporulatie te homogeniseren waardoor 70% van de populatie binnen 8 uur sporen vormt. Ook vertonen deze sporen verhoogde resistentie tegen hitte in vergelijking met wildtype sporen. Transmissie elektronenmicroscopie analyse laat zien dat de *kinA* geïnduceerde sporen een relatief dikkere cortex en coatlaag hebben. Wanneer het proteoom vergeleken wordt tussen wildtype en *kinA* geïnduceerde sporen is te zien dat een set genen die betrokken zijn bij sporulatie opgereguleerd worden en maar ook een set genen die betrokken zijn bij het sporulatie, metabolisme, en stress resistentie juist neerwaarts gereguleerd worden. *KinA* overexpressie verbetert de synchroniteit in de initiatie van sporulatie, maar heeft ook een diepgaand effect op het centrale evenwicht tussen sporevorming, -weerstand en -kieming, doordat het ook de moleculaire compositie van de spore moduleert.

**Hoofdstuk 3** rapporteert de omschakeling van het proteoom gedurende de homogene sporulatie die in hoofdstuk 2 wordt beschreven. Vier modules die gezamenlijk tot expressie komen, module bruin, groen, blauw en geel, zijn met

behulp van een eiwit co-expressie analyse ontdekt. Modules bruin en groen worden geassocieerd met sporulatie. Module blauw wordt geassocieerd met ribosomale en metabole eiwitten. Module geel wordt tegelijkertijd met alle andere drie modules tot expressie gebracht. Opvallend is dat de expressie van een aantal coat eiwitten afnam, naarmate de sporulatie vorderde. Een logische aanname is dat deze coateiwitten een rol spelen in het begeleiden of opbouwen van andere coateiwitten, maar hun rol later overbodig was en ze werden afgebroken. Deze studie laat de grote dynamiek in eiwitexpressie gedurende het sporulatieproces zien in een hoge tijdsresolutie.

In **hoofdstuk 4** is de hoeveelheid DPA en het proteoom van sporen van een stam met hoge hitte resistentie onderzocht. Significant hogere hoeveelheden DPA werden gevonden in sporen van *B. subtilis* A163 in vergelijking met PY79 welke minder hitte resistent is. Ook was de snelheid waarmee DPA vrijkwam uit *B. subtilis* A163 bij 98°C in onze experimenten lager dan die van PY79. In totaal zijn er 2011 en 1901 eiwitten geïdentificeerd in respectievelijk de sporen en vegetatieve cellen van *B. subtilis* A163. 108 eiwitten in sporen en 93 eiwitten in vegetatieve cellen waren differentieel aanwezig in vergelijking met PY79. Daarnaast bleek het morphogene eiwit SpoVM afwezig in A163, wat een mogelijke verklaring kan zijn voor de hoge hitte resistentie en lage DPA afgifte van de A163 sporen.

In **hoofdstuk 5** is de single-pot solid phase-enhanced sample preparation (Sp3) methode succesvol toegepast op de proteoom studie van *B. subtilis* sporen en vegetatieve cellen. Met SP3 zijn een groter aantal membraaneiwitten geïdentificeerd en gequantificeerd. Ook wordt aangetoond dat deze methode de oudere one-pot methode kan vervangen bij *B. subtilis* proteoom studies.

**Hoofdstuk 6** vat de resultaten uit deze thesis samen en bespreekt de op massaspectrometrie gebaseerde proteoom strategieën die zijn gebruikt in deze thesis, de implicaties voor de hitteresistentie van sporen en de toekomstperspectieven die het werk in deze studie biedt.

Edited by Richard de Boer

## List of publications

Tu, Z., Setlow, P., Brul, S., & Kramer, G. (2021). Molecular Physiological Characterization of a High Heat Resistant Spore Forming *Bacillus subtilis* Food Isolate. *Microorganisms*, 9(3), 667.

Tu, Z., R Abhyankar, W., N Swarge, B., van der Wel, N., Kramer, G., Brul, S., & J de Koning, L. (2020). Artificial Sporulation Induction (ASI) by *kinA* Overexpression Affects the Proteomes and Properties of *Bacillus subtilis* Spores. *International journal of molecular sciences*, 21(12), 4315.

Abhyankar, W. R., Wen, J., Swarge, B. N., Tu, Z., de Boer, R., Smelt, J. P. P. M., ... & Brul, S. (2019). Proteomics and microscopy tools for the study of antimicrobial resistance and germination mechanisms of bacterial spores. *Food microbiology*, 81, 89-96.



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