Role of DNA repair in *Bacillus subtilis* spore resistance to high energy and low energy electron beam treatments

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**ABSTRACT**

*Bacillus subtilis* spore inactivation mechanisms under low energy electron beam (LEEB) and high energy electron beam (HEEB) treatment were investigated using seven mutants lacking specific DNA repair mechanisms. The results showed that most of the DNA repair-deficient mutants, including ΔrecA, ΔKu ΔligD, Δexo Δnfo, ΔuvrAB and ΔsbcDC, had reduced resistances towards electron beam (EB) treatments at all investigated energy levels (80 keV, 200 keV and 10 MeV) compared to their wild type. This result suggested DNA damage was induced during EB treatments. The mutant lacking recA showed the lowest resistance, followed by the mutant lacking Ku and ligD. These findings indicated that recA, Ku and ligD and their associated DNA repair mechanisms, namely, homologous recombination and non-homologous end joining, play important roles in spore survival under EB treatment. Furthermore, exoA, nfo, uvrAB, splB, polY1 and polY2, which are involved in nucleotide damage repair/removal, showed different levels of effects on spore resistance under EB treatment. Finally, the results suggested that HEEB and LEEB inactivate *B. subtilis* spores through similar mechanisms. This research will provide a better understanding of how EB technologies inactivate *B. subtilis* spores and will contribute to the application of these technologies as a non-thermal, gentle spore control approach.

1. Introduction

1.1. Bacterial spore inactivation by electron beam technologies

Spore-forming bacteria of the genera *Bacillus* and *Clostridia* spp. are major sources of food spoilage and can cause food-borne diseases (Andersson et al., 1995; Mallozzi et al., 2010). They form resistant bacterial spores, which are the main targets of sterilization. Food industries often try to eliminate them with intensive thermal processing, which unavoidably leads to significant food quality losses. Due to consumer demand for high-quality and safe food products, gentle but efficient spore inactivation methods are needed (Zhang and Mathys, 2019). Electron beam (EB) technologies, including low energy EB (LEEB, ≤ 300 keV) and high energy EB (HEEB, > 300 keV), have been investigated as promising non-thermal microbial decontamination technologies for food and pharma applications (De Lara et al., 2002; Kikuchi et al., 2003; Pillai and Shayanfar, 2018; Sadat and Huber, 2002; Tallentire et al., 2010; Urgiles et al., 2007; Zhang et al., 2018; ISO/ASTM 51818, 2013). For example, EB technologies can decontaminate low moisture food products, e.g., spices, without the introduction of water or steam to the process. Research has shown that EB is more gentle and preserves the quality of food products better compared to other ionizing radiation technologies, e.g., gamma irradiation, and can effectively inactivate bacteria (De Lara et al., 2002; Fan et al., 2017; Fiester et al., 2012; Gryczka et al., 2018; Zhang et al., 2018). Regarding inactivation efficiency, LEEB can inactivate *Bacillus subtilis*, *Bacillus pumilus* and *Geobacillus stearothermophilus* spores with D-values ranging from 2.2 to 3.1 kGy (Zhang et al., 2018). For HEEB, the D-values at 10 MeV ranged from 1.5 to 3.8 kGy for *B. subtilis* and *Bacillus cereus* spores (De Lara et al., 2002).

EB technologies are particle-based ionizing radiation, while the other ionizing radiations, such as gamma and X-rays are photon-based. EB inactivates bacteria by generating electrons and the generated electrons ionize, causing damages to target molecules. Compared to
other ionizing irradiation technologies, e.g., gamma irradiation, EB technologies have many advantages. For example, EB technologies do not use radioactive material, they can have higher dose rates thus decreased treatment time to reach desired irradiation dose, and they can be switched off when not in use (Fan et al., 2017; Silindir and Ozer, 2009; Zhang et al., 2018). However, compared to other well-studied radiation technologies, only limited studies have investigated spore inactivation by EB technologies, and the inactivation mechanisms have not yet been fully elucidated (Fan et al., 2017; Fiester et al., 2012; Zhang et al., 2018).

It has been suggested that similar to other ionizing irradiation, EB technologies also inactivate bacteria and bacterial spores by causing DNA damage (Fiester et al., 2012; Hutchinson, 1985; Moeller et al., 2014; Urgiles et al., 2007; Zhang et al., 2018). DNA damage could occur during the ionizing radiation through direct and indirect effects. Direct effects are caused by energy transfer to target molecules, and indirect effects are induced by reactive oxygen species (ROS), reactive nitrogen species (RNS) and reactive hydrogen species (RHS) produced during the treatment (Friedberg et al., 2005; Goodhead, 1994; Hertwig et al., 2018; Lung et al., 2015; Tahergorabi et al., 2012). For example, radiolysis of water can produce hydroxyl radicals (HO·) and hydrogen peroxide, while superoxide ions (O2·-) will be formed in the presence of dissolved oxygen (Repine et al., 1981).

Ionizing radiation causes different kinds of DNA damage, and likewise, EB treatments could induce similar damage (Goodhead, 1994; Hutchinson, 1985; Moeller et al., 2008b). For example, EB treatments might induce DNA strand breaks, including single-strand breaks (SSB) and double-strand breaks (DSB). SSB occur more often than DSB, and damage is called a DSB only when the distance between two SSB in two strands is within a distance of 10 base pairs (bp) (Krieger, 2016). Moreover, EB treatments might also cause base modifications, which can induce structural changes in the DNA double helix. These kinds of errors are called bulky lesions and can lead to stoppage of DNA replication. Non-bulkly lesions, i.e., lesions without conformation change, can also occur, which can hinder DNA replication or lead to mutations (Lenhart et al., 2012). Furthermore, spore-specific DNA damage, so-called spore photoproduct (SP), which is an intrastrand thymine dimer, might also be produced in spore DNA during EB treatments (Moeller et al., 2008b; Slieman et al., 2000). Furthermore, DNA cross-linking, including DNA-DNA cross-linking or DNA-protein cross-linking, can also occur under high doses of radiation (Krieger, 2016).

It is noteworthy that due to the metabolic dormancy of spores, damage would only be repaired when spores germinate and restart metabolic activities during outgrowth (Leggett et al., 2012; Setlow 1995, 2007, 2014). There are different repair mechanisms in spores that can be activated and repair the damaged DNA. First, the SP produced in spores can be repaired by SP lyase (Spl), which is SP specific and is encoded by the splB gene (Munakata and Rupert, 1974; Setlow, 1992). Moreover, excision repair can take place, which not only repairs the base modification but also SSB. Two excision repair mechanisms are known: nucleotide excision repair (NER) and base excision repair (BER). NER removes the bulky lesions and is an important DNA repair mechanism with high fidelity (Alonso et al., 2013; Friedberg et al., 2005). It does not recognize single base modification but recognizes errors due to structural changes of the DNA. The recognition and excision requires UvrABC excinuclease complex, which is encoded by iuvA, iuvB and iuvC (Friedberg et al., 2005; Lenhart et al., 2012; Sancar, 1996). In contrast to NER, BER repairs non-bulky DNA lesions (Dalhus et al., 2009). The damage is recognized by an enzyme called glycosylase, which also removes the faulty base and leaves an apyrimidinic/apurinic site (AP) in the DNA. The AP will be recognized by enzymes called AP endonucleases, which are encoded by the gene exoA and can remove the AP nucleotides (Lenhart et al., 2012).

Finally, homologous recombination (HR) and non-homologous end joining (NHEJ) can take place to repair DNA DSB. These two mechanisms are important for spore survival because a single unrepaired DSB can be lethal (Alonso et al., 2013; Moeller et al., 2008a; Vlašić et al., 2013). HR is a highly accurate and conserved repair mechanism (Shuman and Glickman, 2007; Vlašić et al., 2013) and is RecA-dependent (Lenhart et al., 2012). A homologous DNA template is used to repair the broken double strand, and due to this, HR is only active during DNA replication (Moeller et al., 2007; Vlašić et al., 2013). In contrast to HR, NHEJ is a specialized DSB repair mechanism that does not require a homologous template and, thus, is the predominant repair mechanism during phases where only one copy of the genome is available (Lenhart et al., 2012; Vlašić et al., 2013). It has been shown that NHEJ in bacteria is mediated by two main proteins called Ku and LigD. The fidelity of NHEJ is low and some nucleotides might be lost (Fleck and Nielsen, 2004; Lenhart et al., 2012). However, because it does not need a homologous template, NHEJ is the preferred pathway when only one copy of the genome is available (Ayora et al., 2011; Lenhart et al., 2012; Shuman and Glickman, 2007).

An SOS response could also be triggered to coordinate DNA repair (Friedberg et al., 2005). The two main players in this system are the RecA protein and the repressor LexA. The SOS response induces DNA repair mechanisms, such as NER, and enhances tolerance against damage (Kreuzer, 2013; Lenhart et al., 2012). Translesion DNA synthesis is part of the SOS system and is one way to tolerate, rather than repair, DNA damage (Friedberg et al., 2005; Yasbin et al., 1990). Specialized DNA polymerases replicate across damaged noncoding bases, which would normally block DNA polymerase (Lenhart et al., 2012; Sutton, 2010). B. subtilis has two specialized Y family DNA polymerases, PolY1 and PolY2 (Sung et al., 2003). Other kinds of DNA repair mechanisms, e.g., mismatch repair, could also be triggered, but they are not the focus of this study and will not be discussed in detail here. For a review of different DNA repair mechanisms of B. subtilis, see Lenhart et al. (2012).

This study compared the resistances of B. subtilis wild type and seven DNA repair-deficient mutants under HEEB and LEEB treatments to understand the spore inactivation mechanisms of EB technologies. The results provide information on what type of DNA damage was induced during the treatment and indicated the important DNA repair mechanisms for bacterial spores to survive LEEB and HEEB irradiation. These findings aid our understanding of EB technologies and could further contribute to the application of these technologies for a milder bacterial spore control, which could ensure food safety while retaining better food quality.

2. Materials and methods

2.1. Bacterial strains, sporulation and sample preparation

Seven B. subtilis mutants that are deficient in different DNA repair mechanisms and their isogenic wild type strain 168 were used in this study. Detailed information is presented in Table 1. A strain (GP1111) with a disrupted polY1 gen was obtained by transposon mutagenesis of B. subtilis; the polY1 gene was disrupted by mini-Tn10 (as described in Commichau et al., 2007a, 2007b; Steinmetz and Richter, 1994). Deletion of the polY2 gene was achieved by transformation with PCR products constructed using oligonucleotides to amplify DNA fragments flanking the target genes and intervening antibiotic resistance cassettes as described previously (for the methodological details see Guérou-Pleury et al., 1995; Wach, 1996), resulting in strain GP1505. GP111 and GP1505 were used to construct the double mutant polY1 polY2 (FF5). Chromosomal DNA of GP111 was transformed into competent cells of B. subtilis GP1505 according to the protocol by Kunst and Rapoport (1995). Transformants were selected on LB agar plates supplemented with 100 μg/ml spectinomycin and 10 μg/ml kanamycin.

For sporulation, the cryo-preserved cultures of all strains were plated on tryptic soy agar (Sigma-Aldrich, Switzerland) plates that contained the appropriate antibiotics (shown in Table 1). From those agar plates, a single colony was picked and inoculated into tryptic soy broth (TSA, Sigma-Aldrich, Switzerland) and incubated at 30°C. 

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Afterward, the incubated cultures were spread on modified Difco sporulation media (mDSM) agar plates, pH 7.2, and incubated at 30°C (Zhang et al., 2018; Nicholson and Setlow, 1990). Further purification was conducted using the buoyant density centrifugation method (Yasbin et al., 1990) with Nycodenz (Axis-Shield, Scotland), and purified spore suspensions were stored at approximately 10⁸ spores/ml) was spread on the surface of a sterile glass slide (Thermo Fisher Scientific, US). All slides were then air-dried at room temperature (approximately 23°C) on a clean bench. The dried samples were stored under cool condition and transported in closed petri dishes for EB treatments.

2.2. EB treatments, dose evaluation and recovery

For LEEB treatment, samples were treated at 80 and 200 keV using a lab-scale LEEB system (EBLab-200, Comet Group, Switzerland). The treatments were at a N₂ atmosphere (O₂ < 210 ppm) with a distance of approximately 18 mm between samples and the emission window (Zhang et al., 2018). Samples were treated at five different nominated doses from 0 to 7 kGy based on their resistances. The absorbed dose of the sample was corrected from the dose measured with Risø B3-12 films (Risø High Dose Reference Laboratory, Denmark) as described in a previous study (Zhang et al., 2018). In short, the surface dose Dₛ (absorbed dose in the first μm of the absorbing medium) was first obtained using the software Risescan (Helt-Hansen et al., 2010), and then, the absorbed doses of the spore samples were calculated based on the obtained Dₛ values and the simulation output on backscattering.

For HEEB treatment, samples were treated at 10 MeV using a Rhodotron TT 300 electron accelerator (IBA Corp, Belgium) at an ambient atmosphere (treatment under a N₂ atmosphere was not feasible due to technical limitations). The nominated treatment doses were set from 0 to 7.5 kGy. The absorbed doses for the HEEB treatments were assessed using alanine pellets (Aerial CRT, France). The pellets were placed together with the samples during the HEEB treatment. Free-radical signals in the alanine pellets after the treatment were measured using an EPR device Miniscope MS-400 (Magnettech, Germany), and the absorbed doses were calculated by comparisons with a standard curve.

After EB treatment, cultivable survivors of treated (N) and untreated samples (N₀) were recovered from the glass slides and enumerated. The spore recovery efficiency from the glass slides was around 30.7% ± 4%. First, the glass slides with treated spore samples were transferred to 50 ml centrifuge tubes, which contained 20 ml phosphate buffered saline recovery solution (PBS, 10 mM, VWR International, United States). The tube was then vortexed for 4 min at full speed to wash the spores off the glass slide. The solution was plated with appropriate dilutions onto tryptic soy agar plates (Sigma-Aldrich, USA) and incubated at 30°C to obtain the survivor counts. The decadic logarithm of the survivor fraction, log₁₀(N/N₀), was plotted against the absorbed dose and the regression was analyzed. The D-value, which reflects the inactivation efficiency, was then calculated from the slope of the fitted linear regression (Zhang et al., 2018). Average D-values (n ≥ 6 for LEEB treatment, n = 3 for HEEB treatment) of different strains or conditions were calculated and statistical analysis was conducted using Student’s t-test (two-tailed, unpaired).

3. Results and discussion

3.1. Absorbed doses of the samples

Accurate dose measurement is essential for acquiring reliable results and needs to be reported for EB inactivation experiments (Pillai and Shayanfar, 2018). The absorbed doses during LEEB and HEEB treatments at each nominal dose are shown in Tables 2 and 3, respectively.

3.2. Spore inactivation curves during LEEB and HEEB treatments

The regression analysis indicated that most inactivation curves under LEEB treatment were fitted with the equation log₁₀ N = log₁₀ N₀ - b D, where N is the number of survivors, N₀ is the number of survivors at zero dose, b is the slope of the inactivation curve, and D is the absorbed dose. The calculated b values for each treatment were used to calculate the Dₙ₅₀ values for each strain or condition. The Dₙ₅₀ values were then used to calculate the survival fraction at a specified dose, which was compared to the inactivation curves under LEEB treatment.

Table 1: Antibiotic resistance information

| Strain | Genotype | Deficient repair mechanism | Reference |
|--------|----------|---------------------------|-----------|
| 168    | Wild type| –                         | Gunka et al. (2012) |
| BP469  | ΔrecA::erm’ | Homologous recombination | Cortesao et al. (2019) |
| BP141  | ΔnuA::kan’ Δglg::kan’ | Non-homologous end joining | Cortesao et al. (2019) |
| GP1503 | trpC2 Δux::aphA3’ Δnfi::cat’ | Base excision repair (BER), AP endonucleases ExoA and Nfo/repair of oxidative DNA damage | Gunka et al. (2012) |
| GP175  | trpC2 Δux::erm’ | Nucleotide excision repair (NER) | Gunka et al. (2012) |
| GP984  | trpC2 Δux::aphA3’ | DNA interstrand cross-link repair; DNA exonuclease | Gunka et al. (2012) |
| BP130  | trpC2 Δpx::spc’ | Spore photoproduct (SP) lyase | Djouiai et al. (2018) |
| GP1111 | trpC2 ΔpolY1::spc’ | Translesion synthesis (TLS-) DNA polymerase Y1 | This study |
| GP1505 | trpC2 ΔpolY2::kan’ | Translesion synthesis (TLS-) DNA polymerase Y2 | This study |
| FFS   | trpC2 ΔpolY1::spc’ ΔpolY2::kan’ | Translesion DNA synthesis (DNA polymerases Y1 and Y2) | This study (Transformation of GP1111 into GP1505) |

*Antibiotic resistance information: erm’, resistant to erythromycin-lincomycin (2 and 25 μg/ml, respectively); kan’/aphA3’, resistant to kanamycin (10 μg/ml); cat’, resistant to chloramphenicol (5 μg/ml); spc’, resistant to spectinomycin (100 μg/ml).
Table 3

Measured absorbed dose for high energy electron beam treatment at 10 MeV.

| Nominal dose (kGy) | 1.5 | 3   | 4.5 | 6   | 7.5 |
|-------------------|-----|-----|-----|-----|-----|
| Dose measured by alanine pellet (kGy) | 1.55 | 3.04 | 4.55 | 6.04 | 7.61 |
| Uncertainty (kGy, k = 2) | 0.070 | 0.137 | 0.205 | 0.273 | 0.345 |

*The uncertainty at k = 2 is close to a 95% confidence interval.

Fig. 1. *Bacillus subtilis* wild type strain 168 as a representative example of spore inactivation curves under low energy and high energy electron beams. Data are shown as the mean ± standard deviation (n = 3).

3.3. Spore resistance and inactivation mechanisms during LEEB treatment

D-values under LEEB treatment, indicating the resistance of the wild type and different mutants, were obtained and compared. Individual D-values under LEEB treatment are shown in Fig. 2. The wild type strain 168 had D-values of 2.4 ± 0.25 kGy at 80 keV and 2.6 ± 0.23 kGy at 200 keV. The resistance of the tested mutants under 80 keV is (from the most resistant to the least resistant) FF5 > GP894 > GP1503 > GP1175 > BP130 > BP141 > BP469. Under 200 keV treatment, the resistance sequence is FF5 > BP130 > GP894 > GP1503 > GP1175 > BP141 > BP469. Results also revealed that except for mutant BP130, all other strains of *B. subtilis* showed similar resistance when comparing $D_{80\text{keV}}$ and $D_{200\text{keV}}$ -values (168: $p = 0.08$, BP469: $p = 0.16$, BP141: $p = 0.07$, GP1503: $p = 0.09$, GP894: $p = 0.03$, GP1175: $p = 0.01$, and FF5: $p = 0.05$). This finding indicates that the kinetic energy levels at the investigated LEEB domain did not influence the inactivation efficiency, and most of the DNA damage was not dependent on the kinetic energy level at the investigated conditions. For BP130, which is the mutant deficient in SP lyase, the D-value obtained was much higher at 80 keV than at 200 keV ($p < 0.01$). The difference between D-values at 80 and 200 keV might indicate that the SP were produced in a different amount at the two applied energy levels, leading to differences in spore survival of SP lyase-deficient mutants.

The results of the sensitivity of investigated mutants compared to the wild type are shown in Fig. 3. All mutants, except for FF5 ($p = 0.887$), showed large decreases in their resistance towards the treatment at 80 keV compared to the wild type ($p < 0.05$). At 200 keV, the resistance of FF5 ($p = 0.135$) and BP130 ($p = 0.688$) were similar to the wild type, while all other mutants were much less resistant ($p < 0.05$). These results indicate that DNA should be one of the targets for LEEB treatment. This finding is consistent with previous research, which showed that mutants lacking a DNA protection mechanism ($\alpha\beta\gamma\delta\zeta$) had reduced resistance towards LEEB treatment compared to the wild type (Zhang et al., 2018).

These results also suggested which repair mechanisms are important for spores to survive LEEB treatments and, thus, give some indications regarding what kind of DNA damage is induced during the treatment. Mutant BP469 (ΔrecA, deficient in HR) was the most sensitive under the LEEB treatment with D-values of 1.08 ± 0.05 kGy at 80 keV and 1.20 ± 0.18 kGy at 200 keV. The next most sensitive mutant was BP141 (ΔKu ΔligD, deficient in NHEJ repair mechanism) with D-values of 1.38 ± 0.10 kGy at 80 keV and 1.58 ± 0.22 kGy at 200 keV. For both mutants, their D-values showed large decreases compared to the wild type, with p-values < 0.001. Since both mutants are deficient in mechanisms involving DNA DSB repair, it is highly possible that DNA DSB is one of the key causes of spore inactivation under LEEB treatment. This type of DNA damage was also found in other ionizing radiation technologies, including X-rays and high-energy protons, where the mutants deficient in HR and NHEJ repair pathways also showed a significant increase in sensitivity towards the treatments (Moeller et al., 2008b, 2012).

The recA gene (mediating the HR repair pathway) was more crucial than the Ku and ligD genes (mediating the NHEJ repair pathway) for spore survival under the investigated LEEB treatment conditions. This finding is in accordance with a previous study, which showed that a *B. subtilis* ΔrecA mutant was more sensitive to X-rays than a ΔykoV (Ku-like gene) ΔykoU (ligase-like gene) mutant (Weller et al., 2002). However, this result does not indicate that HR is more important than NHEJ as the DSB repair pathway for *B. subtilis* under LEEB treatment. Indeed, since *B. subtilis* spores are monogenomic, HR, which requires two homologous chromosomes, would not operate at early stages of germination until the first round of replication, which produces partial duplex chromosomes (Moeller et al., 2008b, 2010; Wang et al., 2006). The reason that the ΔrecA mutant showed extreme sensitivity towards the...
EB treatment might be due to the fact that the recA gene not only encodes for HR-mediated repair but also plays an essential role in triggering the SOS response, which can coordinate DNA repair (Friedberg et al., 2005).

The results showed that GP1503 (deficient in BER) and GP1175 (deficient in NER) had much lower resistances towards LEEB treatment compared to the wild type (p ≤ 0.01) indicating that LEEB also induced DNA bulky lesions and non-bulky lesions. This result is similar for other ionizing irradiations, as previous research showed that mutants lacking BER (via AP endonucleases by Nfo and ExoA) also had significantly decreased resistance compared to the wild type at both 80 keV (p = 0.04) indicating that LEEB treatment also induced DNA interstrand cross-links. The mutant FFS, which is deficient in translesion DNA synthesis, did not show a difference in resistance compared to the wild type at both 80 keV (p = 0.89) and 200 keV (p = 0.14). This result indicates that translesion DNA synthesis, which allows DNA replication despite certain types of DNA damage, does not contribute largely to the survival of spores under LEEB treatment.

3.4. Spore resistance and inactivation mechanisms during HEEB treatment

At the 10 MeV HEEB treatment, the D-value of B. subtilis wild type was 1.5 ± 0.03 kGy. This result is consistent with previous research on HEEB, which also demonstrated a log₁₀ linear relationship between spore survival and dose with D-values in a similar range (De Lara et al., 2002; Fiester et al., 2012; Tallentire et al., 2010). For example, Fiester et al. (2012) reported a D-value of 1.3 ± 0.1 kGy for B. atrophaeus treated at 5 MeV. Notably, D-values between different studies should be compared with care because different sporulation media, different sample preparation and recovery methods can influence spore resistance towards EB treatment (Zhang et al., 2018).

The D-values at 10 MeV, which reflect the resistance of the wild type and mutants towards HEEB treatment, are shown in Fig. 4. The D-values are shown as the mean ± standard deviation (n = 3). All mutants showed different degrees of decreases in D-values compared to the wild type.

UV treatment; however, they were not found to be more sensitive to X-rays (Djouiai et al., 2018; Moeller et al., 2007). The mutant deficient in DNA interstrand cross-link repair (GP894) also had a reduced resistance compared to the wild type at both 80 keV (p = 0.01) and 200 keV (p = 0.04) indicating that LEEB treatment also induced DNA interstrand cross-links. The mutant FFS, which is deficient in translesion DNA synthesis, did not show a difference in resistance compared to the wild type at both 80 keV (p = 0.89) and 200 keV (p = 0.14). This result indicates that translesion DNA synthesis, which allows DNA replication despite certain types of DNA damage, does not contribute largely to the survival of spores under LEEB treatment.



Fig. 3. Fold differences in D-values of investigated Bacillus subtilis mutants compared to wild type strain 168 under electron beam treatments at different energy levels. Color code indicates the fold of differences in D-values compared to the wild type, e.g., BP469 had a D-value that is > 2-fold smaller than the wild type, indicating that BP469 is > 2-fold more sensitive compared to wild type 168. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Fig. 4. D-values of Bacillus subtilis wild type and mutants treated under high energy electron beam at 10 MeV. Treatments were under ambient atmosphere with presence of O₂. The D-values are shown as the mean ± standard deviation (n = 3). All mutants showed different degrees of decreases in D-values compared to the wild type.
towards HEEB treatment with D-values of approximately 1.3 kGy. These mutants have higher resistance compared to the mutants that lack HR, NHEJ, BER and NER repair pathways but still showed reduced resistance compared to the wild type. This result indicates that these genes influence the survival of spores under HEEB treatment but are not as crucial as the other genes related to repair of DNA DSB, bulky and non-bulkely lesions.

3.5. Spore inactivation by LEEB and HEEB

In this study, all LEEB treatments were conducted under N₂ atmosphere with O₂ < 210 ppm to avoid the production of ROS resulting from the encounter of electrons with oxygen during the treatment. ROS are extremely reactive with organic compounds. They can cause DNA base damage and inactive microorganisms, including bacterial spores (Eichner et al., 2015; Hashizume et al., 2013; Mahfoudh et al., 2010; Maness et al., 1999). Therefore, presence of ROS would interfere with the mechanistic study of the direct effects of electrons on spore DNA. Unfortunately, it is not possible to conduct the HEEB treatments under N₂ atmosphere due to technical limitations. Therefore, more ROS may have formed during HEEB treatment compared to LEEB treatment. The presence of ROS might be the reason that lower D-values for HEEB were observed in this study due to the additional inactivation effect of ROS. Previous research actually showed that HEEB and LEEB treatments had similar spore inactivation efficiency at ambient atmosphere (Tallentire et al., 2010), and lower D-values were reported when spores were treated in the presence of oxygen compared to vacuum (Ito and Islam, 1994).

Despite the influence of ROS, the results showed that the resistance sequence of the investigated mutants were almost the same whether the treatments were under HEEB or LEEB. The mutants with a largely reduced resistance compared to the wild type with LEEB treatments were also the most sensitive ones to HEEB, while the ones that showed only slight decreases in resistance under LEEB treatment were also comparatively more resistant to HEEB.

4. Conclusion and recommendation

This study investigated spore inactivation mechanisms during LEEB and HEEB treatment by evaluating the D-values of B. subtilis wild type and seven mutants lacking relevant DNA repair mechanisms. The results revealed that DNA damage is one of the causes responsible for spore inactivation by both LEEB and HEEB treatments. Moreover, the type of DNA damage induced by LEEB and HEEB treatments was found to be similar. Among the different types of DNA damage investigated in this study, DSB is the most lethal one. The B. subtilis mutants that lack the DNA DSB repair mechanisms, including HR and NHEJ, were the most sensitive ones under both LEEB and HEEB treatments. In addition to HR and NHEJ, BER and NER also played important roles in spore survival under HEEB and LEEB treatments. Furthermore, it was revealed that interstrand cross-links and formation of SP might play a role in spore inactivation by EB. Finally, it could be demonstrated that translesion DNA synthesis does not play an important role in the survival of spores after LEEB treatments and may play a small role in spore survival under HEEB treatment.

In future research, it would be interesting to determine the type, nature and level of DNA damage induced during EB treatments. Further, the role of other DNA repair pathways, e.g., mismatch repair, should be investigated to gain further insights into spore inactivation mechanisms by EB technologies. Moreover, it would also be interesting to analyze whether DNA is the only target of LEEB treatment since DNA is not the only target of HEEB treatment (Fiester et al., 2012). Therefore, research on other type of damage that might be induced during EB treatment, e.g., protein, membrane or lipid damage, would be fruitful. Furthermore, investigation on the role of the EB treatment atmosphere on spore inactivation due to the production of reactive species, including ROS, RNS and RHS, could be insightful.

In summary, this study confirmed that DNA should be the major target for LEEB and HEEB technologies to inactivate bacterial spores. It provided more information on the spore inactivation mechanisms of EB technologies and indicated what type of DNA damage is induced during the treatments. It also revealed the roles of different DNA repair mechanisms for spores to survive EB treatments and suggested that LEEB and HEEB technologies share many similarities regarding spore inactivation. This information will help us to understand both technologies more thoroughly and will support their application as non-thermal mild microbial decontamination methods for food and pharma products.

Declarations of competing interest

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