High-Level Heat Resistance of Spores of *Bacillus amyloliquefaciens* and *Bacillus licheniformis* Results from the Presence of a *spoVA* Operon in a Tn1546 Transposon

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Bacterial endospore formers can produce spores that are resistant to 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, designated *spoVA*²mob, present on a Tn1546 transposon in *Bacillus subtilis*, leads to profoundly increased wet heat resistance of *B. subtilis* spores. Such Tn1546 transposon elements including the *spoVA*²mob operon were also found in several strains of *Bacillus amyloliquefaciens* and *Bacillus licheniformis*, and these strains were shown to produce spores with significantly higher resistances to wet heat than their counterparts lacking this transposon. In this study, the locations and compositions of Tn1546 transposons encompassing the *spoVA*²mob operon were also found in several strains of *Bacillus amyloliquefaciens* and *Bacillus licheniformis*, and these strains were shown to produce spores with significantly higher resistances to wet heat than their counterparts lacking this transposon. In this study, the locations and compositions of Tn1546 transposons encompassing the *spoVA*²mob operons in *B. amyloliquefaciens* and *B. licheniformis* were analyzed. Introduction of these *spoVA*²mob operons into *B. subtilis* 168 (producing spores that are not highly heat resistant) rendered mutant 168 strains that produced high-level heat resistant spores. Such Tn1546 transposon elements including the *spoVA*²mob operon were also found in several strains of *Bacillus amyloliquefaciens* and *Bacillus licheniformis*, and these strains were shown to produce spores with significantly higher resistances to wet heat than their counterparts lacking this transposon. In this study, the locations and compositions of Tn1546 transposons encompassing the *spoVA*²mob operons in *B. amyloliquefaciens* and *B. licheniformis* were analyzed. Introduction of these *spoVA*²mob operons into *B. subtilis* 168 (producing spores that are not highly heat resistant) rendered mutant 168 strains that produced high-level heat resistant spores, demonstrating that these elements in *B. amyloliquefaciens* and *B. licheniformis* are responsible for high level heat resistance of spores. Assessment of growth of the nine strains of each species between 5.2°C and 57.7°C showed some differences between strains, especially at lower temperatures, but all strains were able to grow at 57.7°C. Strains of *B. amyloliquefaciens* and *B. licheniformis* that contain the Tn1546 elements (and produce high-level heat resistant spores) grew at temperatures similar to those of their Tn1546-negative counterparts that produce low-level heat resistant spores. The findings presented in this study allow for detection of *B. amyloliquefaciens* and *B. licheniformis* strains that produce highly heat resistant spores in the food chain.

Keywords: spores, heat resistance, Tn1546 transposon, *spoVA* operon, genome analysis
INTRODUCTION

The ubiquitous presence of bacterial spore formers in nature can be largely attributed to their ability to produce endospores (spores) that can survive harsh environmental conditions (Nicholson et al., 2000; Setlow, 2006). Bacterial spores can enter the food chain from many different sources, for example via soil, dust, and biofilms (Heyndrickx, 2011). The intrinsic resistance properties of spores may 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 (Postollec et al., 2012). 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 (Scheldeman et al., 2006; Wells-Bennik et al., 2016).

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. atroheus, and B. sonorensis, which are phylogenetically close, yet distinguishable (Logan and Vos, 2009). These species can generally grow between temperatures of 30–50°C, with reported growth temperatures of B. licheniformis up to 58°C (Warth, 1978). 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 (te Giffel et al., 1996; Oomes et al., 2007; Lima et al., 2011; Lücker et al., 2013; Miller et al., 2015). These species are for instance well-known contaminants of raw materials used in bread making (Rosenkvist and Hansen, 1995; Sorokulova et al., 2003), and the spores can potentially even survive the bread baking process (Valerio et al., 2015). After spore survival, germination, and outgrowth, vegetative cells of B. amyloliquefaciens, B. subtilis or B. licheniformis can result in spoiled food products. B. subtilis, for instance has been reported to be present in cocoa (Lima et al., 2011) leading to spoiled chocolate drinks, B. licheniformis may be present in milk and milk powders, leading to spoilage of heat treated dairy products (Gopal et al., 2015), and B. amyloliquefaciens may spoil bread, resulting in ropy bread by degradation of starch and the formation of extracellular polysaccharides (Sorokulova et al., 2003; Valerio et al., 2012, 2015). Certain strains of B. licheniformis can produce a toxin, lichenysin A, that can cause foodborne illness (Salkinoja-Salonen et al., 1999; Nieminen et al., 2007; Logan, 2012). Lichenysin is a non-ribosomally synthesized lipo-peptide that is heat-stable (Konz et al., 1999). Due to the pathogenic potential of strains of B. licheniformis, it is critical to control these spores in the food chain (Madslen et al., 2013).

Notable differences have been observed with respect to the spore wet heat resistance properties of strains within the B. subtilis group (Kort et al., 2005; Oomes et al., 2007; Lima et al., 2011; Berendsen et al., 2015). Following a detailed analysis of the heat resistance of spores of 14 strains belonging to the B. subtilis group, strains could be divided in two groups based on spore heat resistance (Berendsen et al., 2015). For B. subtilis strains, it was recently demonstrated that spores with high-level heat resistance contain a Tn1546 transposon, encompassing a spoVA operon that is directly responsible for this phenotype (designated spoVA<sup>2mob</sup>, where mob indicates the presence on a mobile genetic element; Berendsen et al., 2016a). In addition, we observed high-level heat resistance of spores of B. licheniformis and B. amyloliquefaciens strains that carried the Tn1546 transposon; the spores of these strains showed heat resistance levels similar to as those of spores of B. subtilis strains with a Tn1546 transposon (Berendsen et al., 2015, 2016a).

In this study, we report the presence and composition of Tn1546 transposon homologs of B. subtilis which were found in strains of B. amyloliquefaciens and strains of B. licheniformis that produced highly heat resistant spores. This was performed by genome analysis or PCR detection. 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. In addition, the growth temperatures of all B. amyloliquefaciens and B. licheniformis strains (nine each) with or without the Tn1546 transposons were assessed.

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. This included nine strains of B. amyloliquefaciens isolated from natural and food environments, nine strains of B. licheniformis from food environments, and two strains of B. subtilis. The genome sequences were available for the two B. subtilis strains, all B. amyloliquefaciens strains, and four B. licheniformis strains (Berendsen et al., 2016a,b; Krawczyk et al., 2016). For B. amyloliquefaciens strains B425 and B4140, the heat inactivation kinetics of spores were described previously (Berendsen et al., 2015) and for all other B. amyloliquefaciens and B. licheniformis strains, the heat inactivation kinetics of spores were previously determined (Berendsen et al., 2016a). Strains of B. subtilis 168 and B. subtilis B4146 were included in the genome analysis as reference strains, and the heat resistances of the strains of these strains were previously analyzed (Berendsen et al., 2015).

Genome Analysis

Multiple sequence alignments were made for protein sequences of conserved genes that were present in single copy in all genomes using MUSCLE (Edgar, 2004). The core genome phylogenetic tree was constructed using PHYML (Guindon and Gascuel, 2003). To investigate the presence of the Tn1546 transposon and the encoded spoVA (designated spoVA<sup>2mob</sup>) operon that mediates high-level heat resistance, an orthology matrix was constructed using Ortho-MCL (Li et al., 2003) 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 low-level heat resistance (Supplementary Data Sheet 1). The genomic organization of
TABLE 1 | Strains used in this study.

| Strain no | Species          | Description                  | Tn1546 | Genome sequence References |
|-----------|------------------|------------------------------|--------|----------------------------|
| B425      | B. amyloliquefaciens | Isolated from sterilized milk | +      | LQYP00000000 Berendsen et al., 2015; Krawczyk et al., 2016 |
| B4140     | B. amyloliquefaciens | Isolated from pizza          | –      | LQYO00000000 Berendsen et al., 2015; Krawczyk et al., 2016 |
| 10A5      | B. amyloliquefaciens | Known as 10A5; NRRL B-14383, isolated from soil | –(PCR) No | Priest et al., 1988 |
| FZB42     | B. amyloliquefaciens | Known as 10A6; FZB42, isolated from plant soil | –      | NC_009725 Chen et al., 2007 |
| 10A18     | B. amyloliquefaciens | Known as 10A18; CL8004       | –(PCR) No | Zahler et al., 1987 |
| DSM7      | B. amyloliquefaciens | Known as DSM7, isolated from soil | +      | FNS97644 Ruckert et al., 2011 |
| DSM1060   | B. amyloliquefaciens | Known as DSM1060             | –(PCR) No | Priest et al., 1987 |
| 101       | B. amyloliquefaciens | Received as 101              | –(PCR) No | Berendsen et al., 2016a |
| SB42      | B. amyloliquefaciens | Received as SB42             | –(PCR) No | Berendsen et al., 2016a |
| B4089     | B. licheniformis   | Known as E5/T12, isolated from pea soup | –      | LKPM00000000 Oomes et al., 2007 |
| B4090     | B. licheniformis   | Known as T1, isolated from pea soup | +      | LQYL00000000 Oomes et al., 2007; Krawczyk et al., 2016 |
| B4091     | B. licheniformis   | Known as T29, isolated from mushroom soup | –      | LQYM00000000 Oomes et al., 2007; Krawczyk et al., 2016 |
| B4092     | B. licheniformis   | Isolated from buttermilk powder | +      | LQKY00000000 Krawczyk et al., 2016 |
| B4094     | B. licheniformis   | Isolated from camomile tea | +      | LKPN00000000 Berendsen et al., 2016a |
| B4121     | B. licheniformis   | Isolated from sateh pastry | –      | LKPC00000000 Berendsen et al., 2016a |
| B4123     | B. licheniformis   | Isolated from sateh pastry | –      | LKPP00000000 Berendsen et al., 2016a |
| B4124     | B. licheniformis   | Isolated from pancakes       | –      | LKPO00000000 Berendsen et al., 2016a |
| B4125     | B. licheniformis   | Isolated from pancakes       | –      | LPKO00000000 Berendsen et al., 2016a |
| B4062     | B. subtilis        | Type strain 168              | –      | NC_000964 Kunst et al., 1997 |
| B1416     | B. subtilis        | Isolated from curry sauce    | +      | NZ_JXR01000000 Berendsen et al., 2015, 2016a,b This study |
| 168-spoVA2mob (B4090) | B. subtilis | 168 amyE::spoVA2mob (B4090) specR | – | – | This study |
| 168-spoVA2mob(DSM7) | B. subtilis | 168 amyE::spoVA2mob (DSM7) specR | – | – | This study |

the Tn1546 transposon was visualized using the Artemis comparison tool (ACT), and using microbial genomic context viewer (MGcV) (Carver et al., 2005, 2012; Overmars et al., 2013). For the identified Tn1546 transposons, operon predictions were performed using FGENESB (http://www.softberry.com). Additionally, manual sequence comparisons and searches for pseudogenes were performed for all genes in the transposon.

Generic PCR primers were used, as described previously, for the detection of the Tn1546 encoded genes tnpA, spoVAC2mob and cls in the five strains of B. amyloliquefaciens for which no genome sequence information was available (i.e., strains 10A5, FZB42, 10A18, 101 and SB42; Berendsen et al., 2016a).

### Comparison of Heat Inactivation Kinetics of Spores

For all strains the heat resistances of their spores (produced at 37°C) was previously determined using capillary tubes, at three different temperatures, using at least five time points, as described in Berendsen et al. (2015) and Berendsen et al. (2016a). For comparison of strain specific heat resistance of spores, the z-values (i.e., the increase in temperature required to achieve an additional log unit reduction) were calculated as described previously (van Asselt and Zwietering, 2006). Furthermore, the D-values (i.e., the time needed at that temperature to decrease the spore count 10-fold) reported previously, were used to calculate reference D-values (Dref) at the reference temperature of 110°C with the corresponding 95% prediction intervals, as presented in Supplementary Table 1 (van Asselt and Zwietering, 2006).

### Cloning of the spoVA2mob Operon

The spoVA2mob operon, including the predicted promotor region as present in strain B. licheniformis B4090 and B. amyloliquefaciens DSM7 was cloned into plasmid pDG1730, using a procedure as previously described (Berendsen et al., 2016a). The obtained constructs were transformed to B. subtilis 168 and integrated in the amyE locus as described in Berendsen et al. (2016a), to verify the role of this operon in increased heat resistance of spores. Spores were prepared for strains 168 amyE::spoVA2mob (DSM7), 168 amyE::spoVA2mob (B4090) and 168 as described above, and the heat resistance of spores of these strains was assayed by heating at 100°C for 1 h, followed by plating, incubation and enumeration of survivors.
Determination of the Growth Temperature

All strains were cultured from the −80°C glycerol stocks in brain heart infusion broth (BHI, Oxoid). For assessing growth at different temperatures, 24-well plates were filled with 1.5 ml of BHI agar and surface inoculated with 20 µl of turbid individual overnight cultures (>10^7 cfu ml⁻¹, i.e., > 2 × 10^5 cells on the surface of the agar in each well). Growth or no growth on the agar surface was assessed on day 2, 6, 13, 23, and 30 at the following temperatures: 5.2, 11.5, 14.5, 30.5, 36.3, 45.9, 55.1, and 57.7°C. Growth was scored visually, based on the formation of colonies or a lawn on the agar surface in the wells. The day on which growth was first observed was noted. Two biological replicates were performed, and for each biological replicate, two technical replicates were performed.

RESULTS

Genome Mining for the Tn1546 Transposon

For B. subtilis strains, it has been demonstrated that the presence of a Tn1546 transposon is responsible for high-level heat resistance of the spores (Berendsen et al., 2016a). 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) using primers for these three target genes showed positive results for *B. amyloliquefaciens* strains B425 and DSM7 but did not reveal the transposon in the other five strains. 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 (Supplementary Table 1). The heat resistance of spores was displayed as a reference decimal reduction time of spores per strain at the reference temperature of 110°C (D110°C-c-values) in relation to the presence or absence of the Tn1546 transposon (Supplementary Table 1).

Two strains of *B. amyloliquefaciens*, namely B425 and DSM7, contained the Tn1546 transposon. These strains produced spores that required significantly longer heating times (unpaired *t*-test *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. Strains *B. licheniformis* B4090, B4092, and B4094 contained the Tn1546 transposon, and the spores of these strains all required longer heating times (unpaired *t*-test *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.

### The spoVA<sup>2mob</sup> Operon Is Responsible for Increased Heat Resistance of Spores

We previously showed that the introduction of the *spoVA<sup>2mob</sup>* operon originating from *B. subtilis* strain B4067 into laboratory strain *B. subtilis* 168 resulted in the formation of high-level heat resistant spores by this strain (Berendsen et al., 2016a). 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 operons were also introduced individually into *B. subtilis* 168. The *B. subtilis* 168 mutants carrying the *spoVA<sup>2mob</sup>* genes of *B. licheniformis* B4090 and the *spoVA<sup>2mob</sup>* genes of *B. amyloliquefaciens* produced spores with significantly higher heat resistances than the parent strains after heating at 100°C for 60 min (Figure 2). The spores of *B. subtilis* 168 were inactivated to a level below the detection limit, i.e., more than 8 log units reduction, indicative of a low level of heat resistance of spores. The spores of *B. subtilis* 168 *amyE::spoVA<sup>2mob</sup>* (containing the *spoVA<sup>2mob</sup>* operon of *B. licheniformis* B4090) and spores of *B. subtilis* 168 *amyE::spoVA<sup>2mob</sup>* (containing the *spoVA<sup>2mob</sup>* operon of *B. amyloliquefaciens* DSM7) showed survival of 4.0 log<sub>10</sub> unit (±0.4), and 1.7 log<sub>10</sub> unit (±0.5), respectively, indicating a high level of heat resistance of spores. The control strain *B. subtilis* 168 *amyE::spoVA<sup>2mob</sup>* producing high level heat resistant spores (containing the *spoVA<sup>2mob</sup>* operon of *B. subtilis* B4067) (Berendsen et al., 2016a), showed survival of 2.8 log<sub>10</sub> unit (±0.05).

### 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 inserted in *yitF* and between *yxjA* and *yxjB* (Berendsen et al., 2016a). 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 location(s) 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.

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. amyloliquefaciens* strains B425 and DSM7 and in *B. licheniformis* strain B4090.

### Determination of Vegetative Growth at Different Temperatures

None of the 18 *B. amyloliquefaciens* and *B. licheniformis* strains were able to grow at 5.2°C within 30 days. At 11.5°C, six
out of nine *B. amyloliquefaciens* strain and five out of nine *B. licheniformis* strains showed growth. At 14.5°C, all but one strain showed growth. All strains grew at the highest temperature tested (57.7°C), and at all other temperatures, growth of strains was observed except for strain 101 at 55.1°C. In the case of the nine *B. amyloliquefaciens* and nine *B. licheniformis* strains, high level heat resistance of spores due to the presence of the Tn1546 transposon was not correlated with the ability to grow at different temperatures (Table 2).

**DISCUSSION**

*B. licheniformis* strains B4090, B4092, and B4094 contained a single copy of the Tn1546 transposon with a single *spoVA*^mob^ 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. The number of *spoVA*^mob^ operons have previously been found to correlate with the level of heat resistance of spores in *B. subtilis*; strains carrying three copies produced spores with the highest level of heat resistance (Berendsen et al., 2016a). For *B. subtilis*, it has been shown that the Tn1546 transposon was found at different locations in the genome, all leading to high-level heat resistance of spores (Berendsen et al., 2016a). 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.

The presence of intact *tnpA* and *tnpR* genes in *B. amyloliquefaciens* DSM7 and B425, and *B. licheniformis* B4090 suggests that active transposition of the Tn1546 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* (Arthur et al., 1993). 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 (Berendsen et al., 2016a), or via the uptake of external DNA via natural competence (Kovacs et al., 2009).

The spoVA<sub>2mob</sub> operons present on the Tn1546 transposons differ from the spoVA operons (designated spoVA<sup>1</sup>) that are encoded on the chromosomes of B. subtilis (Tovar-Rojo et al., 2002), B. amyloliquefaciens and B. licheniformis. The SpoVA proteins encoded in the spoVA<sup>1</sup> operon are required for uptake of DPA during the sporulation process, and sporulation cannot be completed upon deletion or disruption of these genes in B. subtilis 168 (Tovar-Rojo et al., 2002). In addition, the SpoVA proteins are involved in the release of Ca-DPA during the germination process (Vepachedu and Setlow, 2004, 2007), with the SpoVAC protein functioning as a mechano sensitive channel during germination (Velasquez et al., 2014). The SpoVAD protein has a binding pocket, whereby it can directly bind DPA (Li et al., 2012). Both the spoVA<sup>1</sup> and the spoVA<sub>2mob</sub> operons contain genes encoding SpoVAC, SpoVAD and SpoVAEb, while the other genes in the operons are different. Presumably the presence of the spoVA<sub>2mob</sub> encoded proteins results in the increased uptake of DPA into the spore core, as was previously found for B. subtilis (Berendsen et al., 2016a).

It is known that environmental conditions during sporulation, such as temperature, matrix and medium composition, can influence the heat resistance of spores of the B. subtilis group (Cazemier et al., 2001; Melly et al., 2002; Rose et al., 2007). To allow for a direct comparison of the resistance properties of spores of the different strains, the applied sporulation conditions of all B. licheniformis and B. amyloliquefaciens strains were the same (Berendsen et al., 2015, 2016a). 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<sup>2+</sup>, Mn<sup>2+</sup>, Mg<sup>2+</sup> and K<sup>+</sup>) results in higher resistances of spores to heat (Cazemier et al., 2001). Furthermore, the temperature during sporulation can influence the heat resistance of B. subtilis spores (Melly et al., 2002). In line with these findings, the heat resistances of spores of a B. licheniformis strain have been reported to be higher upon sporulation at 45°C, with a modeled optimum at 49°C, than at lower temperatures such as 20°C (Baril et al., 2012). Overall, the environmental conditions during sporulation, the presence or absence of genetic elements such as the spoVA<sub>2mob</sub> operon, and the storage conditions of spores will ultimately determine the heat resistance properties of spores. It is therefore conceivable that spores produced under laboratory conditions do not necessarily reach the same levels of heat resistance as spores found in foods (van Zuijlen et al., 2010; Lima et al., 2011).

It is generally assumed for bacterial spore formers that higher optimal growth temperatures of vegetative cells correlates positively with spore heat resistance (Nicholson et al., 2000). In this study, some differences were seen between strains with respect to their abilities to grow at different temperatures, but no consistent pattern was seen for strains that contain the Tn1546 element and produce high level heat resistant spores versus the ones that do not harbor the element and produce low-level heat resistant spores. All strains were able to grow at 57.7°C. All genes present on the Tn1546 transposon are under the control of sporulation-specific sigma factors K or G (Berendsen et al., 2016a) and are thus only expressed during late stages of

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**TABLE 2 | Determination of ability to grow at different temperatures for nine strains of B. amyloliquefaciens and nine strains of B. licheniformis.**

| Species         | Strain  | Tn1546 transposon | Growth observed at temperature<sup>a</sup> |
|-----------------|---------|-------------------|------------------------------------------|
|                 |         |                   | 5.2°C | 11.5°C   | 14.5°C | 30.5°C | 36.3°C | 45.9°C | 55.1°C | 57.7°C |
| *B. amyloliquefaciens* | 10A5    | No                | –     | + + +    | + + +   | + + +   | + + +   | + + +   | + + +   | + + +   |
|                 | F2B42   | No                | –     | – – –    | – – –   | – – –   | – – –   | – – –   | – – –   | – – –   |
|                 | 10A18   | No                | –     | + + +    | + + +   | + + +   | + + +   | + + +   | + + +   | + + +   |
|                 | 101     | No                | –     | + + +    | + + +   | + + +   | + + +   | + + +   | + + +   | + + +   |
|                 | SB42    | No                | –     | + + +    | + + +   | + + +   | + + +   | + + +   | + + +   | + + +   |
|                 | B4140   | No                | –     | + + +    | + + +   | + + +   | + + +   | + + +   | + + +   | + + +   |
|                 | DSM1060 | No                | –     | – ++ +   | ++ + +  | ++ + +  | ++ + +  | ++ + +  | ++ + +  | ++ + +  |
|                 | DSM7    | Yes               | –     | – ++ +   | ++ + +  | ++ + +  | ++ + +  | ++ + +  | ++ + +  | ++ + +  |
|                 | B425    | Yes               | –     | + + +    | + + +   | + + +   | + + +   | + + +   | + + +   | + + +   |
| *B. licheniformis* | B4089   | No                | –     | – – –    | + + +   | + + +   | + + +   | + + +   | + + +   | + + +   |
|                 | B4091   | No                | –     | – – –    | + + +   | + + +   | + + +   | + + +   | + + +   | + + +   |
|                 | B4121   | No                | –     | – – –    | + + +   | + + +   | + + +   | + + +   | + + +   | + + +   |
|                 | B4123   | No                | –     | + + +    | + + +   | + + +   | + + +   | + + +   | + + +   | + + +   |
|                 | B4124   | No                | –     | + + +    | + + +   | + + +   | + + +   | + + +   | + + +   | + + +   |
|                 | B4125   | No                | –     | + + +    | + + +   | + + +   | + + +   | + + +   | + + +   | + + +   |
|                 | B4090   | Yes               | –     | + + +    | + + +   | + + +   | + + +   | + + +   | + + +   | + + +   |
|                 | B4092   | Yes               | –     | + + +    | + + +   | + + +   | + + +   | + + +   | + + +   | + + +   |
|                 | B4094   | Yes               | –     | + + +    | + + +   | + + +   | + + +   | + + +   | + + +   | + + +   |

<sup>a</sup>Growth in: 2 days (+ + +), 6 days (+ +), 13 days (+), 23 days (+), and no growth within 30 days (−).
sporulation. It is therefore in line with expectations that the genes on the Tn1546 transposon, which determine spore heat resistance, do not influence the ability of vegetative cells to grow at high temperatures as these genes are not expressed during vegetative growth.

CONCLUSIONS

Variation in heat resistance of spores exists between strains of different spore forming species but also within species (Oomes et al., 2007; Orsburn et al., 2008; Lima et al., 2011; Berendsen et al., 2015). In this study, a genomic analysis revealed the presence of Tn1546 transposons in two strains of B. amylobiiquefaciens and in three strains of B. licheniformis. The presence of this transposon, containing the spoVA2mob operon, correlated with high-level heat resistance of spores. Strains producing low or high level heat resistant spores showed similar temperature ranges for growth. 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 role of the spoVA2mob operon in spore heat resistance can be used for specific detection of strains of the B. subtilis group that produce high-level heat resistant spores. 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 (Caspers et al., 2011; Alkema et al., 2016). The ability to detect certain strains of B. subtilis group that have the ability to produce high level heat resistant spores can aid control of these sporeformers in the food chain.

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AUTHOR CONTRIBUTIONS

EB and RK collected the data. EB, RK, AD, and JB analyzed the data. EB, OK, and MW wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2016.01912/full#supplementary-material

Supplementary Datasheet 1 | Orthology matrix constructed using Ortho-MCL with the genomes of the four B. amylobiiquefaciens 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.

Supplementary Table 1 | Calculated reference D-value (Dref) at a reference temperature (Tref) of 110°C, and z-value, of spores of nine strains of B. amylobiiquefaciens and spores of nine strains of B. licheniformis (Berendsen et al., 2015, 2016a). In these studies, all strains were sporulated on the same media using the same incubation, namely using Nutrient Agar (Difco, 23g/L) plates supplemented with CaCl2 (1 mM), KCl (13 mM), MgSO4 (1 mM) and MnSO4 (0.13 mM), with a pH of 7.0, and incubation for 7 days at 37°C to allow for sporulation (Berendsen et al., 2015). Subsequently spores were harvested from the plates and washed three times with sterile water (5000 g, 10 min, 4°C) (Berendsen et al., 2015).
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