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ORIGINAL ARTICLE

A mobile genetic element profoundly increases heat resistance of bacterial spores

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Bacterial endospores are among the most resilient forms of life on earth and are intrinsically resistant to extreme environments and antimicrobial treatments. Their resilience is explained by unique cellular structures formed by a complex developmental process often initiated in response to nutrient deprivation. Although the macromolecular structures of spores from different bacterial species are similar, their resistance to environmental insults differs widely. It is not known which of the factors attributed to spore resistance confer very high-level heat resistance. Here, we provide conclusive evidence that in *Bacillus subtilis,* this is due to the presence of a mobile genetic element (Tn*1546*-like) carrying five predicted operons, one of which contains genes that encode homologs of SpoVAC, SpoVAD and SpoVAEb and four other genes encoding proteins with unknown functions. This operon, named *spoVA*^{2mob}, confers high-level heat resistance to spores. Deletion of *spoVA*^{2mob} in a *B. subtilis* strain carrying Tn*1546* renders heat-sensitive spores while transfer of *spoVA*^{2mob} into *B. subtilis* 168 yields highly heat-resistant spores. On the basis of the genetic conservation of different *spoVA* operons among spore-forming species of *Bacillaceae*, we propose an evolutionary scenario for the emergence of extremely heat-resistant spores in *B. subtilis*, *B. licheniformis* and *B. amyloliquefaciens*. This discovery opens up avenues for improved detection and control of spore-forming bacteria able to produce highly heat-resistant spores.
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Introduction

The bacterial endospore is one of the most resistant life forms on earth with astounding longevity that may exceed thousands of years (Cano and Borucki, 1995; Vreeland *et al.,* 2000). Endospores can survive not only exposure to extremes of temperature but also other stresses such as desiccation, radiation and disinfectants (Setlow, 2006). Spore-forming species belonging to the Firmicutes have broad biotechnological applications in fermentation processes, gut health promotion (probiotics), crop protection and increasing crop yields, as carriers for vaccine antigens, and in the production of a range of useful chemicals, enzymes and fuels (Duc *et al.,* 2003; Cutting, 2011). However, some of the species are pathogenic and their spores have a pivotal role in the spread of infection (for example, *Bacillus anthracis*, *Clostridium difficile*, *Clostridium perfringens*, *Clostridium tetani*, *Clostridium botulinum*; Ivanova *et al.,* 2003; Read *et al.,* 2003; Rupnik *et al.,* 2009; Peck *et al.,* 2011). Pathogenic sporeformers are estimated to cause over one million cases of foodborne illness in the USA alone (for example, due to *C. perfringens* and *B. cereus*; Scallan *et al.,* 2011). Furthermore, several non-pathogenic bacterial sporeformers are a cause of major financial losses to the food industry through the presence of heat-resistant spores that survive processes such as pasteurization or even sterilization, leading to reduced shelf life, food spoilage and subsequent food waste (Scheldeman *et al.,* 2006; Scallan *et al.,* 2011; Wells-Bennik *et al.,* 2016).

The tremendous importance of spores has prompted considerable effort to understand the molecular mechanisms responsible for their resistance properties (Nicholson *et al.,* 2000; Errington, 2003; Gould, 2006; Sunde *et al.,* 2009). It is known that different features of the spore, including components in the core, cortex, coat and membranes, contribute to resistance properties (Setlow, 2006; Brul *et al.,* 2011). However, it remains unknown why spores of some strains are able to withstand extreme heat treatments, whereas others succumb quickly (Oomes *et al.,* 2007; Lima *et al.,* 2011; Berendsen *et al.,* 2015b).

In this study, we investigated determinants of high-level heat resistance of spores of *B. subtilis* strains that were isolated from diverse sources. Therefore, the genome sequences and the heat-resistance properties of spores of these strains were determined. A comparative genomics approach revealed the presence of

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a transposon only in strains producing high-level heat-resistant spores. The genes in the transposon were shown to contribute to high-level heat resistance of spores and their occurrence was assessed in other species belonging to the family of *Bacillaceae*.

Materials and methods

Strains, sporulation and establishing spore heat resistance

For 18 strains of *B. subtilis*, 9 strains of *B. amyloliquefaciens* and 9 strains of *B. licheniformis*, the heat resistance of spores was characterized as described previously (Berendsen *et al.,* 2015b; Supplementary Table 1). Detailed spore heat-inactivation kinetics were previously determined for 11 strains of *B. subtilis* and two strains of *B. amyloliquefaciens* (Berendsen *et al.,* 2015b). For the other strains, spores were prepared and detailed inactivation kinetics were determined. The heat resistance of spores was visualized by plotting the calculated decimal reduction time (*D*-value) at a given temperature (for example, 100 °C or 112.5 °C) for spores of different strains (Berendsen *et al.,* 2015b).

The genome sequences of 8 *B. subtilis* strains were publicly available, and the genomes of the other 10 *B. subtilis* strains were sequenced (Supplementary Table 2; Berendsen *et al*., 2016). In addition, the genomes of two strains of *B. amyloliquefaciens* and all nine strains of *B. licheniformis* were sequenced (Supplementary Table 2).

Phenotype-genotype matching and genome analysis

To compare the genome content of members of the *Bacillaceae* family, orthology matrices were constructed on the basis of the predicted protein content of the strains using Ortho-MCL (Li *et al.,* 2003). Three different orthology matrices were constructed, namely, one for the 18 strains of *B. subtilis* (Supplementary Dataset 1), another for the strains of *B. licheniformis,* the *B. amyloliquefaciens* strains with available genome sequences, *B. subtilis* strain 168 and strain B4146 (Supplementary Dataset 2), and a third one for 103 spore-forming members of the *Bacillaceae* (Supplementary Dataset 3; Li *et al.,* 2003). The latter orthology matrix contained 35 strains belonging to the *B. subtilis* group, 33 strains of *B. cereus,* 5 strains of *Anoxybacillus flavithermus,* 23 strains of *Geobacillus* spp., 2 strains *of Caldibacillus debilis*, 1 strain of *B. sporothermodurans* and 4 strains of *B. thermoamylovorans* (Supplementary Table 2).

Phenotype-genotype matching was performed for the 18 *B. subtilis* strains using Phenolink (Bayjanov *et al.,* 2012), with low- or high-level heat resistance of spores as the phenotypic input, and the orthology matrix (Supplementary Dataset 1) as genotypic input. The genomic locations of target genes were visualized using Artemis (Carver *et al.,* 2012) and Artemis Comparison Tool (Carver *et al.,* 2005). For *B. subtilis* strains, detailed gene and operon predictions within the Tn*1546* transposon were made using FGENESB (www.softberry.com) and predictions were manually inspected. Specific insertion locations of the transposon and the number of transposon elements present per strain was verified by PCR using primers as listed in Supplementary Table 3.

A maximum likelihood core genome phylogenetic tree was constructed on the basis of the predicted protein sequences of all genes that are conserved in a single copy in all 103 *Bacillaceae* strains that were selected. Protein alignments were made using MUSCLE (Edgar, 2004) and the phylogenetic trees were constructed using PHYML (Guindon and Gascuel, 2003). The number and organization of *spoVA* genes in the genomes was verified using the orthology matrix of the 103 strains. To find potential functional equivalents, a Hidden Markov Model was constructed per orthologous group, that was used to search against all genomes (Johnson *et al.,* 2010). Protein sequences were extracted from the 103 genomes of spore-forming *Bacillaceae* for predicted SpoVAC and SpoVAD. Protein alignments and phylogenetic protein trees were prepared as described above, and manually inspected for evolutionary relatedness of the proteins. The operon structures were verified for all *spoVA* genes. In addition, it was determined whether the genomic location of the *spoVA* operon was on the chromosome or on a plasmid, and it was established whether the operon was part of transposable genetic elements.

Carry over of transposon

Natural transfer of the Tn*1546* transposon was achieved by generalized transduction from strain B4067, which produces high-level heat-resistant spores, to recipient strain 168-sp^R which produces low-level heat-resistant spores (Supplementary Figure 1). Details of this procedure are given below. Strain B4067 carries a prophage in the genome (locus tags B4067_4636 to B4067_4698, comprising 39 kb) that was induced with mitomycin C (1 μg ml^{-1}, Sigma, Zwijndrecht, The Netherlands) to produce phages as described previously (Moineau *et al.,* 1994). The phages were isolated for DNA sequencing as follows. Briefly, the lysed culture was centrifuged (10 min, 6000 *g*) and the supernatant was filter-sterilized using a filter with a pore size of 0.22 μm (Merck Millipore, Amsterdam, The Netherlands). The filtered supernatant was incubated with RNAse (10 μg ml^{-1}, Sigma) and DNAse (1 μg ml^{-1}, Sigma) at 37 °C for 1 h. Subsequently, NaCl (1 M) and polyethylene glycol (10%) were added to the phages, followed by incubation for 18 h at 4 °C. The phages were centrifuged (10 min, 6000 *g*) and re-suspended in phage buffer (100 mM NaCl, 5 mM $CaCl_2$, 1 mM $MgSO_4$, 0.01% gelatin, pH 7.5). DNA was isolated from the phages followed by DNA sequencing as described previously (Krawczyk *et al.,* 2015). The sequences of the DNA isolated from the phages and the coverage were visualized on the *B. subtilis* B4067 genome using

Artemis (Carver *et al.,* 2012) and found to contain the whole genome sequence of the strain.

Transfer of the Tn*1546* transposon element from strain B4067 to strain 168-sp^R was achieved as follows. Upon induction of the prophage in strain B4067 by adding mitomycin C (1 µg ml^{-1}), a phage lysate was obtained. This lysate was mixed with cells of recipient strain *B. subtilis* 168-sp^R and incubated for 1 h at 37 °C on a nitrocellulose filter (0.2 µm pore size, Nalgene, Rochester, NY, USA) that was placed on a Luria Broth plate (as described by Auchtung *et al.,* 2005). The recipient cells (with some assumed to have received the Tn*1546* transposon element) were transferred to sporulation plates and spores were prepared as described above. The resulting spores were subjected to a heat treatment of 100 °C for 60 min. This high heat treatment allowed for the survival of spores that were produced by cells of strain 168-sp^R that had incorporated a DNA element encompassing the Tn*1546* transposon in the genome, while spores of cells that received DNA elements unrelated to high-level spore heat resistance were fully inactivated and not recovered. In addition to selection on the basis of heat resistance of spores, resulting strains were also selected on the basis of antibiotic resistance (due to the *spec* marker, 100 µg ml^{-1}) and tryptophan deficiency. The donor strain B4067 could not grow in the presence of spectinomycin in the concentrations used. The presence of the Tn*1546* transposon in the resulting colonies of 168-sp^R was verified by PCR (primers are listed in Supplementary Table 3). One of the colonies containing the Tn*1546* transposon was selected (designated 168HR, NIZO culture collection strain B4417) and the genome sequence was determined as described previously (Krawczyk *et al.,* 2015).

Gene deletion and cloning

Specific deletion mutants (Supplementary Table 1) were constructed in strain 168HR using the *cre/lox* system, as previously described, with slight alterations (Lambert *et al.,* 2007; Yan *et al.,* 2008). The PCR fragment *lox66*-P32-*cat*-*lox71* cassette from pNZ5319 was fused by overhang PCR with the flanking regions of the genes to be deleted (primers are listed in Supplementary Table 3). The fused fragments were cloned into pNZ5319 using the *Swa*I/*Ecl*136II restriction sites (Lambert *et al.,* 2007). Following this strategy, the gene *yitF* was deleted from *B. subtilis* 168, and in strain 168HR, deletion of the entire Tn*1546* transposon and predicted operons and genes in Tn*1546* was achieved (Supplementary Table 1). Deletions of target genes or operons by replacement with the *lox66*-P32-*cat*-*lox71* cassette in mutants were verified by PCR. The *spoVA*^{2mob} operon from *B. subtilis* B4067 was cloned into pDG1730 (Guerout-Fleury *et al.,* 1996), for ectopic expression from the *amyE* locus in *B. subtilis* 168. The construct was integrated into the *amyE* locus of *B. subtilis* 168, yielding strain 168 *amyE*::*spoVA*^{2mob}. Heat resistance of spores for the constructed strains was assessed by exposure to 100 °C for 1 h as described above.

Spore characterization

The dipicolinic acid (DPA) contents of spores were determined for *B. subtilis* strain 168 and 168HR, as described previously (Kort *et al.,* 2005). For analysis of proteins of spores of strain 168HR, total protein was extracted by bead beating (4 rounds, 40 s, 5 m s^{-1}) of 0.5 ml of spore suspension (1×10^{10} colony forming units (CFU) ml^{-1}), followed by addition of 1 ml Urea (8 M) Tris (10 mM) at pH 8 and incubation at room temperature for 1 h. From the total protein extract, 10 µg was digested in-solution with trypsin upon reduction and alkylation. The resulting peptide fragments were purified and concentrated, and the peptide mixture was analyzed by nanoflow C18 reversed phase liquid chromatography (Bruker Daltonics, Breda, The Netherlands). For *B. subtilis* 168 and 168HR, the dimensions of the spore core and cortex were measured by imaging of cross sections of spores using transmission electron microscopy, as described previously (Lima, 2012). Measurement of the dimensions of sporoplast and core volume were performed using ImageJ (Schneider *et al.,* 2012). The spore dimensions of the cortex and core were determined for 308 individual spores for *B. subtilis* 168 and 254 individual spores for *B. subtilis* 168HR.

Results and discussion

To find the cause of extreme heat resistance in spores, the genomes of 18 strains of *B. subtilis* with either high or low spore heat-resistance properties were analyzed. Nine of these strains were isolated from diverse food products and produced spores that easily survived prolonged periods of boiling (10.5 h at 100 °C needed for one decimal reduction), while the other nine, including laboratory strain 168, produced spores that were much more readily heat-inactivated (only 2.9 min at 100 °C led to one decimal reduction) (Figure 1a). The analysis of the genomes of all 18 strains (Supplementary Table 4 and Dataset 1) revealed that only the highly heat-resistant strains contained a unique transposon Tn*1546*, related to the class II cointegrative Tn*3*-type transposon first described in *Enterococcus faecium* conferring antibiotic resistance (Arthur *et al.,* 1993), with integration in the genomic locus *yitF* (BSU10970) in all cases (Figure 1b). The backbone of the transposon contains the transposase *tnpA* (showing 93% similarity at the nucleotide level with *tnpA* in *E. faecium,* but fragmented in all *B. subtilis* strains), a resolvase *tnpR* (present in only two *B. subtilis* strains), two 38 bp imperfect inverted repeats at the ends of the transposon and a direct repeat of 5 bp at the site of integration. Although the Tn*1546* elements present in *B. subtilis* strains vary in length from 12 kb to 16 kb, they all

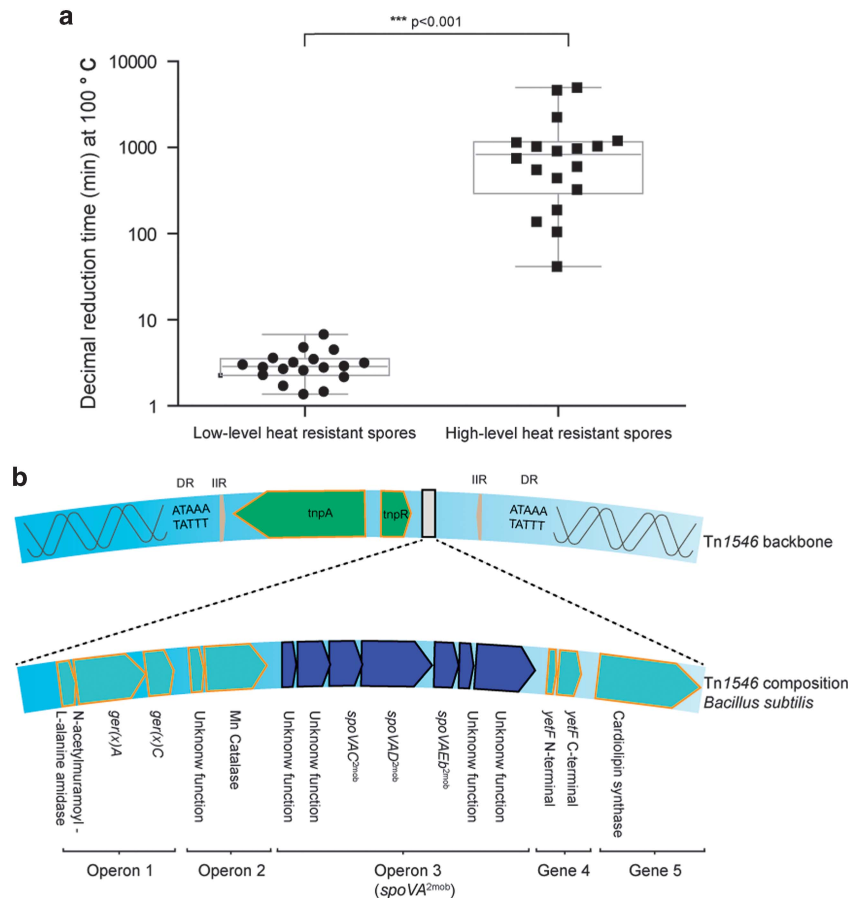

Figure 1 (**a**) Time needed to achieve one decimal reduction at 100 °C of spores of 18 strains of *B. subtilis* (assessed for two independent spore crops). On the basis of heat resistance of spores, strains belonged to one of two significantly different groups. One group contained nine strains with low-level heat-resistant spores (168, B4055, B4056, B4057, B4058, B4059, B4060, B4061, B4143), the other group contained nine strains with high-level heat-resistant spores (B4067, B4068, B4069, B4070, B4071. B4072, B4073, B4145, B4146). (**b**) Overview of the Tn*1546*-like transposon exclusively present in *B. subtilis* strains producing high-level heat-resistant spores.

contained the same five predicted transcriptional units (gene organization, putative functions and domains are shown in Figure 1b). As an example, the genes in the Tn*1546* element present in strain B4146 can be found with the locus tags B4146_1165 to B4146_1182. The Tn*1546* transposon found in *B. subtilis* includes the following genes: operon 1 encompasses a gene encoding a putative *N*-acetylmuramoyl-L-alanine amidase, *ger(x)A* and *ger(x)C*; operon 2 contains a gene with unknown function and a gene encoding a putative manganese catalase; operon 3 (designated *spoVA*^{2mob}) carries one gene of unknown function with a predicted DUF1657 domain, one gene of unknown function with a predicted YhcN/YlaJ domain, *spoVAC*^{2mob}, *spoVAD*^{2mob}, *spoVAEb*^{2mob}, one gene of unknown function with a predicted DUF1657 domain and one gene of unknown function with a predicted DUF421-domain and a DUF1657 domain; gene 4 encodes a YetF N-terminal part and a YetF C-terminal part; and lastly gene 5 encodes a putative cardiolipin synthetase. Each of the five predicted transcriptional units is preceded by a sporulation-specific binding site for sigma factor G (σ^G) or K (σ^K), which are known to target RNA polymerase to specific promotor sequences that drive gene expression during spore development. Operons 1 and 2 were predicted to be under control of σ^K, and *spoVA*^{2mob}, gene 4 and gene 5 were predicted to be under control of σ^G.

Genes encoded by the Tn*1546* transposon were directly responsible for high-level heat resistance of spores as evidenced by the introduction of Tn*1546* into the model laboratory strain *B. subtilis* 168. Active transposition of the Tn*1546*-like transposon was not possible as only remnants of the transposase gene (*tnpA*) were found in all nine strains that produce high-level heat-resistant spores, suggesting that the active transfer of Tn*1546*-like transposon is prone to evolutionary decay. Therefore, natural transfer of this element to *B. subtilis* 168 was achieved by generalized transduction, upon induction of a prophage (locus tags B4067_4636 to B4067_4698) in strain B4067 which produces spores with high-level heat resistance. A transductant was selected that produced spores with significantly higher heat resistance than spores of strain 168. Heat treatment of spores of this strain, designated 168HR, for 1 h at 100 °C resulted in less than 100-fold reduction in viable counts, while viable spores of strain 168 were reduced more than 10 billion-fold

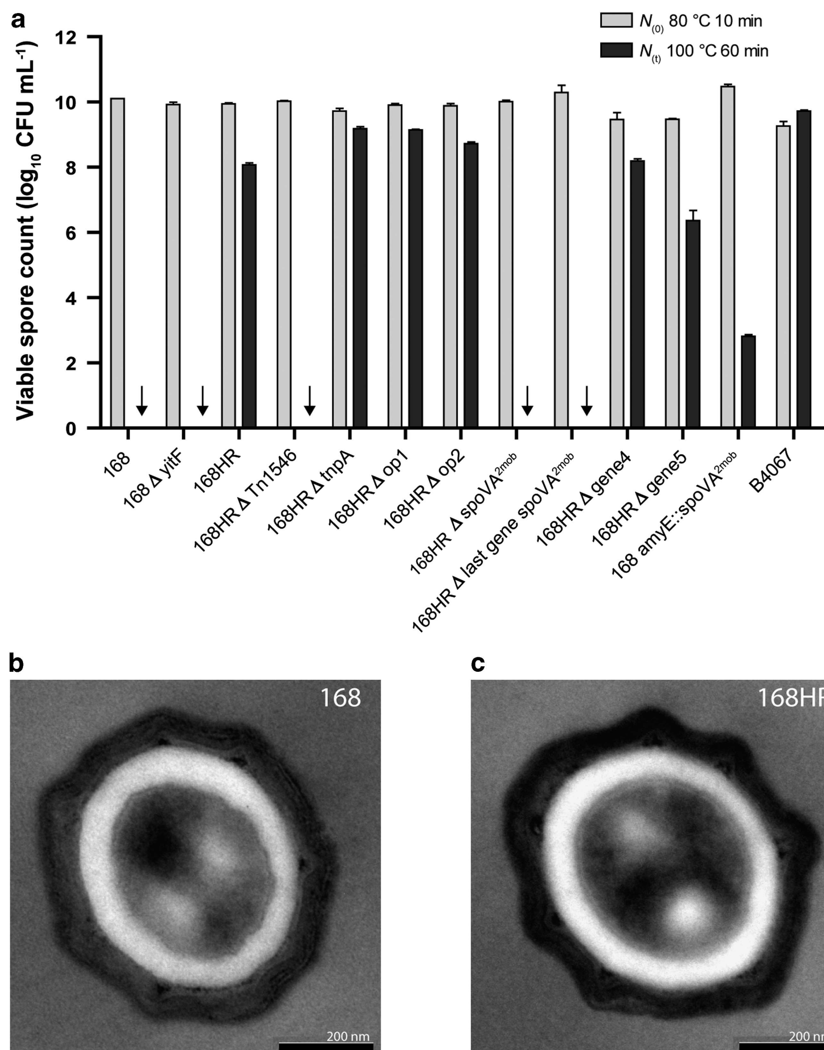

Figure 2 (**a**) Survival of spores of *B. subtilis* strains. The initial counts of spores were determined following heating for 10 min at 80 °C (gray bars). Survival of spores after 60 min at 100 °C is indicated by black bars. A downward arrow means that counts were below the detection limit, i.e., 1.7 log units. Heating was applied to spores of the following strains: 168, 168Δ*yitF*, 168HR (which is 168 including the Tn*1546* transposon encompassing five operons), 168HR without Tn*1546* (168HRΔ*Tn1546*), 168HR without *tnpA* (168HRΔ*tnpA*), 168HR without operon 1 (168HRΔop1), 168HR without operon 2 (168HRΔop2), 168HR without *spoVA*^{2mob} (168HRΔ*spoVA*^{2mob}), 168HR without gene 4 (168HR*Δgene 4*), 168HR without gene 5 (168HR*Δgene 5*), strain 168 *amyE::spoVA*^{2mob}, in which *spoVA*^{2mob} was inserted on the *amyE* locus, and strain B4067, a food isolate producing high-level heat-resistant spores. (**b** and **c**) Representative pictures of transmisison electron microscopy cross sections of spores of *B. subtilis* strain 168 and 168HR, respectively.

(Figure 2a). Sequencing of the genome of strain 168HR showed the presence of a 100 kb DNA fragment from B4067 that was recombined between *metC* and *yitA* with the Tn*1546*-like transposon inserted in *yitF* (Supplementary Figure 1). To exclude the potential effect of other mutations in the 100 kb region on the heat resistance of spores, the Tn*1546*-like transposon was deleted from the 168HR strain to verify its role in high-level heat resistance. Subsequent deletion of the Tn*1546* transposon from strain 168HR rendered spores that were much more sensitive to heat treatment than those of 168HR, and similar to those of strain 168 (Figure 2a). The appearance of spores of strains 168 and 168HR was very similar and their core/sporoplast ratios were not significantly different (Figures 2b and c); thus, genes on the Tn*1546* transposon do not seem to confer major structural changes. The core/sporoplast ratios were not significantly different for the analyzed spores of 168 and 168HR, with ratios of 0.52 ± 0.06 and 0.55 ± 0.07, respectively. Average dimensions of the spore core were $107\,453 \pm 24\,635$ nm^2 and $115\,363 \pm 26\,063$ nm^2, for 168 and 168HR, respectively. The average dimensions of the spore cortex were $97\,088 \pm 19\,098$ nm^2 and $94\,668 \pm 20\,591$ nm^2, for 168 and 168HR, respectively. Moreover, disruption of *yitF* due to Tn*1546* insertion does not have a role in increased heat resistance of spores, with spores of constructed strain 168Δ*yitF* showing similar heat resistance characteristics as spores of the parental strain 168 (Figure 2a).

The third operon on the Tn*1546* element, carrying genes that encode SpoVA homologs and four other genes (designated *spoVA*^{2mob}), was demonstrated

to confer high-level heat resistance of spores. After deletion of only the *spoVA*^{2mob} operon from strain 168HR (that is, 168HRΔ*spoVA*^{2mob}), the spores could be heat-inactivated under the same conditions as demonstrated for spores of strain 168 (Figure 2a). In addition, introduction of the *spoVA*^{2mob} operon into the *amyE* locus of strain 168 rendered a strain (168 *amyE::spoVA*^{2mob}) that produces spores with high-level heat resistance. Survival of these spores upon heating for 60 min at 100 °C was not quite as high as survival of spores of *B. subtilis* 168HR (containing the *spoVA*^{2mob} operon as part of the Tn*1546* transposon inserted in *yitF*), but significantly higher than survival of spores of *B. subtilis* 168; the latter were not recovered after this heat treatment, that is, showing more than 10 log units reduction (with a calculated reduction of 17.4 log units) (Figure 2a). The impact of the *spoVA*^{2mob} element on spore heat resistance is much greater than some of the previously reported factors influencing *Bacillus* spore heat resistance, such as altered temperature, pH, salts and matrix composition during sporulation, which may lead to up to 10-fold increases in the times required to inactivate spores (Cazemier *et al.,* 2001; Rose *et al.,* 2007; Baril *et al.,* 2012).

The other four transcriptional units present on the Tn*1546* element were not required for high-level heat resistance as the spores retained high-level heat resistance following deletion of each of these transcriptional units in strain 168HR (Figure 2a). Sporulation-specific expression of all transcriptional units in the Tn*1546* transposon except for the fourth transcriptional unit containing the fragmented gene *yetF,* was seen in strain 168HR and food isolate B4067 by RNA sequence analysis (data not shown). The encoded products may also determine spore properties other than heat resistance. Some of the encoded proteins were detected in extracts of spores of strain 168HR using mass spectrometry, revealing peptide fragments of the Mn catalase homolog and of proteins encoded by the first and the last gene on the *spoVA*^{2mob} operon (Supplementary Table 5).

Additional evidence for the crucial role of *spoVA*^{2mob} genes in high-level heat resistance of spores came from detailed genome analysis of the *B. subtilis* isolates from foods. The level of spore heat resistance was found to correlate with the number of *spoVA*^{2mob} operons present in the chromosome. Spores of nine strains (168, B4055, B4056, B4057, B4058, B4059, B4060, B4061, B4143) that lack the *spoVA*^{2mob} operon showed one decimal reduction in viable count after 0.2 min at 112.5 °C. Of the nine strains that produce high-level heat-resistant spores, one strain (B4146) carried one *spoVA*^{2mob} operon on a Tn*1546*-like transposon element inserted in *yitF,* and the average time to achieve one decimal reduction of its spores was 1.2 min at 112.5 °C. Strains B4067, B4068, B4069, B4070, B4071, B4072, B4073 and B4145 contained the same element inserted in *yitF* and a second *spoVA*^{2mob} operon on a Tn*1546*-like transposon between the two divergently transcribed genes *yxjA* (BSU39020) and *yxjB* (BSU39010) and their spores needed even longer average heating times of 8.8 min at 112.5 °C for the same inactivation. In addition to these two Tn*1546*-like transposons, three strains (B4067, B4070 and B4145) contained a third *spoVA*^{2mob} operon, which was flanked by genes of another mobile genetic element, but further genomic context could not be determined. Spores of these strains required as much as 25.6 min on average at 112.5 °C for one decimal reduction of viable counts (Figure 3b).

Despite extensive studies on many *spo* genes of *B. subtilis* 168, knowledge of the precise function of individual *spoVA*-encoded proteins is rather limited. The *spoVA* operon of *B. subtilis* 168 (for clarity reasons named *spoVA*^{1}) encompasses *spoVAA*, *spoVAB*, *spoVAC*, *spoVAD*, *spoVAEb*, *spoVAEa* and *spoVAF*, of which the first five genes are essential for completion of sporulation (Tovar-Rojo *et al.,* 2002). Only the functions of SpoVAC^{1} and SpoVAD^{1}, which are associated with the inner membrane of the spore, are known (Vepachedu and Setlow, 2005; Li *et al.,* 2012; Velasquez *et al.,* 2014). Structural analysis of SpoVAD^{1} revealed a binding pocket that is important for uptake of pyridine-2,6-dicarboxylic acid (known as DPA) during sporulation (Li *et al.,* 2012). SpoVAC^{1} was recently shown to function as a mechanosensitive channel during germination, with increased probability of opening at increased membrane tension (Velasquez *et al.,* 2014). The *spoVA*^{2mob} operon mediating high-level heat resistance carries *spoVAC*, *spoVAD* and *spoVAEb* (hereafter called *spoVAC*^{2mob}, *spoVAD*^{2mob} and *spoVAEb*^{2mob}) and four genes with unknown functions (shown in Figure 3a). The SpoVAC, SpoVAD and SpoVAEb proteins encoded in the *spoVA*^{1} and *spoVA*^{2mob} loci share 55%, 49% and 59% amino acid identity, respectively. Given the known roles of SpoVAC^{1} and SpoVAD^{1} in DPA uptake during sporulation, we hypothesize that proteins encoded by the *spoVA*^{2mob} operon have an important auxiliary role in this process, ultimately leading to higher heat resistance of spores. This was indeed the case: the introduction of the *spoVA*^{2mob} operon in strain 168 resulted in 50% higher DPA concentrations in *B. subtilis* spores. Spores of strains 168HR and 168 *amyE::spoVA*^{2mob} contain 63.1 ± 2.3 and 58.1 ± 0.1 µg DPA per mg dry weight, respectively, both significantly higher than the concentration in spores of strain 168 (40.1 ± 2.3 µg DPA per mg dry weight). Interestingly, high levels of DPA were previously reported in spores of a *B. subtilis* strain with high-level heat resistance (isolated from foods) (Kort *et al.,* 2005), and this phenomenon can now be linked to the presence of *spoVA*^{2mob} genes. At present, it has not been established which gene or which combinations of genes on the *spoVA*^{2mob} operon are essential and sufficient to convey high-level heat resistance of spores, but we did find that deletion of the last gene of unknown function fully

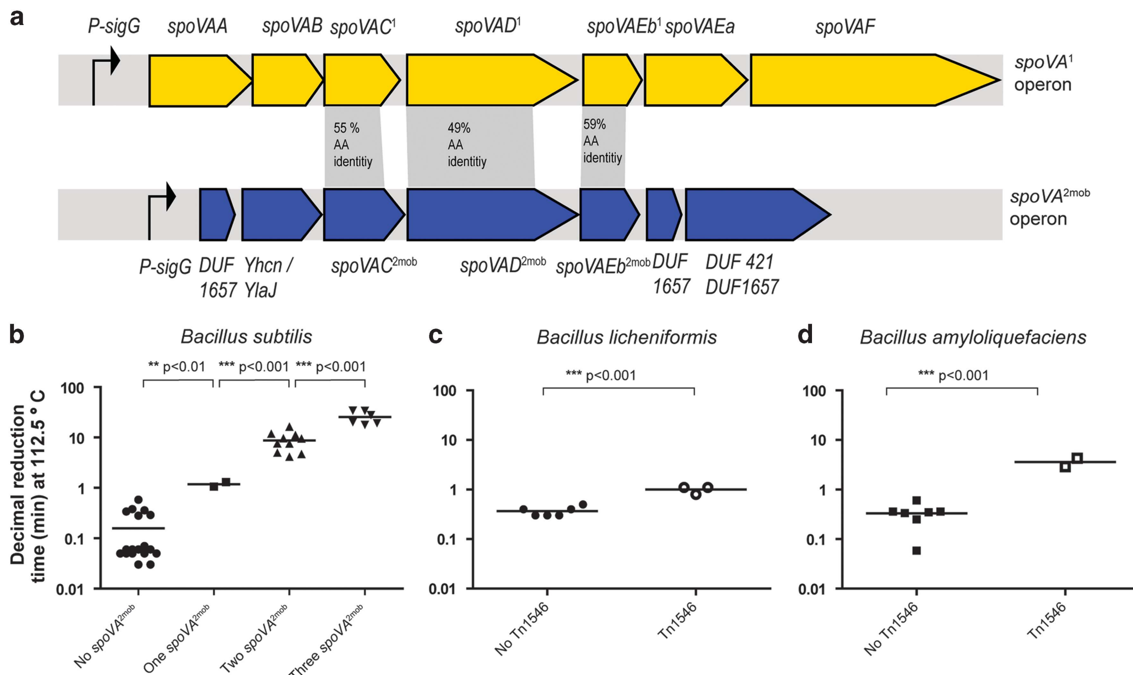

Figure 3 (**a**) Overview of the native *spoVA* operon (*spoVA*¹) in *B. subtilis* 168 and the *spoVA*2mob operon found in *B. subtilis* strains producing spores with high-level heat resistance. (**b**) The calculated time to achieve a decimal reduction at 112.5 °C for spores of strains of *B. subtilis* that possess zero, one, two or three *spoVA*2mob operons. (**c**) The calculated time to achieve a decimal reduction at 112.5 °C of spores of nine strains of *B. licheniformis.* Three strains possess one Tn*1546* transposon (including the *spoVA*2mob operon), and spores of these strains had significantly higher heat resistances than those of the six strains that did not contain this transposon. (**d**) The calculated time to achieve a decimal reduction at 112.5 °C of spores of nine strains of *B. amyloliquefaciens.* Two strains possess at least one Tn*1546* transposon (including the *spoVA*2mob operon), and produce spores with significantly higher heat resistances than the seven strains that did not carry the transposon.

abolished high-level heat resistance of spores (Figure 2a), indicating at least its essential role. The last gene of the *spoVA*2mob operon encodes a protein that is predicted to be membrane bound by three transmembrane segments, and contains a DUF421 domain and a DUF1657 domain. Homologs of this protein were neither found in *B. subtilis* 168 nor in other *Bacillus* spp. unless they carried *spoVA*² or *spoVA*2mob operons. Two other genes in the *spoVA*2mob operon encode proteins with DUF1657 domains, for which no homologs were found in *B. subtilis* 168 and other *Bacillus* spp. unless they contained the *spoVA*² or *spoVA*2mob operon. The DUF421 encoded in the last gene in the *spoVA*2mob operon was found in other predicted proteins in the absence of DUF1657; in *B. subtilis* 168, this domain was encoded by five different genes, namely, *yetF, yrbG, ykjA, ydfR* and *ydfS*, but their functions have not been established or predicted. It is not clear at this stage what roles the proteins containing these domains have in heat resistance of spores.

It is conceivable that the Tn*1546* transposon found in the *B. subtilis* group originates from *B. cereus* pXO1-like plasmids that can carry this transposon including the *spoVA*2mob operon (Figure 4; Rasko *et al.,* 2007), given similarities in gene presence and GC content (Supplementary Table 6) and because the Tn*3*-like transposon requires a plasmid intermediate for active transposition (Arthur *et al.,* 1993). It is not clear whether the presence of the *spoVA*2mob operon in *B. cereus* strains has an influence on the heat resistance of these spores. Limited sequence variation in key genes in the *spoVA*2mob operon found in the *B. subtilis* group strains suggests that genomic incorporation of the Tn*1546* transposon, including the *spoVA*2mob operon, involves a recent evolutionary event.

Genes encoding SpoVAC, SpoVAD and SpoVAEb are conserved among spore-forming *Bacillaceae* and *Clostridium* spp. (Galperin *et al.,* 2012), and were also present in the analyzed genomes of 103 *Bacillaceae* species (Figure 4). Three types of *spoVA* operons could be distinguished: *spoVA*¹, *spoVA*² and *spoVA*2mob (where mob indicates presence on a mobile genetic element). The division between *spoVA*¹ and *spoVA*² is based on the difference in operon structure (Figure 3a) and separate clustering of the SpoVAC and SpoVAD proteins in the evolutionary trees (Supplementary Figure 2).

Both the *spoVA*¹ and *spoVA*² operons are present in the spore-forming *Geobacillus* spp., *Anoxybacillus flavithermus*, and species of the *B. cereus* group *sensu strictu*, but not as parts of mobile genetic elements (Figure 4). Interestingly, all evaluated strains belonging to the *B. subtilis* group possess the *spoVA*¹ operon while lacking the *spoVA*² operon. However, some strains gained *spoVA*2mob on the Tn*1546* transposon (Figure 4). The determining role of the *spoVA*2mob element in high-level spore heat resistance was experimentally confirmed for strains of *B. licheniformis* and *B. amyloliquefaciens* (Figures 3c and d).

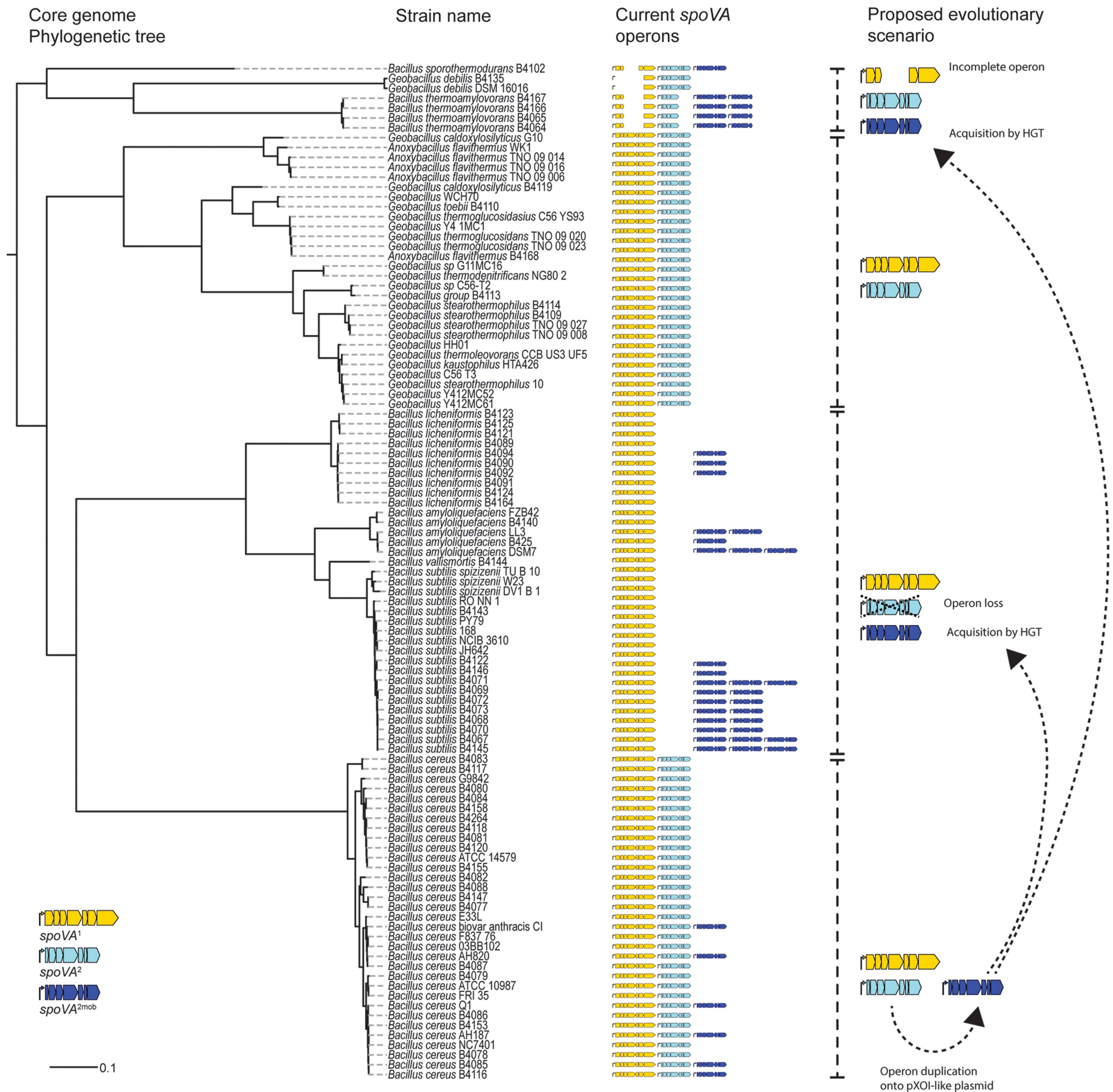

Figure 4 Maximum likelihood core genome phylogenetic tree of 103 spore-forming *Bacillaceae*, with indication of the number and type of *spoVA* operons present in the genomes, and proposed evolutionary scenarios. Three types of *spoVA* operons were identified in this analysis and are indicated in the tree. First, a *spoVA*^{1} operon, encompassing *spoVAA*, *spoVAB*, *spoVAC*^{1}, *spoVAD*^{1}, *spoVAEb*^{1}, *spoVAEa* and *spoVAF*. Second, a *spoVA*^{2} operon, encompassing a gene with a predicted DUF1657 domain, a gene with a YhcN/YljA domain, *spoVAC*^{2}, *spoVAD*^{2}, *spoVAEb*^{2}, a gene with a predicted DUF1657 domain and a gene with a predicted DUF 421 domain and DUF1657 domain. Third, a *spoVA*^{2mob} operon, which is a duplication of the *spoVA*^{2} operon, but present on a mobile genetic element, e.g., Tn*1546* in *B. subtilis* strains. The proposed evolutionary scenarios were based on protein trees of SpoVAC and SpoVAD and the genomic context of the *spoVA* operons. Strains of *B. cereus*, *Geobacillus* spp. and *A. flavithermus* all carry *spoVA*^{1} and *spoVA*^{2} operons. Six strains of *B. cereus* carry *spoVA*^{2mob} on a pXO1-like plasmid, as part of a Tn*1546* transposon. Members of the *B. subtilis* group (*B. subtilis*, *B. vallismortis*, *B. amyloliquefaciens*, *B. licheniformis*) lost the *spoVA*^{2} operon during evolution, but the *spoVA*^{2mob} operon re-entered in some strains as part of a Tn*1546* transposon. Similarly, *spoVA*^{2mob} entered strains of *B. thermoamylovorans* and *B. sporothermodurans*. Incomplete *spoVA*^{1} and *spoVA*^{2} operons were observed in strains of *B. thermoamylovorans*, *B. sporothermodurans* and *C. debilis*.

Interestingly, the genomes of species notorious for very high-level heat resistance of their spores, namely *B. thermoamylovorans*, *B. sporothermodurans* and *Caldibacillus debilis* (Scheldeman *et al.,* 2005, 2006; Berendsen *et al.,* 2015a), showed diverse compositions of their *spoVA* operons. The exact roles of the *spoVA*^{1}, *spoVA*^{2} and *spoVA*^{2mob} operons in determining spore properties in these species remains to be established.

Horizontal gene transfer has an important role in bacteria to acquire resistance against selective pressures (Ochman *et al.,* 2000). The transfer of the *spoVA*[2mob] operon to sporeformers occurs in the vegetative growth phase, subsequently leading to production of highly heat-resistant spores that can survive heat treatments routinely used in food processing. The acquisition of the *spoVA*[2mob] operon in food isolates may take place during growth in a food-processing environment, but it is also possible that such events occur during growth in other niches, such as soil or compost. The competitive advantage of acquisition of these genes may also be related to properties other than merely heat resistance of spores.

Conclusions

This study shows that horizontal gene transfer can profoundly affect heat resistance characteristics of spores. Our finding that the *spoVA*[2mob] operon on a Tn*1546*-like transposon has an important role in high-level heat resistance of *Bacillus* spores offers new opportunities for dealing with the problem of highly heat-resistant spores in food and health. Studying phenotypic properties of strains other than the well-studied laboratory strain in conjunction with analysis of their genomes proved to be a powerful approach to match phenotypes with underlying genetic traits.

Conflict of Interest

The authors declare no conflict of interest.

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