High-level heat resistance of spores of *Bacillus amyloliquefaciens* and *Bacillus licheniformis* results from the presence of a *spoVA* operon in a Tn1546 transposon.

Erwin M. Berendsen  
Rosella A. Koning  
Jos Boekhorst  
Anne de Jong  
Oscar P. Kuipers  
Marjon H.J. Wells-Bennik
Abstract

Bacterial endospore formers can produce spores that are resistant against many food processing conditions, including heat. Some spores may survive heating processes aimed at production of commercially sterile foods. Recently, it was shown that a spoVA operon present on a Tn1546 transposon in *Bacillus subtilis* profoundly increases the wet heat resistance of spores. In this study, the presence of the Tn1546 transposon was assessed in nine strains of *Bacillus amyloliquefaciens* and nine strains of *Bacillus licheniformis*, and was found to be naturally present in several strains. Its effect on the wet heat resistance of spores was investigated. The presence of the Tn1546 transposon homologues and spoVA operon was confirmed by whole genome sequencing and PCR detection. Strains of *B. amyloliquefaciens* and *B. licheniformis* carrying a Tn1546 transposon produced spores with significantly higher resistance to wet heat than their counterparts that lacked this transposon. The spoVA operons encoded on the Tn1546 transposons of *B. licheniformis* and *B. amyloliquefaciens* were cloned into *B. subtilis* 168, and resulted in strains that produced high-level heat resistant spores. The finding that spoVA genes on a transposon determine heat resistance properties of spores of strains belonging to the *B. subtilis* group can contribute to improved control of these spores in the food chain.
High-level heat resistance of spores of *B. amyloliquefaciens* and *B. licheniformis*

Introduction

Bacterial spore formers can survive harsh environmental conditions due to the formation of endospores (spores) and they are ubiquitously found in nature (27, 40). Since bacterial spores are widely present in nature, they can enter the food chain from many different sources, for example via soil (13). The intrinsic resistance properties of spores potentially result in survival during food processing, in which heating is one of the most commonly applied treatments to reduce bacterial loads. Such treatments put selective pressure on the microflora that is present, allowing for survival of those strains that produce spores with high heat resistance (32). Surviving spores may germinate upon exposure to certain environmental triggers, and can subsequently resume vegetative growth, potentially resulting in food pathogenicity or food spoilage, depending on the species (39).

Spores of mesophilic species belonging to the *B. subtilis* group are commonly found in various food ingredients and food products. The *B. subtilis* group encompasses the species *B. subtilis, B. amyloliquefaciens, B. licheniformis, B. vallismortis, B. mojavensis, B. atropheus* and *B. sonorensis*, which are phylogenetically close, yet distinguishable (22). These species can generally grow between temperatures of 30°C to 50°C, with reported growth temperatures of *B. licheniformis* up to 58°C (51). The spores of *B. subtilis, B. amyloliquefaciens* and *B. licheniformis*, are commonly found in various food ingredients and food products including cocoa, herbs, spices, bread, soups, milk and milk powders (20, 23, 26, 29, 42). These species are for instance well-known contaminants of raw materials used in bread making (36, 41), and the spores can potentially even survive the bread baking process (44). After spore survival, germination, and outgrowth, vegetative cells of *B. amyloliquefaciens, B. subtilis* or *B. licheniformis* can result in spoiled bread, by degradation of starch and the formation of extracellular polysaccharide, resulting in ropy bread (41, 44, 45). Certain strains of *B. licheniformis* can produce a toxin, lichenisyn A, that can lead to foodborne illness (21, 28, 38). Lichenisyn is a non-ribosomally synthesized lipo-peptide that is heat-stable (14). Due to the pathogenic potential of strains of *B. licheniformis*, it is critical to control these spores in the food chain (24).

The resistances of spores toward wet heat treatments that are applied in food processing can vary significantly between strains within the *B. subtilis* group (4, 15, 20, 29). A detailed analysis of the heat resistance of spores for fourteen strains of the *B. subtilis* group revealed the presence of two distinct groups of strains that produce spores with significantly different heat resistances of spores (4). For *B. subtilis* strains, we recently demonstrated that the high-level heat resistance of spores resulted from the presence of a *spoVA* operon (designated *spoVA*\(^{mob}\), where mob indicates the presence on a mobile genetic element) that is encoded on a Tn1546 transposon (3). In another study, we
observed high-level heat resistance of spores of two *B. amyloliquefaciens* strains (B4140 and B425), with spores of strain B425 showing heat resistance levels similar to those of spores of *B. subtilis* strains with a Tn1546 transposon (4). Given the fact that *B. subtilis*, *B. amyloliquefaciens* and *B. licheniformis* are closely related, variation in resistance of spores to wet heat in the latter two species may also result from the presence or absence of a transposon.

In this study, we evaluated presence or absence of the Tn1546 transposon homologue that mediates heat resistance of spores in *B. subtilis* in nine strains of *B. amyloliquefaciens* and nine strains of *B. licheniformis*. This was performed by either genome analysis or by PCR detection. In addition, the detailed spore heat inactivation kinetics were determined for all eighteen strains, and phenotypic data on spore heat resistance correlated with the presence or absence of the Tn1546 transposon. The *spoVA*<sup>2mob</sup> operons found in *B. amyloliquefaciens* and *B. licheniformis* were introduced into *B. subtilis* to assess their role in spore heat resistance.

### Materials and methods

#### Bacterial strains used in this study

The strains used in this study for genomic and phenotypic analyses are listed in Table 1. In this study, nine strains of *B. amyloliquefaciens* and nine strains of *B. licheniformis* were analyzed. For *B. amyloliquefaciens* strains B425 and B4140, the heat inactivation kinetics of their spores were described previously (4). Strains of *B. subtilis* 168 and *B. subtilis* B4146 were included in the genome analysis, and were previously analyzed for heat resistance of spores (4).

#### Genome analysis

Multiple sequence alignments were made for protein sequences of conserved genes that were present in single copy in all genomes using MUSCLE (10). The core genome phylogenetic tree was constructed using PHYML (12). To verify the presence of the Tn1546 transposon and the encoded *spoVA* (designated *spoVA*<sup>2mob</sup>) operon, an orthology matrix was constructed using Ortho-MCL (18) with the genomes of the four *B. amyloliquefaciens* strains, the nine *B. licheniformis* strains, *B. subtilis* B4146 as a strain that produces spores with high heat resistance and *B. subtilis* 168 as a reference strain, producing spores of normal heat resistance. The genomic organization of the Tn1546 transposon was visualized using Artemis, the Artemis comparison tool (ACT), and using microbial genomic context viewer (MGcV) (5, 6, 31). For the identified Tn1546 transposons, operon predictions were performed using FGENESB (www.softberry.com). Additionally, manual sequence comparisons and searches for pseudogenes were performed for all genes in the transposon.
Table 1. Strains used in this study.

<table>
<thead>
<tr>
<th>Strain No</th>
<th>Species</th>
<th>Description</th>
<th>Tn1546</th>
<th>Genome sequence</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>B425</td>
<td><em>B. amyloliquefaciens</em></td>
<td>Isolated from sterilized milk</td>
<td>Yes</td>
<td>LQYP00000000</td>
<td>(4)</td>
</tr>
<tr>
<td>B4140</td>
<td><em>B. amyloliquefaciens</em></td>
<td>Isolated from pizza</td>
<td>No</td>
<td>LQYO00000000</td>
<td>(4)</td>
</tr>
<tr>
<td>10A5</td>
<td><em>B. amyloliquefaciens</em></td>
<td>Known as 10A5; NRRL B-14393, isolated from soil</td>
<td>No (PCR)</td>
<td>No</td>
<td>(34)</td>
</tr>
<tr>
<td>FZB42-</td>
<td><em>B. amyloliquefaciens</em></td>
<td>Known as 10A6; FZB42, isolated from plant soil</td>
<td>No</td>
<td>NC_009725</td>
<td>(9)</td>
</tr>
<tr>
<td>10A18</td>
<td><em>B. amyloliquefaciens</em></td>
<td>Known as 10A18; CU8004</td>
<td>No (PCR)</td>
<td>No</td>
<td>(52)</td>
</tr>
<tr>
<td>DSM7-</td>
<td><em>B. amyloliquefaciens</em></td>
<td>Known as DSM7, isolated from soil</td>
<td>Yes</td>
<td>FN597644</td>
<td>(37)</td>
</tr>
<tr>
<td>DSM1060</td>
<td><em>B. amyloliquefaciens</em></td>
<td>Known as DSM1060</td>
<td>No (PCR)</td>
<td>No</td>
<td>(33)</td>
</tr>
<tr>
<td>101</td>
<td><em>B. amyloliquefaciens</em></td>
<td>Received as 101</td>
<td>No (PCR)</td>
<td>No</td>
<td>This study</td>
</tr>
<tr>
<td>SB42</td>
<td><em>B. amyloliquefaciens</em></td>
<td>Received as SB42</td>
<td>No (PCR)</td>
<td>No</td>
<td>This study</td>
</tr>
<tr>
<td>B4089</td>
<td><em>B. licheniformis</em></td>
<td>Known as E5/T12, isolated from pea soup</td>
<td>No</td>
<td>LKPM00000000</td>
<td>(29)</td>
</tr>
<tr>
<td>B4090</td>
<td><em>B. licheniformis</em></td>
<td>Known as T1, isolated from pea soup</td>
<td>Yes</td>
<td>LQYL00000000</td>
<td>(29)</td>
</tr>
<tr>
<td>B4091</td>
<td><em>B. licheniformis</em></td>
<td>Known as T29, isolated from mushroom soup</td>
<td>No</td>
<td>LQYM00000000</td>
<td>(29)</td>
</tr>
<tr>
<td>B4092</td>
<td><em>B. licheniformis</em></td>
<td>Isolated from butter-milk powder</td>
<td>Yes</td>
<td>LQYK00000000</td>
<td>This study</td>
</tr>
<tr>
<td>B4094</td>
<td><em>B. licheniformis</em></td>
<td>Isolated from camomile tea</td>
<td>Yes</td>
<td>LKPN00000000</td>
<td>This study</td>
</tr>
<tr>
<td>B4121</td>
<td><em>B. licheniformis</em></td>
<td>Isolated from sateh pastry</td>
<td>No</td>
<td>LKPO00000000</td>
<td>This study</td>
</tr>
<tr>
<td>B4123</td>
<td><em>B. licheniformis</em></td>
<td>Isolated from sateh pastry</td>
<td>No</td>
<td>LKPP00000000</td>
<td>This study</td>
</tr>
<tr>
<td>B4124</td>
<td><em>B. licheniformis</em></td>
<td>Isolated from pancakes</td>
<td>No</td>
<td>LKPQ00000000</td>
<td>This study</td>
</tr>
<tr>
<td>B4125</td>
<td><em>B. licheniformis</em></td>
<td>Isolated from pancakes</td>
<td>No</td>
<td>LKP00000000</td>
<td>This study</td>
</tr>
<tr>
<td>B4062</td>
<td><em>B. subtilis</em></td>
<td>Type strain 168</td>
<td>No</td>
<td>NC_000964</td>
<td>(17)</td>
</tr>
<tr>
<td>B4146</td>
<td><em>B. subtilis</em></td>
<td>Isolated from curry sauce</td>
<td>Yes</td>
<td>NZ_JXHR01000000</td>
<td>(4)</td>
</tr>
<tr>
<td>168-spoVA&lt;sup&gt;2mob&lt;/sup&gt; (B4090)</td>
<td><em>B. subtilis</em></td>
<td>168 amyE::spoVA&lt;sup&gt;2mob&lt;/sup&gt; (B4090) specR</td>
<td>-</td>
<td>-</td>
<td>This study</td>
</tr>
<tr>
<td>168-spoVA&lt;sup&gt;2mob&lt;/sup&gt; (DSM7)</td>
<td><em>B. subtilis</em></td>
<td>168 amyE::spoVA&lt;sup&gt;2mob&lt;/sup&gt; (DSM7) specR</td>
<td>-</td>
<td>-</td>
<td>This study</td>
</tr>
</tbody>
</table>

Generic PCR primers were designed for the detection of the Tn1546 encoded genes *tnpA*, *spoVAC<sup>2mob</sup>* and *cls* in the other strains of *B. amyloliquefaciens*. The primers were designed based on aligned nucleotide sequences of the corresponding genes found in *B. subtilis*, *B. licheniformis* and *B. amyloliquefaciens* using MUSCLE (10). These primers (Table 2) were used for verification of the presence of these genes in the five strains of *B. amyloliquefaciens* (10A5, FZB42, 10A18, 101 and SB42) for which no genome sequence was available.
Chapter 4

**Table 2.** Primers used in this study. Primers were designed for detection of tree genes of the Tn1546 transposon: *tnpA*, *spoVAC<sup>2mob</sup>* and cardiolipin synthase (*cls*). The expected PCR fragment sizes for *tnpA*, *spoVAC* and *cls*, are 181 bp, 192 bp, and 687 bp, respectively. For the *B. amyloliquefaciens* strains 101, SB42, DSM1060, 10A5, and 10A18 a PCR was performed on genomic DNA for the detection of Tn1546 encoded *tnpA*, *spoVAC* and *cls* genes. The *spoVA<sup>2mob</sup>* operon was cloned from B4090 and DSM7 into pDG1730 (11) using the primers EMB1-F and EMB23-R.

<table>
<thead>
<tr>
<th>Primer used for</th>
<th>Name primer</th>
<th>DNA sequence 5’ to 3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>Universal <em>spoVAC&lt;sup&gt;2mob&lt;/sup&gt;</em></td>
<td>Uni-<em>spoVAC</em>-F</td>
<td>TGAGCAACGGCAGAATTC</td>
</tr>
<tr>
<td>Universal <em>spoVAC&lt;sup&gt;2mob&lt;/sup&gt;</em></td>
<td>Uni-<em>spoVAC</em>-R</td>
<td>ACCTAGGGCAATACAAATC</td>
</tr>
<tr>
<td>Universal <em>cls</em></td>
<td>Uni-<em>cls</em>-F</td>
<td>TGAGCAATCTGGGTGCTTCAG</td>
</tr>
<tr>
<td>Universal <em>cls</em></td>
<td>Uni-<em>cls</em>-R</td>
<td>ATCAAGGGTGCTCAACTCTG</td>
</tr>
<tr>
<td>Universal <em>tnpA</em></td>
<td>Uni-<em>tnpA</em>-F</td>
<td>CAGCGTGGCTACAGCTTAC</td>
</tr>
<tr>
<td>Universal <em>tnpA</em></td>
<td>Uni-<em>tnpA</em>-R</td>
<td>CGTTCGTTCCCTAGCTTC</td>
</tr>
<tr>
<td>EMB23</td>
<td>EMB1-F</td>
<td>CCGCGGATCTGGGAAAGGTTATTATCG</td>
</tr>
<tr>
<td>EMB23</td>
<td>EMB23-R</td>
<td>CCGCGGATCTGGGAAAGGTTATTATCG</td>
</tr>
</tbody>
</table>

**Heat inactivation kinetics of spores**

To verify whether the presence of the Tn1546 transposon influences the heat resistance of spores, spores were prepared as described previously and subjected to heat treatments. For the *B. amyloliquefaciens* strains B4140 and B425, the inactivation kinetics were previously determined, and those data were included in the current analysis (4). For all other *B. amyloliquefaciens* and *B. licheniformis* strains, spore crops were prepared. The initial spore concentrations were determined by heating at 80°C for 10 minutes, followed by pour plating in nutrient agar, and incubation for 5 days at 37°C. Spores were suspended at a concentration of 1 x 10<sup>8</sup> CFU/mL in peptone physiological salt. The heat inactivation kinetics of spores were determined for one spore crop per strain, at three different temperatures, using at least five time points. The inactivation temperatures were selected on the basis of the resistance of the spores of a strain (4). The inactivation data obtained were fitted with a log linear model, and decimal reduction times (*D*-values) were calculated using equation 1.

Equation 1:  \[ \log N(t) = \log N(0) - \frac{t}{D} \]

The *D*-values at 110°C were plotted versus temperature to visualize the strain variation in heat resistance of spores in relation to the presence or absence of the Tn1546 transposon. Additionally, for all strains and groups of strains the *z*-value (i.e. the increase in temperature required to achieve an additional log unit reduction), and the reference *D*-value (*D*<sub>ref</sub>) at reference temperature (*T*<sub>ref</sub>) 110°C were calculated (46).

**Cloning of the *spoVA<sup>2mob</sup>* operon**

The *spoVA<sup>2mob</sup>* operon, including predicted promotor region, was cloned from *B. licheniformis* B4090 and *B. amyloliquefaciens* DSM7 into plasmid pDG1730, as
High-level heat resistance of spores of B. amyloliquefaciens and B. licheniformis

previously described (3). The obtained constructs were transformed to B. subtilis 168 and integrated in the amyE locus, to verify the role of this operon in increased heat resistance of spores. Spores were prepared for strains 168 amyE::spoVA\textsuperscript{mob} (DSM7), 168 amyE::spoVA\textsuperscript{mob} (B4090) and 168 as described above, and the heat resistance of spores of these strains was assessed by heating at 100°C for 1 hour, followed by plating, incubation and enumeration of survivors.

Results and discussion

Genome mining for the Tn1546 transposon

For B. subtilis strains, it has been demonstrated that the presence of a Tn1546 transposon leads to a profound increase in heat resistance of the spores (3). Here, the presence of the Tn1546 transposon was assessed in nine strains of B. amyloliquefaciens and nine strains of B. licheniformis. The genome sequences of all nine strains of B. licheniformis were available and in three of these strains the Tn1546 transposon was found (namely, strains B4090, B4092 and B4094) (Figure 1). The genome sequences of four strains of B. amyloliquefaciens were available, and the Tn1546 transposon was found in two of these strains of B. amyloliquefaciens (namely in B425 and DSM7) (Figure 1). The predicted protein for the transposase TnpA, which is part of the Tn1546 transposon, was found in orthologous group OG3133 for all of the strains that carry the Tn1546 transposon. Genome sequences were not available for the other five strains of B. amyloliquefaciens. PCR-based detection of the genes tnpA, spoVAC and cls (that are present on the transposon and very well conserved) did not reveal the transposon in these five strains, whereas the PCRs using primers for these three target genes were positive for B. amyloliquefaciens strains B425 and DSM7. In short, two out of nine strains of B. amyloliquefaciens and three out of nine strains of B. licheniformis contained the Tn1546 transposon.

Heat resistance of spores is related to the presence of the Tn1546 transposon

The heat resistances of spores of B. amyloliquefaciens and B. licheniformis were assessed in relation to the presence or absence of the Tn1546 transposon. Detailed heat inactivation kinetics of spores were obtained for seven strains of B. amyloliquefaciens (including the two strains carrying the Tn1546 transposon) and nine strains of B. licheniformis (Table 3). The inactivation kinetics of spores of B. amyloliquefaciens strains B4140 and B425 have been reported previously (4) and are included in the current analysis (Figure 2B). The heat resistance of spores was visualized by plotting the reference decimal reduction time of spores per strain at the reference temperature of 110°C ($D_{110°C}$-values) in relation to the presence or absence of the Tn1546 transposon (Table 3). The heat resistance of spores was plotted for nine strains of B. licheniformis (Figure 2A) and nine strains of B. amyloliquefaciens (Figure 2B).
Two strains of *B. amyloliquefaciens*, namely B425 and DSM7, contained the Tn1546 transposon. These strains produced spores that required significantly longer heating times (p<0.001) at 110°C, i.e. approximately 15 times, to achieve one decimal reduction, than spores of the other seven strains without this transposon. To illustrate the difference in heat resistance of spores: heating at 110°C for 5 minutes results in 0.6 log reduction for strains without the Tn1546 transposon, whereas the group with Tn1546 shows more than 10.5 log reduction. Strains *B. licheniformis* B4092 and B4094 contained the Tn1546 transposon, and the spores of these strains all required...
High-level heat resistance of spores of *B. amyloliquefaciens* and *B. licheniformis*

longer heating times (p<0.001) (2.5 times) to reach a decimal reduction than the spores of the six *B. licheniformis* strains that did not possess the Tn1546 transposon. Heating at 110°C for 5 minutes results in a calculated 3.4 log reduction for the strains carrying the Tn1546 transposon, whereas the other group without the Tn1546 transposon would show 8.5 log reduction.

The number of spoVA<sup>2mob</sup> operons in *B. subtilis* was found to correlate with the level of heat resistance of spores; strains carrying three copies produced spores with the highest level of heat resistance (3). *B. licheniformis* strains B4090, B4092 and B4094 contained a single copy of the Tn1546 transposon with a single spoVA<sup>2mob</sup> operon. The heat resistances of spores of *B. licheniformis* with or without this operon were significantly different, but relatively modest. For *B. amyloliquefaciens*, spores of strains B425 and DSM7 showed comparable high-levels of heat resistance, which were significantly higher than those of the spores of other *B. amyloliquefaciens* strains. Strain DSM7 contains three Tn1546 transposable elements, and it is likely that strain B425 also contains multiple copies, however this remains to be established.

![Figure 2](image.png)

**Figure 2.** Spore heat inactivation kinetics at 110°C of A) Nine strains of *B. licheniformis* and B) Nine strains of *B. amyloliquefaciens*. The closed circles and squares indicate the absence of a Tn1546 transposon in the strains of *B. licheniformis* and *B. amyloliquefaciens*, respectively. The open circles and squares indicate the presence of at least one Tn1546 transposon in the strains of *B. licheniformis* and *B. amyloliquefaciens*, respectively.

*The spoVA<sup>2mob</sup> operon is responsible for increased heat resistance of spores*

The introduction of the spoVA<sup>2mob</sup> operon originating from *B. subtilis* strain B4067 in laboratory strain *B. subtilis* 168, has been shown to result in high-level heat resistance of spores (3). To establish whether the spoVA<sup>2mob</sup> operons present in the Tn1546 transposons of *B. licheniformis* B4090 and *B. amyloliquefaciens* DSM7 have a functional role, these were introduced into *B. subtilis* 168. The *B. subtilis* 168 mutants carrying the spoVA<sup>2mob</sup> genes from *B. licheniformis* B4090 and *B. amyloliquefaciens* produced spores with significantly higher heat resistance than the parent strains: after heating at 100°C
for 60 minutes, the spores of \textit{B. subtilis} 168 \textit{amyE}::\textit{spoVA} \textit{2mob} (containing the \textit{spoVA} \textit{2mob} operon of \textit{B. licheniformis} B4090) showed survival of 4.0 log\textsubscript{10} unit (± 0.4), and \textit{B. subtilis} 168 \textit{amyE}::\textit{spoVA} \textit{2mob} (containing the \textit{spoVA} \textit{2mob} operon of \textit{B. amyloliquefaciens} DSM7) showed survival of 1.7 log\textsubscript{10} unit (± 0.5), whereas the spores of \textit{B. subtilis} were inactivated below the detection limit. The control strain \textit{B. subtilis} 168 \textit{amyE}::\textit{spoVA} \textit{2mob} (containing the \textit{spoVA} \textit{1} operon of \textit{B. subtilis} B4067) (3), showed survival of 2.8 log\textsubscript{10} unit (± 0.05).

The \textit{spoVA} \textit{2mob} operon differs from the \textit{spoVA} \textit{1} operon (designated \textit{spoVA} \textit{1}) that is encoded on the chromosome of \textit{B. subtilis} (43), \textit{B. amyloliquefaciens} and \textit{B. licheniformis}. The SpoVA proteins encoded in the \textit{spoVA} \textit{1} operon are required for uptake of DPA during the sporulation process and upon deletion or disruption of these genes in \textit{B. subtilis} 168, sporulation is not completed (43). In addition, the SpoVA proteins are involved in the release of Ca-DPA during the germination process (49, 50), with the SpoVAC protein functioning as a mechano sensitive channel during germination (48). The SpoVAD protein has a binding pocket whereby it can directly bind DPA (19). Both the \textit{spoVA} \textit{1} and the \textit{spoVA} \textit{2mob} operons contain genes encoding for SpoVAC, SpoVAD and SpoVAEb, while the other genes in the operons are different.
It is known that environmental conditions during sporulation, such as temperature, matrix and medium composition, can influence the heat resistance of spores of the \textit{B. subtilis} group (8, 25, 35). To allow for a direct comparison of the resistance properties of spores of the different strains, the sporulation conditions were kept constant in this study. Factors that are known to influence spore heat resistance include the composition of the sporulation medium, and it is known that the addition of salts (Ca\textsuperscript{2+}, Mn\textsuperscript{2+}, Mg\textsuperscript{2+} and K\textsuperscript{+}) results in higher resistances of spores to heat (8). Furthermore, the temperature during sporulation is an important factor that influences heat resistance of \textit{B. subtilis} spores (25). In line with these findings, the heat resistance of spores of a \textit{B. licheniformis} strain was higher upon sporulation at 45°C, with a modeled optimum at 49°C, than at lower temperatures such as 20°C (2). Overall, the environmental sporulation conditions, the presence or absence of genetic elements such as the \textit{spoVA\textsuperscript{2mob} operon}, and the storage conditions will ultimately determine the heat resistance properties of spores.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure3.png}
\caption{A) Overview of the \textit{spoVA\textsuperscript{2mob} operon}, as initially found in \textit{B. subtilis} strain B4146. The \textit{spoVA\textsuperscript{2mob} operon} has a predicted sigma G binding site upstream of the first gene. The operon consists of seven genes: a gene of unknown function with a predicted DUF1657 domain, a gene of unknown function with a predicted Yhcn/YlaJ domain, \textit{spoVA\textsubscript{C}}, \textit{spoVA\textsubscript{D}}, \textit{spoVA\textsubscript{Eb}}, a gene of unknown function with a predicted DUF1657 domain, and a gene of unknown function with a predicted DUF421 and a DUF1657 domain. B) Spore counts after heating at 80 °C for 10 minutes and at 100 °C for 60 minutes for strains of \textit{B. subtilis} 168, \textit{B. subtilis} 168 \textit{amyE::spoVA\textsuperscript{2mob}} (B4067, data from (3)), \textit{B. subtilis} 168 \textit{amyE::spoVA\textsuperscript{2mob}} (B4090), and \textit{B. subtilis} 168 \textit{amyE::spoVA\textsuperscript{2mob}} (DSM7). The downward arrow indicates that the spores were inactivated below detection limit.}
\end{figure}
Chapter 4

It is therefore conceivable that spores produced under laboratory conditions do not necessarily reach the heat resistance levels of spores that are present in food products (20, 47).

**Detailed analysis of the Tn1546 transposon**

The composition of the Tn1546 transposon in *B. licheniformis* strains B4090, B4092 and B4094 is shown in Figure 1A. In these strains, the Tn1546 transposon is highly similar to the one found in *B. subtilis* B4146 (Figure 1A). The transposons found in *B. subtilis* and *B. licheniformis* consist of genes that are required for transposition, and furthermore contain three operons and two single genes. The Tn1546 transposon found in the *B. amyloliquefaciens* strains DSM7 and B425 was smaller than the transposon found in *B. subtilis* and *B. licheniformis*. In both *B. amyloliquefaciens* strains, the first two operons were absent, possibly due to a site-specific recombination event, as a recombinase gene and a hypothetical gene were present at that genomic location.

The evolutionary relatedness of the different strains and species was visualized in a maximum likelihood core genome phylogenetic tree, based on concatenated protein sequences of conserved genes present in single copy in all genomes (Figure 1B). The species *B. amyloliquefaciens*, *B. licheniformis* and *B. subtilis* clustered in separate branches of the phylogenetic tree. For *B. amyloliquefaciens*, the strains with the Tn1546 transposon clustered together, whereas this was not the case for *B. licheniformis* strains carrying the Tn1546 transposon.

The genomic locations of the Tn1546 transposons were different for *B. subtilis*, *B. amyloliquefaciens* and *B. licheniformis*. In *B. subtilis*, the transposon was found at two genomic locations, namely in *yitF* and between *yxjA* and *yxjB* (3). In *B. amyloliquefaciens*, three Tn1546 transposons were found in strain DSM7 at three different genomic locations, namely between a gene encoding for a fructose-1,6-bisphosphatase and a hypothetical gene, between two hypothetical genes, and between a hypothetical gene and *rapK*. For *B. amyloliquefaciens* strain B425 it was not possible to determine the genomic locations and copy number of the Tn1546 transposon, since contig breaks were present on both sides of the Tn1546 transposon. In *B. licheniformis* strains B4090, B4092 and B4094, a single Tn1546 transposon was found integrated in a gene that encodes a D-alanyl-D-alanine carboxypeptidase. For *B. subtilis*, it has been shown that different insertion locations of the Tn1546 transposon led to high-level heat resistance of *B. subtilis* spores (3). It remains to be established whether the location of the insertion of the transposon in the genome of *B. amyloliquefaciens* and *B. licheniformis* plays a role in the level of heat resistance of spores.
Detailed analysis revealed that some genes in the Tn1546 transposon were mutated and present as pseudogenes in the transposon of some strains. The genes tnpA and tnpR in the Tn1546 transposon (which are required for active transposition) were intact and present in B. licheniformis strain B4090, and in B. amyloliquefaciens strains B425 and DSM7. This suggests that active transposition of the element may be possible for these strains, although active transposition of the Tn1546 transposon is believed to require a plasmid intermediate, as has been described in Enterococcus faecium (2). Interestingly, B. amyloliquefaciens DSM7, containing the intact tnpA and tnpR genes, contained three Tn1546 transposons. The encoded proteins required for transposition potentially allowed for internal transposition within the chromosome of strain DSM7. In B. subtilis strain B4146 and in B. licheniformis strains B4092 and B4094, the transposition genes are absent or not intact, suggesting that active transposition of the Tn1546 transposon is not likely to occur in these strains. This does not mean that the transposons cannot be transferred; transfer of genetic material including the Tn1546 transposon can be mediated by other transfer mechanisms, such as phage transduction, as described previously for B. subtilis (3), or via the uptake of external DNA via natural competence (16).

Conclusions
Variation in heat resistance of spores exists between strains of different spore forming species (4, 20, 29, 30). In this study, a genomic analysis revealed the presence of Tn1546 transposons in three strains of B. licheniformis and in two strains of B. amyloliquefaciens. The presence of this transposon, containing the spoVA2mob operon, correlated with high-level heat resistance of spores. A functional role of the spoVA2mob operon in increasing the heat resistance of spores was demonstrated by cloning these operons in B. subtilis 168, resulting in spores with high-level heat resistance. Clearly, mere identification of the species of spores in food products does not provide information on the heat resistance levels of these spores. The knowledge obtained in this study on the spoVA2mob operon can be used for specific detection of strains of the B. subtilis group that produce spores with high-level heat resistance. Multiple DNA based methods can be used for the detection of such genetic elements, such as whole genome sequencing and specific PCR detection, among others (1, 7). The data presented in this study can be used for improved control of bacterial spores in the food chain.

Acknowledgements
The authors have declared that no competing interests exist. The research was funded by TI Food and Nutrition, a public-private partnership on pre-competitive research in food and nutrition. The funding organization had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.
Chapter 4

Supplementary Dataset 1

Orthology matrix constructed using Ortho-MCL with the genomes of the four *B. amyloliquefaciens* strains, the nine *B. licheniformis* strains, *B. subtilis* B4146 as a strain that produces spores with high heat resistance and *B. subtilis* 168 as a reference strain that produces spores with normal heat resistance.

References

High-level heat resistance of spores of *B. amyloliquefaciens* and *B. licheniformis*


