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Multifactorial resistance of *Bacillus subtilis* spores to low-pressure plasma sterilization

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ABSTRACT Common sterilization techniques for labile and sensitive materials have far-reaching applications in medical, pharmaceutical, and industrial fields. Heat inactivation, chemical treatment, and radiation are established methods to inactivate microorganisms, but pose a threat to humans and the environment and can damage susceptible materials or products. Recent studies have demonstrated that cold low-pressure plasma (LPP) treatment is an efficient alternative to common sterilization methods, as LPP's levels of radicals, ions, (V)UV-radiation, and exposure to an electromagnetic field can be modulated using different process gases, such as oxygen, nitrogen, argon, or synthetic (ambient) air. To further investigate the effects of LPP, spores of the Gram-positive model organism *Bacillus subtilis* were tested for their LPP susceptibility including wild-type spores and isogenic spores lacking DNA-repair mechanisms such as non-homologous end-joining (NHEJ) or abasic endonucleases, and protective proteins like α/β-type small acid-soluble spore proteins (SASP), coat proteins, and catalase. These studies aimed to learn how spores resist LPP damage by examining the roles of key spore proteins and DNA-repair mechanisms. As expected, LPP treatment decreased spore survival, and survival after potential DNA damage generated by LPP involved efficient DNA repair following spore germination, spore DNA protection by α/β-type SASP, and catalase breakdown of hydrogen peroxide that can generate oxygen radicals. Depending on the LPP composition and treatment time, LPP treatment offers another method to efficiently inactivate spore-forming bacteria.

IMPORTANCE Surface-associated contamination by endospore-forming bacteria poses a major challenge in sterilization, since the omnipresence of these highly resistant spores throughout nature makes contamination unavoidable, especially in unprocessed foods. Common bactericidal agents such as heat, UV and γ radiation, and toxic chemicals such as strong oxidizers: (i) are often not sufficient to completely inactivate spores; (ii) can pose risks to the applicant; or (iii) can cause unintended damage to the materials to be sterilized. Cold low-pressure plasma (LPP) has been proposed as an additional method for spore eradication. However, efficient use of LPP in decontamination requires understanding of spores' mechanisms of resistance to and protection against LPP.

KEYWORDS *Bacillus subtilis*, spore resistance, plasma inactivation, decontamination

Protective structures of endospores and survival strategies

In some Bacillota, the differentiation of growing cells into spores is induced by poor nutrient conditions, and these spores are dormant and resistant and can survive for years. The ability of *Bacillus subtilis* spores to survive long-term dormancy is due to a combination of protective structural components (passive protection), spore-specific attributes (core dehydration), and efficient DNA-repair mechanisms (active protection). Spores have multiple layers, and from the outside to inside (Fig. 1), these are the coats and crust, outer membrane, peptidoglycan (PG) cortex, PG germ-cell wall, inner

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This paper is dedicated to the memory of our colleague Wayne L. Nicholson, who did so much important work on spore resistance and left us much too soon.

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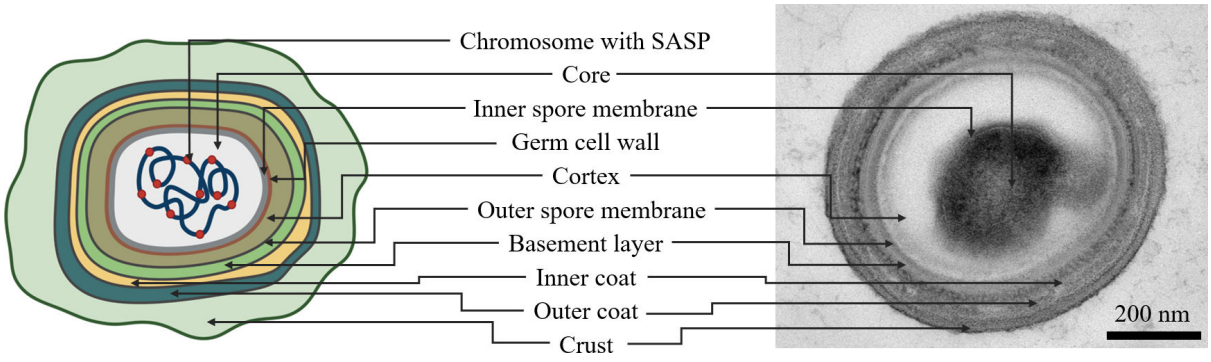

FIG 1 Schematic drawing and TEM (transmission electron microscopic) image of the multiple layers of a spore characteristic of *B. subtilis*. Red dots represent the binding of the major SASP to the spore DNA.

membrane (IM), and the central core (1–3). The coat/crust layers are spores' first barrier to the environment and are essential in resistance to PG hydrolytic enzymes. Coat layers can also play a role in spore UV resistance since they may contain pigments that absorb UV, and also contain large amounts of protein which can detoxify oxidative chemicals and radicals (4, 5). The spore cortex PG is similar but not identical to vegetative cell wall PG and is crucial in establishing spores' low core water content, as low as 25% of core wet weight (wt) (6–8). This core feature is crucial in some spore resistance properties as well as spore dormancy and lack of core enzyme action, as at least one core protein is immobile in dormant spores (9). Under the cortex is the germ-cell PG, with a structure identical to vegetative cell PG, and then the IM, which is a strong permeability barrier to chemicals that may damage core DNA or proteins, and IM lipids have very low mobility (2, 7, 9–11). Finally, the core contains nucleic acids, enzymes, and ribosomes and a small molecule called dipicolinic acid (DPA) in a 1:1 chelate with Ca^{2+} (CaDPA) which comprises ~25% of core dry wt and contributes to core water loss and spore UV resistance (12). The α/β-type small acid-soluble spore proteins (SASP) saturate spore DNA, changing its conformation and increasing its UV and γ-radiation resistance. Because of the spore cores' low water content, DNA damage can accumulate during dormancy and is only repaired during outgrowth that follows germination (1, 12). The DNA photoproducts generated in spores by UV at 254 nm are as follows: cyclobutane pyrimidine dimers (CPDs), pyrimidine 6–4 pyrimidones (6–4 PPs), and by far most abundantly the 5-thyminyl-5,6-dihydrothymine adduct, the latter termed the spore photoproduct (SP) (13); in contrast, 254 nm irradiation generates only CPDs and PPs in vegetative cells. Spore DNA's novel UV photochemistry is due to DNA's saturation with α/β-type SASP which converts DNA from a B-like to an A-like helix in which SP formation is favored over CPDs or PPs (1, 14–16). Additional DNA lesions are caused by γ radiation or chemical agents, most notably DNA single and/or double-strand breaks. For the maintenance of an intact genome, spores have multiple DNA-repair systems that act after germination is completed (1, 12). SP removal is by SP lyase, which is made in the developing spore late in sporulation and in outgrowth cleaves any SP to two thymine residues without DNA backbone cleavage (12, 17, 18). Among radiation-induced DNA damage, species of reactive oxygen (ROS) and/or nitrogen (RNS) generated by UV or chemicals can also form adducts in DNA. In order to maintain genome integrity, all DNA damage can be repaired by five repair pathways. Base excision repair (BER) starts with detection of altered bases by N-glycosylases, which cleave N-glycosylic bonds and remove the altered base. This leaves a highly mutagenic gap termed an abasic site, and either apyrimidinic or apurinic (AP) sites can lead to single-strand break formation (19). Subsequently, AP-specific endonucleases like ExoA, Nfo, or DNA polymerase PolX recognize and cleave the 5′ and 3′ backbone at the AP site, and additional processing by exonucleases prepares DNA for the final fill of the gap by DNA polymerase and ligase (12, 20). While BER preferentially deals with non-bulky DNA lesions, nucleotide excision repair

(NER) is used for the repair of bulky adducts. NER enzymes include the conserved UvrABC excision endonuclease (excinuclease) complex. This complex's activity is regulated by the SOS system and removes 10–15 nucleotides containing the damaged nucleotide to repair this lesion (12, 20, 21). In the case of misincorporated bases during DNA recombination or replication, DNA mismatch repair can specifically recognize and correct errors (22). Finally, for highly detrimental DNA lesions like double-strand breaks, *B. subtilis* has two major repair pathways: homologous recombination (HR) and non-homologous end joining (NHEJ). This repair involves a series of reactions including enzymatic steps that form a crossover junction and ultimately endonucleolytic resolution. Several enzymes of the "Rec"-class, such as RecA are essential in this process, and NHEJ deals with double-strand breaks in the absence of a homologous DNA template (11, 19).

Utilization of plasma as decontamination agent

A variety of commercial disinfection methods are used to inactivate spores, but there are limitations due to corrosive, uneconomic, or hazardous properties (23). Hence, to ensure strict hygiene standards in the food and pharmaceutical industry, suitable decontamination methods are needed to avoid spore-derived diseases, material corrosion, and food spoilage. Plasma, the fourth state of matter, has bactericidal and sporicidal properties and thus an extensive range of decontamination approaches. Plasma discharges are defined as partially ionized gas consisting of a mixture of free radicals, various charged particles, an electric field, and neutral as well as excited atoms and photons in the ultraviolet (UV) and vacuum ultraviolet (VUV) range (24). There are two plasma categories: thermal and non-thermal. Non-thermal plasmas are most applicable for biomedical purposes and are generated either under (cold) atmospheric pressure (CAP), low-pressure (LPP), or high pressure. LPP generation utilizes a vacuum and thus generates a homogeneous plasma discharge, making it particularly suitable for sterilization of complex 3D structures and large surfaces of vacuum-stable materials such as glass and metal (25). In addition to the pressure, the gas mixture in plasma generation plays a major role in the inactivation efficiency, as the amount and complexity of the bactericidal components, such as the radical concentration or emitted UV radiation changes. In LPPs, argon plasma, for instance, emits at wavelengths of 104.8 nm and 106.7 nm, and the number of reactive species such as atomic or molecular oxygen is very low, as argon is an inert gas (26). However, synthetic air plasma, a 4:1 mixture of nitrogen and oxygen emits photons ranging from 130 nm to 400 nm and thus in the VUV, UV-A, UV-B, and UV-C ranges (Fig. 2). Compared to LPPs made with noble gases, synthetic

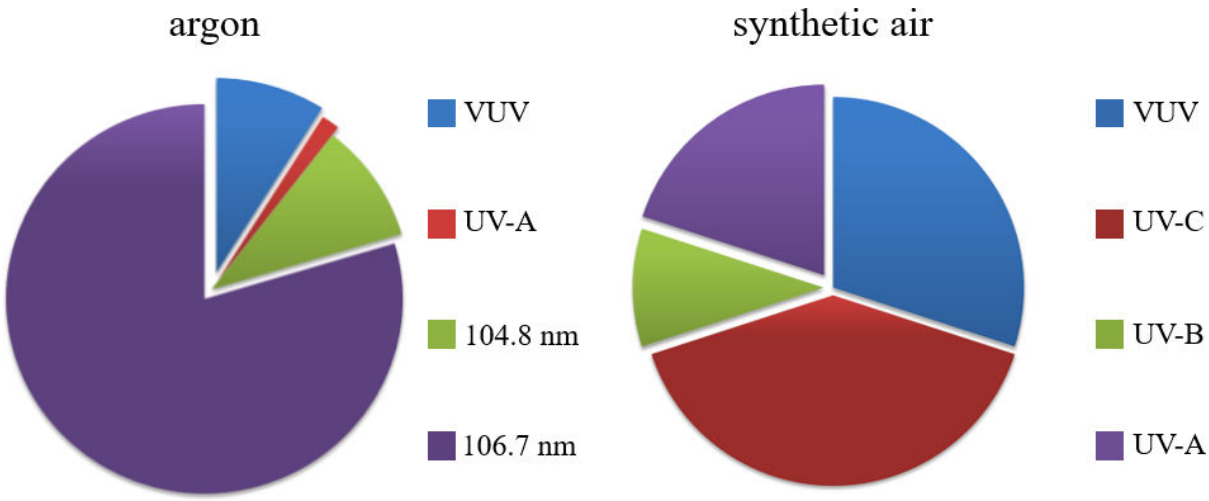

FIG 2 Comparison of argon and (synthetic) ambient air LPP in emitted photons; argon LPP mainly emits photons in the VUV and below while ambient air LPP mainly emits photons in the UV-C range followed by VUV and UV-A. Modified after reference (26), details on the absolutely calibrated spectra can be seen in Fig. S1.

air LPPs generate more ROS and RNS including atomic or molecular radicals which have bactericidal and sporicidal effects (26). Inactivation of spore-forming microorganisms, and in particular their spores, by non-thermal plasmas has attracted research interest and seems likely to be a multipurpose application (27–29). The work reported in this communication was to gain a comprehensive understanding of mechanisms of spore inactivation by LPP and spore LPP resistance, and in the latter, specifically examining roles of known spore protective structures and spore-specific and universal DNA-repair systems. Hopefully, a deeper understanding of how endospores resist and repair LPP-mediated damage may facilitate optimization of non-thermal LPP for spore inactivation.

RESULTS

Role of feed gas

In this study, two sets (Tables 1 and 2) of isogenic *B. subtilis* strains lacking major spore-specific protective structures and DNA-repair mechanisms were exposed to non-thermal plasma under low-pressure conditions. To assess the role of factors in LPP resistance, spores lacking spore-specific and/or universal protection attributes were systematically tested for survival in LPP with either argon or synthetic air (N_2/O_2 = 16:4), all in comparison with survival of spores of the appropriate wild-type (WT) strains (Fig. S1 to S17). Sporicidal/germicidal components of plasma include charged particles, reactive species, an electric field, and photons in the UV and/or (V)UV range. The composition and quantities of these components are mainly dependent on the pressure and feed gas used. In a previous study, Fiebrandt et al. analyzed the photon fluxes generated by different gases within a double inductively coupled plasma (DICP) reactor and their correlation with spore survival (26). Molecular argon emits photons mainly in the (V)UV range and below, while synthetic air gives emission in the VUV, UV-C, UV-B, and UV-A range (Fig. 2), and the authors concluded that a 30-second treatment with synthetic air LPP is more efficient at spore inactivation than an argon LPP (26). Analysis of the WT strains (PS832 and 168) used in our study confirmed these findings (Table 3).

Role of preventive spore protection properties

The resistance of spores to stress stimuli can be attributed to both active and passive factors. While DNA protection is passively accomplished by the coat proteins and SASP, DNA damage is repaired only in outgrowth following germination in an active manner

TABLE 1 List of isogenic *Bacillus subtilis* strains deficient in DNA-repair mechanisms[a]

Strain	Genotype	Repair deficiency	Reference
168	WT	None	Laboratory strain
BP130	*trpC2* Δ*splB*; Spc^R	SP lyase/monomerization of SPs	(30)
BP141	*trpC2* Δ*ligD* Δ*ku::aphA3*	Multifunctional DNA ligase LigD/NHEJ	(31)
RM023	*trpC2* Δ*ligD* Δ*ku* Δ*splB*; Kan^R Spc^R	SP lyase and LigD Ku/monomerization of SPs and NHEJ	BP141 → BP130 generated in this study
GP1175	*trpC2* Δ*uvrAB*; Erm^R	Exonuclease/NER repair	(32)
RM020	*trpC2* Δ*uvrAB* Δ*splB*; Erm^R Spc^R	SP lyase and exonuclease/removal of SPs and NER	GP1175 → BP130 generated in this study
PERM639	Δ*ywjD::lacZ*; Erm^R	UVDE/repair and protection of UV-damaged DNA	(33)
RM021	*trpC2* Δ*ywjD::lacZ* Δ*splB*; Erm^R; Spc^R	UVDE and SP lyase/repair and protection of UV-damaged DNA and removal of SPs	PERM639 → BP130 generated in this study
GP1503	*trpC2* Δ*exoA::aphA3* Δ*nfo*; Cat^R	AP endonucleases ExoA and Nfo/BER	(32)
BP469	*trpC2* Δ*recA*; Erm^R	RecA protein/HR	(31)
RM022	*trpC2 pMUTIN4::yqjH (polY1)* Δ*yqjW (polY2)*; Em^R Kan^R	DNA polymerases Y1 and Y2 repair of DNA damage	GP1111 → GP1505 generated in this study

[a]Arrows indicate constructions made by transformation. Antibiotic resistances: Cat^R, resistant to chloramphenicol (5 µg/mL); Spc^R, resistant to spectinomycin (100 µg/mL); Tet^R, resistant to tetracycline (10 µg/mL); Erm^R, resistant to erythromycin (1 µg/mL); and Kan^R, resistant to kanamycin (10 µg/mL). AP, apurinic/apyrimidinic and UVDE, UV damage endonuclease.

TABLE 2 List of isogenic *Bacillus subtilis* strains lacking protective components and essential DNA-repair systems[a]

Strain	Genotype	Deficient protective mechanism(s)	Reference
PS832	WT, prototroph; Trp+ revertant of strain 168	None	(34)
PS283	Δ*sspA*; Cat^R	α-Type SASP/DNA protection	(35)
PS338	Δ*sspB*; Cat^R	β-Type SASP/DNA protection	(35)
PS483	Δ*sspE*; Cat^R	γ-Type SASP	(36)
PS356	Δ*sspA* Δ*sspB*; Cat^R	α- and β-type SASP/DNA protection	(37)
PS482	Δ*sspA* Δ*sspB* Δ*sspE*; Cat^R	α-, β-, and γ-type SASP/DNA protection	(38)
PS1899	Δ*dacB*; Cat^R	Carboxypeptidase DacB/higher core water	(34)
PS2211	Δ*dacB* Δ*sspA* Δ*sspB*; Cat^R	DacB, α/β-type SASP/higher core water, and DNA protection	(34)
PS3394	Δ*cotE*; Tet^R	CotE protein/outer coat	(39)
PS3395	Δ*cotE* Δ*sspA* Tet^R Δ*sspB*; Tet^R	CotE and α/β-type SASP/outer coat and DNA protection	(28, 39)
FB122	Δ*sleB* Δ*spoVF*; Spc^R Tet^R	DPA/higher spore core water	(40)
PS3664	Δ*sleB* Δ*spoVF* Δ*sspA*, Δ*sspB*; Spc^R Tet^R	DPA and α/β-type SASP/higher spore core water and DNA protection	(41)
PS3747	Δ*cotE* Δ*sleB* Δ*spoVF* Δ*sspA* Δ*sspB*; Tet^R Spc^R Cat^R	CotE, DPA and α/β-type SASP/outer coat, higher core water and DNA protection	(41)
PS2558	Δ*katX*; Cat^R	Major catalase KatX/protection against oxidative stress	(42)
PS2559	Δ*katX* Δ*sspA* Δ*sspB*; Cat^R	KatX, α/β-type SASP/protection against oxidative stress and DNA protection	(42)
PS3722	Δ*ligD* Δ*ku*; Erm^R	DNA ligase LigD and DNA binding protein Ku/NHEJ	(43)
PS3751	Δ*ligD* Δ*ku* Δ*sspA* Δ*sspB*; Erm^R Cat^R	LigD, α/β-type SASP/NHEJ and DNA protection	(43)
PS2318	Δ*recA*; Mls^R	RecA/HR	(44)
PS2319	Δ*recA*; Δ*sspA*, Δ*sspB*; Mls^R	RecA α/β-type SASP/HR and DNA protection	(44)
PERM454	Δ*exoA* Δ*nfo*; Tet^R Neo^R	AP endonucleases ExoA and Nfo/BER	(45)
PERM450	Δ*exoA* Δ*nfo* Δ*sspA*, Δ*sspB*; Tet^R Neo^R	ExoA, Nfo and α/β-type SASP/BER and DNA protection	(45)

[a]Antibiotic resistances: Cat^R, resistant to chloramphenicol (5 µg/mL); Spc^R, resistant to spectinomycin (100 µg/mL); Tet^R, resistant to tetracycline (10 µg/mL); Erm^R, resistant to erythromycin (1 µg/mL); Mls^R, resistant to macrolide antibiotics; Neo^R, resistant to neomycin (10 µg/mL); and Kan^R, resistant to kanamycin (10 µg/mL).

(12). α/β-Type SASP are essential for spore survival when faced with numerous DNA-damaging agents (1, 2, 12), and spores lacking major α/β-type SASP show significantly higher LPP sensitivity compared to WT spores (Table 3). Interestingly, spores with the Δ*sspA sspB* genotype exposed to argon gas plasma are more susceptible than those in synthetic air plasma. The low hydration state of the core is also important in spore resistance and is provided by *dacB* affecting cortex PG synthesis as well as CaDPA accumulation driven by its synthesis in sporulating cells' mother cell compartment (Table 3). Note that the *sleB* mutation in the *spoVF* strain is to ensure the stability of the CaDPA-less spores (46). The absence of CaDPA or DacB individually increased LPP sensitivity compared to that of WT spores (Table 3). The WT spore sensitivity in synthetic air or argon LPP appears identical, suggesting that increased core water content enhances the formation of intracellular ROS and RNS damage. Spores lacking CotE also exhibited higher sensitivity to synthetic air and argon LPP, indicating that the outer coat contributes significantly to spore plasma resistance, although the precise mechanism of this protection is not clear. Possible causes of this protection include the following: (i) neutralization of plasma generated ROS/RNS by reaction with the abundant coat proteins, much as these proteins protect spores against inactivation by toxic chemicals such as hypochlorite which might damage spores' inner membrane or even core components such as DNA (1, 2); and (ii) shielding the genome against UV in plasma by spore coat pigments (6).

Impact of active repair mechanisms in radiation and plasma-induced cell damage

In contrast to passive protection which is based on prevention, active processes include the enzymatic removal and repair of damage. Here, the spore-specific catalase KatX is a key factor in decomposition of ROS such as H_2O_2, and *katX* spores are more susceptible to synthetic air LPP, presumably due to higher intracellular ROS levels caused by the shift in the spectrum of emitted photons (Table 3). Nicholson et al. observed that UV-A mainly leads to the formation of ROS (47). Data from the latter study correlated inactivation

TABLE 3 Spore resistance to synthetic air and argon LPP: lethal dose (lethal to 90% of the population) LD_{90} values (lethal LPP exposure time in seconds leading to 90% spore inactivation) of the spores lacking major damage preventing, detoxification, or removal/repair factors[b]

Genotype[a]	LD_{90}—synthetic air LPP	LD_{90}—argon LPP
WT	19.1 ± 2.6	31.2 ± 4.7
sspA	6.5 ± 1.0**	6.9 ± 1.1**
sspB	6.3 ± 0.9**	11.4 ± 2.3**
sspE	16.4 ± 1.5	24.5 ± 3.0
sspA sspB1	4.4 ± 0.9***	3.8 ± 0.3***
sspA sspB sspE	4.2 ± 0.5***	4.2 ± 0.7***
dacB	3.5 ± 0.5***	7.6 ± 1.6**
dacB sspA sspB	3.1 ± 0.4***	4.7 ± 0.9***
sleB spoVF	5.5 ± 0.9**	9.4 ± 1.2**
sleB spoVF sspA sspB	5.4 ± 1.0**	4.6 ± 1.1**
cotE	4.7 ± 0.5***	9.5 ± 1.5**
cotE sspA sspB	4.0 ± 0.8***	4.8 ± 0.9***
cotE sleB spoVF sspA sspB	3.2 ± 0.3***	2.4 ± 0.2***
katX	4.4 ± 0.7***	12.3 ± 2.0**
katX sspA sspB	3.3 ± 0.4***	3.6 ± 0.7***
exoA nfo	6.9 ± 1.1**	13.6 ± 1.2**
exoA nfo sspA sspB	4.5 ± 0.8***	4.1 ± 0.5***
ligD ku	3.9 ± 0.4***	9.0 ± 1.4***
ligD ku sspA sspB	3.3 ± 0.4***	3.8 ± 0.6***
recA	5.0 ± 0.8**	5.2 ± 1.0***
recA sspA sspB	3.2 ± 0.7***	3.6 ± 0.4***

[a]All strains have PS832 background.
[b]Significant differences from WT spores are indicated by *P* values shown as follows: *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$.

efficiency by synthetic air LPP with UV-A-mediated ROS formation. Active removal of plasma-induced damage in spores is accomplished by repair of DNA lesions following spore germination. The role of BER in the survival of LPP stress has been investigated using an *exoA nfo* deficient mutant which lacks two of the three apurinic/apyrimidinic endodeoxyribonucleases (AP endonucleases) (11). AP sites are generated by plasma treatment, whereupon the BER system replaces missing bases and prevents mutations and dysfunctional DNA replication (48). The absence of most AP endonucleases results in spores with a significantly lower LD_{90} value compared to WT spores, without noticeable differences between the two LPPs (Tables 3 and 4). Lack of major α/β-type SASP also results in decreased spore LPP resistance, which could be caused by an increased naked surface area of the DNA, offering more potential interaction sites for UV and reactive species. In addition to AP sites, photoproducts are generated by damaging radiation, which require repair to prevent cell death. An important repair mechanism upon germination of *B. subtilis* spores is the enzyme spore photoproduct lyase that monomerizes SP dimers (12, 17, 18), and much work has shown that SP formation by UV-C is serious damage. Hence, synthetic air LPP emitting higher UV-C is more effective than argon LPP in spore killing, as argon LPP emits mainly in the VUV range (Fig. 2) and is less efficient in SP formation. Spores' DNA-repair mechanisms also include NER, HR, and NHEJ. The role of NER was investigated by LPP treatment of spores lacking *uvrAB* and *ywjD*, products of which are endonucleases cleaving UV-mediated photoproducts (33). Both these enzymes seem to have important roles in LPP resistance based on the significantly lowered LD_{90} values, with *uvrAB* spores more susceptible than *ywjD* spores independent of the type of LPP gas. A knockout of the HR system by deletion of *recA* decreased spore resistance to LPP treatment, with synthetic air LPP more efficient than argon LPP. This is consistent with the idea that LPP-mediated damage (independent of the feed gas composition) is repaired by HR. However, with multiple strand breaks, HR becomes less efficient, and NHEJ takes over which uses the homodimeric Ku and

TABLE 4 Spore resistance to synthetic air and argon LPP: LD_{90} values (lethal LPP exposure time in seconds leading to 90% spore inactivation) of the spores lacking major DNA-repair mechanisms[b]

Genotype[a]	LD_{90}—synthetic air LPP	LD_{90}—argon LPP
WT	18.5 ± 2.8	48.0 ± 4.4
splB	4.5 ± 0.7**	6.0 ± 0.7***
uvrAB	7.6 ± 1.5**	9.8 ± 1.5***
splB uvrAB	3.4 ± 0.5***	2.6 ± 0.3***
ywjD	10.9 ± 1.3*	19.3 ± 3.6***
splB ywjD	3.9 ± 0.6***	3.9 ± 0.6***
ligD ku	9.7 ± 1.6*	10.6 ± 1.1***
ligD ku splB	2.1 ± 0.4**	4.3 ± 0.6***
recA	4.6 ± 0.4**	7.6 ± 1.2***
exoA nfo	13.1 ± 1.8*	10.8 ± 2.0***
polY1 polY2	8.9 ± 1.2**	19.7 ± 2.6***

[a]All strains have 168 background.
[b]Significant differences from WT spores are indicated by *P* values shown as follows: *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$.

LigD proteins, which are both important for LPP resistance based on high LPP susceptibility of *ligD Ku* deficient spores. A previous study suggested SplB and RecA as major photoproduct repair proteins with YwjD of less importance (33), which is consistent with data from the current study (Table 4). The combined effects on spore sensitivity of the various mutations, from largest to least, were as follows: *recA* > *ligD Ku* > *splB* > *uvrAB* > *exoA nfo* > *polY1 polY2* > *ywjD* (Fig. 3). Interestingly, there are reports describing the following: (i) a less relevant contribution of HR in UV-C- and UV-B-mediated damage, rather than in UV-A damage and (ii) the highest production of dimers in the UV-C range compared to UV-A and UV-B (47, 49). Notably, strains lacking *splB* and *ywjD* exposed to synthetic air LPP were more affected in survival than those treated with argon LPP. Thus, spores exposed to air LPP either break thymine dimers (the SPs) or replace them by NER or photoproduct lyase instead of replacing whole sequences by recombination repair mechanisms.

Interaction between prevention and repair mechanisms

For uncovering interactive or cumulative effects in LPP survival, strains with different deficiencies were tested. For this purpose, various single mutants were tested as well as with additional mutations in either α/β-type SASP or *splB*. In general, with strains lacking additional SASP as well as the protective function in question, a much greater increase in sensitivity was observed (Fig. 4). Spores lacking KatX and CotE had higher susceptibility than the respective single mutants lacking α/β-type SASP. Most strains exposed to argon LPP showed a higher sensitivity, indicating that almost all of the protective and repair mechanisms tested are essential for survival of LPP emitting in the low wavelength range. In addition, the importance of spore core water content seems to be independent of the LPP type. In general, the results emphasized the importance of SASP in LPP (especially argon) resistance and highlighted this component and the genome as major targets for LPP inactivation of spores. Similar effects were observed for the contribution of the spore photoproduct lyase (Table 4; Fig. 3). Thus, the absence of SP results in an increase in LPP sensitivity, particularly with spores defective in NER and NHEJ; presumably removal of SP is an essential step prior these repair mechanisms and is more important in argon LPP.

DISCUSSION

Since plasmas have sporicidal and bactericidal properties, their use for sterilization of medical instruments or implants, complex 3D structures, food pasteurization and packaging, and many more have arisen (25, 45, 50, 51). Although LPPs are known to

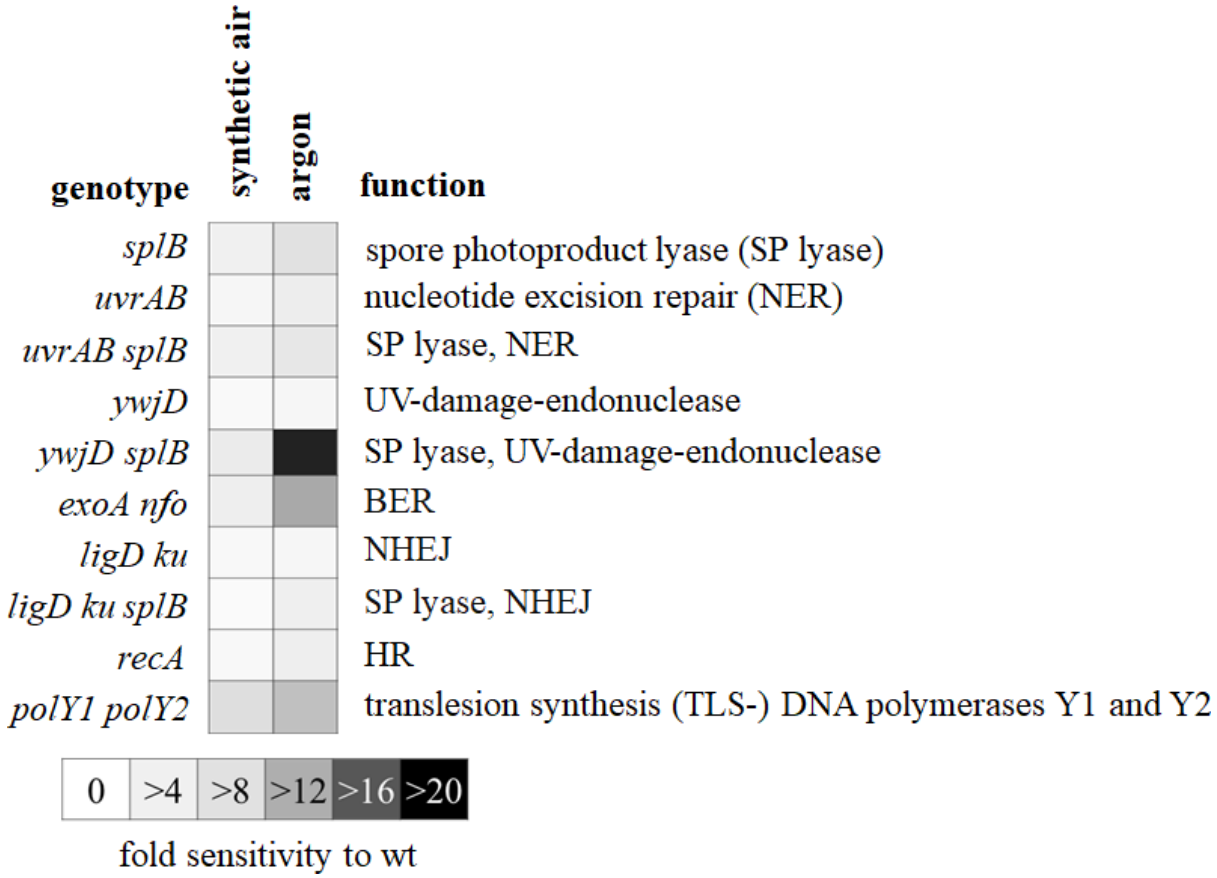

FIG 3 Relative LPP sensitivity of *B. subtilis* spores lacking major DNA-repair mechanisms. Data show a comparison between synthetic air and argon LPP. Values were calculated by building a quotient of the mean LD_{90} data for the WT and respective mutant spores.

efficiently inactivate bacteria and filamentous fungi, detailed biological processes and understanding of bacterial spore inactivation have remained relatively unexplored.

Plasma inactivation efficiency is dependent on emitted photons which generate (V)UV and UV as well as radical and reactive species, reactive oxygen nitrogen species (RONS) (26, 29). Etching by these RONS can be defined as atom-by-atom erosion and is considered one of the main inactivation factors because it leads to cracks within the spore protein coat while UV radiation induces photo-oxidation of biomolecules such as nucleic acids (52–55). Thus, by covering the VUV and UV range from 130 nm to 400 nm, a higher sporicidal effect could be achieved by synthetic air. Consequently, argon plasma is less effective in sterilization because of high intensities in emission at 106.7 nm and lesser etching characteristics, as confirmed in a study by Raguse et al. (54). It is important to note that O_2 and/or N_2 gas plasma generates more reactive species, such as O, N, or NO radicals which could impact microbial inactivation. In contrast, plasmas generated in noble gases do not produce reactive species. Instead, UV or VUV emission is produced as the main active sporicidal component, indicating a different mode of action compared to N_2/O_2 gas mixtures. However, the enhanced inactivation efficiency in N_2/O_2 compared to noble gases is only due to different emission spectra and not due to the abundance of RONS (51, 53, 55, 56).

Due to the conformational change in the condensed A-like conformation of the DNA saturated with α/β-type SASP, the DNA presumably has less sites available for UV and/or ROS damage, suggesting that the genome is more readily damaged by argon LPP. Spores lacking only one of the two major (α/β-type) SASP are also more sensitive to plasma inactivation, but more resistant than spores lacking both of these SASP. However, spores lacking the one γ-type SASP (*sspE* mutant) are not affected by LPP regardless of the

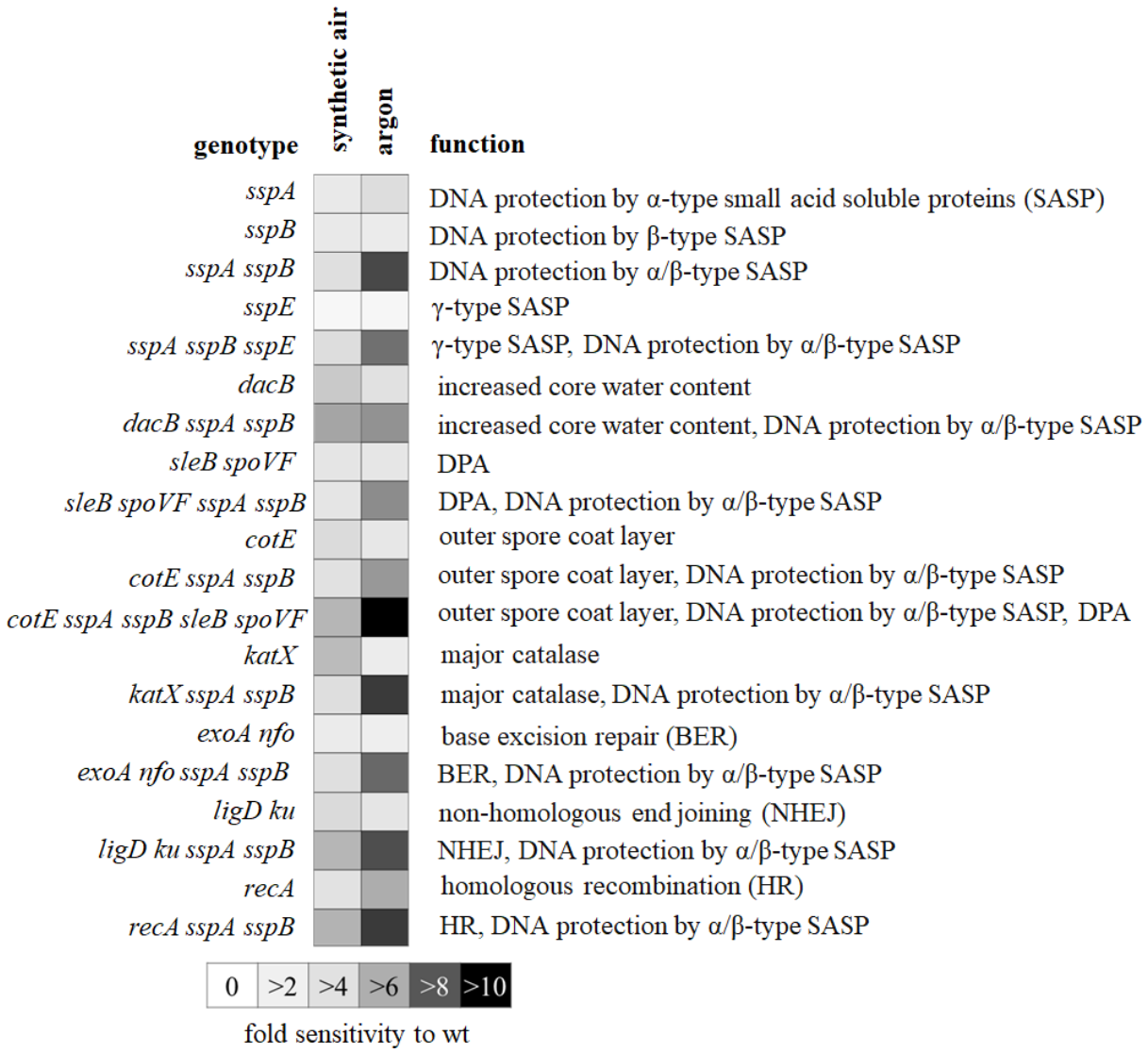

FIG 4 Relative LPP sensitivity of *B. subtilis* spores lacking major protection and prevention mechanisms. Data show a comparison between synthetic air and argon gas LPP. Values were calculated by building a quotient of the mean LD_{90} data for the WT and respective mutant spores.

applied feed gas, indicating this protein makes no contribution to LPP plasma resistance, consistent with this major protein not binding to DNA (1, 18, 36). Identical results were observed by Moeller et al. when investigating resistance to monochromatic UV-C at 254 nm, where α/β-type SASP were found to be major factors, whereas γ-type SASP were much less important (57).

This study investigated the resistance characteristics as well as DNA-repair mechanisms of *B. subtilis* spores upon LPP treatment in a comprehensive way. These characteristics can be divided into three subgroups: prevention, protection, and (DNA) repair. Photons in the UV-C region (254 nm) emitting in different levels using synthetic air (high) and argon (low) LPP caused DNA damage due to the production of RONS. The gas combination of nitrogen and oxygen LPP showed elevated spore inactivation capabilities, even resulting in etching of the outer spore layers and producing reactive species (54). Comparing both gas mixtures shows that synthetic air is more efficient in terms of spore inactivation. Most of the deletion mutants investigated show comparatively good resistance to exposure to lower wavelengths, which are produced to a large extent in argon LPP. A very important component in spore LPP resistance seems

to be DNA-associated SASP. In addition, the results showed that LPP-mediated DNA damage is not repaired by a single repair mechanism, as spores required multiple repair mechanisms including prior removal of photoproducts to properly repair lesions in the genome. To verify plasma sterilization as an innovative sterilization technique, additional studies must be performed to evaluate other plasma gas mixtures and the role of ROS/RNS for other plasma sources where they might be crucial for inactivation.

MATERIALS AND METHODS

Spore production and purification

The *B. subtilis* strains used for this study are listed in Tables 1 and 2 and were chosen for their lack of DNA-repair mechanisms and/or protective structures. Strains lacking DNA-repair systems are isogenic with the WT 168, while strains with mutations in protective components are derived from the PS832 laboratory 168 strain. For production of spores from each strain, 200 µL of an overnight culture were inoculated into 200 mL of double-strength liquid Schaeffer sporulation medium (58), and appropriate antibiotics were added. Inoculated cultures were shaken to give vigorous aeration at 37°C for 72 hours, and spores were harvested in 40 mL of sterile double deionized water ($_{dd}H_2O$). To ensure high purity, spores were washed in multiple steps using $_{dd}H_2O$ and shaking with at least 10 sterile glass beads (d = 3 mm) for ease of resuspension during vortexing and to break up clumps of debris from lysis of mother cells. Pure spores were obtained by repeating the washing procedure until a purity of >99% spores free from cell debris and vegetative cells was achieved, as confirmed by phase contrast microscopy. Pure spore solutions were then stored at 4°C.

Sample preparation

Glass plates with 12 cavities (76 × 60 × 4 mm; pressed glass; Marienfeld Superior—Paul Marienfeld GmbH & Co. KG, Lauda-Königshofen, Germany) were sterilized at 220°C for 4 hours. Each cavity was filled with 25 µL containing 2×10^7 spores/mL of the various strains, allowing spores to be deposited as homogenous monolayers. After air-drying overnight (at least 15 hours) at room temperature (20 ± 2°C), samples were LPP treated.

Plasma setup and sample treatment

The low-pressure plasma system used was a DICP reactor consisting of a cylindrical 25-L stainless-steel vessel. The system is described in detail in reference (26) and has been well characterized with respect to plasma properties and spore inactivation efficiency. The top and bottom of the device are quartz glass making the center visible for the operator. The DICP discharges are driven by two copper coils at the top and the bottom, powered by a matchbox of 5 kW at 13.56 MHz maximum. A system containing a roots pump plus a rotary vane pump is essential to generate a low-pressure environment of at least 5 Pa with gas flows up to 160 sccm (standard cubic centimeters/minute) flow rate. The sample carrier was placed in the middle of the discharge on thin glass holders (Fig. 5). LPP exposure used a power (P) of 500 W and a pressure (p) of 5 Pa for treatment times from 15 to 120 seconds. Samples were also subjected to 120 seconds of vacuum only as a control. Two gas mixtures were used: argon (Ar 100 sccm) and synthetic air containing a mixture of nitrogen and oxygen (N_2:O_2 16:4 sccm).

Spore survival assay

For determination of spores surviving plasma treatment, colony forming units (CFU) of treated samples were calculated. Plasma exposed spores were harvested by covering each well of the glass carrier with 50 µL of a sterile 10% aqueous polyvinyl alcohol (PVA) solution (Merck KGaA, Darmstadt, Germany) and air-drying for 24 hours. The PVA layer

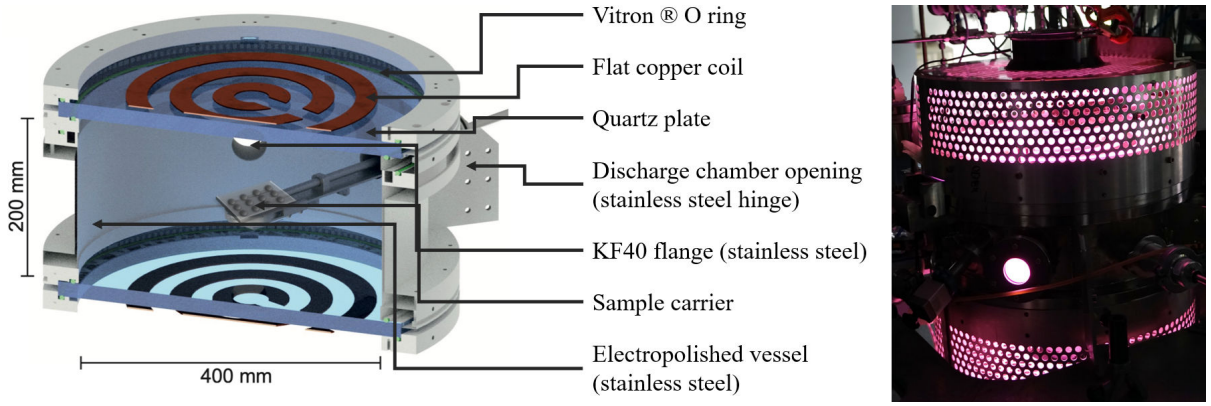

FIG 5 Schematic overview and a picture of the DICP reactor including the sample carrier on the right.

with the adhered treated spores was then peeled off, transferred to a sterile tube, and dissolved in 1 mL sterile distilled water as described (49). To determine CFU, samples were diluted in sterile distilled water, plated on LB media, and incubated overnight at 37°C according to a standardized protocol (58). PVA has been shown to be non-toxic and non-hazardous to bacterial spores, and their germination, outgrowth, and the subsequent growth of vegetative cells are not affected, and recovery rates of >95% viable spores are reported (49, 59)

Numerical and statistical analysis

Spore survival of spores was calculated from dilutions of LPP treated and untreated spores as CFU on LB agar plates after overnight incubation at 37°C. To determine surviving fractions of *B. subtilis* spores, the ratio N_t/N_0 was calculated (N_t = CFU of LPP exposed spores, N_0 = CFU of untreated vacuum control). Dose effect curves were obtained by plotting survival fraction against plasma treatment time (Fig. S1 to S17). Inactivation efficiency was expressed as the LD_{90} (lethal LPP exposure time in seconds) giving killing of 90% of the spore population determined by linear regression of the survival fraction (data from Fig. S1 to S17). Data points are given as average of $n = 3$ with denoted standard deviations.

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Erika Muratov, Investigation, Validation, Writing – original draft | Florian P. Rosenbaum, Investigation, Methodology, Validation, Writing – original draft, Writing – review and editing | Felix M. Fuchs, Investigation, Methodology, Supervision, Writing – review and editing | Nikea J. Ulrich, Investigation, Supervision, Writing – review and editing | Peter Awakowicz, Supervision, Writing – review and editing | Peter Setlow, Visualization, Writing – review and editing | Ralf Moeller, Conceptualization, Investigation, Methodology, Project administration, Supervision, Writing – review and editing

ADDITIONAL FILES

The following material is available online.

Supplemental Material

Fig. S1-S18, Table S1 (AEM01329-23-s0001.pdf). Supplemental figures and table.

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