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REVIEW ARTICLE

Bacterial spore structures and their protective role in biocide resistance

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Summary

The structure and chemical composition of bacterial spores differ considerably from those of vegetative cells. These differences largely account for the unique resistance properties of the spore to environmental stresses, including disinfectants and sterilants, resulting in the emergence of spore-forming bacteria such as *Clostridium difficile* as major hospital pathogens. Although there has been considerable work investigating the mechanisms of action of many sporicidal biocides against *Bacillus subtilis* spores, there is far less information available for other species and particularly for various Clostridia. This paucity of information represents a major gap in our knowledge given the importance of Clostridia as human pathogens. This review considers the main spore structures, highlighting their relevance to spore resistance properties and detailing their chemical composition, with a particular emphasis on the differences between various spore formers. Such information will be vital for the rational design and development of novel sporicidal chemistries with enhanced activity in the future.

Introduction

When cells of certain Gram-positive bacteria, for example *Bacillus* and *Clostridium* spp., encounter environmental stresses such as nutrient starvation, they form a dormant structure termed an endospore (simply referred to as a spore in this review). Bacterial spores can survive in this dormant state for many years (Kennedy 1994), with some studies suggesting that they may even persist for millions of years (Cano and Borucki 1995). Faced with the challenge of surviving prolonged periods of dormancy, spores have evolved many mechanisms to protect themselves from damage, which also serve to protect them from modern disinfection/sterilization procedures (Setlow 2006). It is this highly resistant characteristic that makes them such a problem in the food industry, where *Bacillus cereus* is commonly responsible for food-borne diseases (Bottone 2010), and in healthcare settings where the spore-forming *Clostridium difficile* is a major cause of hospital-acquired diarrhoea (Lyerly *et al.* 1988; Wilcox and Fawley 2000).

It is therefore of interest to investigate how bacterial spores withstand environmental stress, including their ability to resist disinfectants and sterilants. Much of the work on spore resistance to date has centred on spores of *Bacillus subtilis*, owing principally to the ease with which this organism may be genetically manipulated (Nicholson *et al.* 2000; Setlow 2006), as well as the relatively early availability of its complete genome sequence (Kunst *et al.* 1997). This review provides an update on what is known about spore structures, highlighting their detailed composition where known and noting any similarities/differences between *Bacillus* and *Clostridium* spores in particular. Consideration will also be given to any known resistance factors in the spore structure itself.

Spore-former life cycle

The process of sporulation is classically divided into seven stages (Hitchins and Slepecky, 1969; Piggot and Coote 1976; Errington 1993; McDonnell 2007; Fig. 1) and is basically identical for Bacilli and Clostridia, except

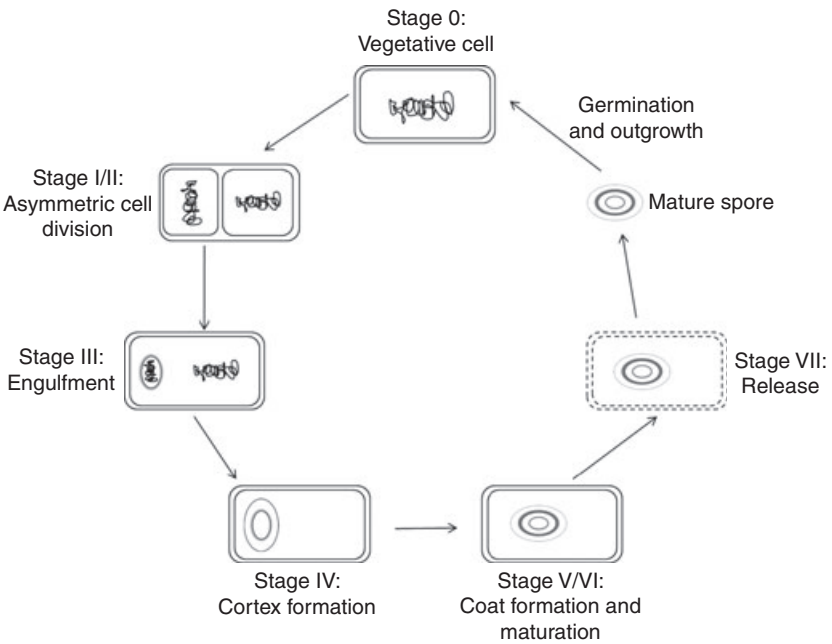

Figure 1 Key morphological changes that take place during sporulation. Modified from McDonnell (2007).

that Clostridia undergo a considerable cell lengthening during sporulation and visible clubbing on development of the forespore (Fitz-James and Young 1969). Normal vegetative cell growth can be defined as stage 0 with regard to sporulation, and is followed by stage I/II, where the vegetative cell undergoes asymmetric cell division, forming two compartments, the smaller of which is termed the prespore, separated by a septum; stage I – presentation of the cell DNA as an axial filament – was originally defined by Ryter (1965), but is generally no longer recognized as a defined stage (Piggot and Coote 1976; Errington 1993). During stage III, the prespore is engulfed by the mother cell to form a distinct cell termed the forespore bound by the inner and outer forespore membranes. Stage IV sees the synthesis of the spore cortex, composed of peptidoglycan (PG), between the inner and outer forespore membranes, which is followed by stage V, spore coat formation. During stages IV and V, the mother cell also synthesizes a very abundant spore-specific molecule, pyridine-2,6-dicarboxylic acid [dipicolinic acid (DPA)]. This accumulates in the forespore and is accompanied by a reduction in the forespore water content. Spore maturation takes place during stage VI, where the coat material becomes denser in appearance. The final stage (VII) sees the lysis of the mother cell and release of the mature spore structure (Figs 1 and 2). The mature spore structure protects the dormant micro-organism from external influences until the conditions once more become favourable for vegetative cell growth. The dormant spore is then re-activated and undergoes germination and outgrowth.

The transition from dormant spore to vegetative cell involves three separate phases: activation, germination and outgrowth. Activation can be triggered by appropriate conditions of heat, pH or chemical exposure and renders the dormant spore poised to enter germination, thus breaking its dormant state (Keynan and Evenchik 1969). Activation is a reversible process that does not necessarily commit the spore to germination and outgrowth, and activated spores retain most properties of the dormant spore (Keynan and Evenchik 1969). In contrast, once a spore is committed to germinate, the spore can no longer return to its dormant state (Gould 1969). Germination can be initiated in response to various stimuli, often varying depending on the species. These include, but are not limited to, metabolizable nutrient germinants, such as specific amino acids and sugars (although these germinants' metabolism is not required for their triggering of germination), nonmetabolizable germinants such as some ionic species, cationic surfactants and chelates (in particular, a Ca:DPA chelate), and some physical treatments such as high pressures (Gould 1969; Setlow 2003). 'Outgrowth' is defined as all developmental events taking place after germination, including initiation of metabolism and macromolecular synthesis, swelling of the spore, emergence (where the outer spore layers are shed) and growth of the new cell, and represents a return of the spore to vegetative cell growth (Strange and Hunter 1969).

Spore structure

The structure (Fig. 2) and chemical composition of the spore differ considerably from those of the vegetative cell. These differences largely account for the unique spore resistance to environmental stresses, including disinfectants and sterilants (Setlow 2006). They are considered in further detail below and, unless stated otherwise, the discussion refers to spores of *B. subtilis*.

Exosporium

The exosporium is the outermost structure of many bacterial spores, in particular those of the *B. cereus* group, which also includes *Bacillus anthracis* and *Bacillus thuringiensis* (Todd *et al.* 2003; Redmond *et al.* 2004), but is also found in some other Bacilli and Clostridia, including the pathogenic *Cl. difficile* (Lawley *et al.* 2009; Permpoonpattana *et al.* 2011). The presence of an exosporium is by no means universal, and this structure may be either absent or greatly reduced in many species, including *B. subtilis* (Waller *et al.* 2004); this has resulted in a lack of information regarding its composition (Todd *et al.* 2003). Based on studies with *B. cereus*, the exosporium is composed principally of protein (43–52% of dry weight), but also contains lipids (15–18% of dry weight) and carbohydrates (20–22% of dry weight), as well as a minor (around 4%) component described as ash, which contained both calcium and magnesium as well as some undetermined components (Matz *et al.* 1970; Beaman *et al.* 1971). The exosporium protein component is notable for its low level, or lack, of the sulfur-containing amino acids cysteine (a prominent component of the spore coat) and methionine, as well as histidine and tyrosine. Of the lipid component, diphosphatidylglycerol (cardiolipin) represented the only detectable phospholipid (~30% of total lipids); the majority were neutral lipids, and there were at least 19 fatty acids, 40% of which were normal C16 and C18 fatty acids. Of the remaining fatty acids, nine were straight chained (seven saturated and two unsaturated), seven were branch chained (four *iso*- and three *anteiso*-), and one was unidentified (Matz *et al.* 1970; Beaman *et al.* 1971). The exosporium polysaccharide component was made up of glucose, glucosamine and rhamnose, with a very small amount of ribose (which was attributed to RNA contamination of exosporium preparations). Whilst the exosporia of clostridial spores have been described (Hodgkiss *et al.* 1967; Mackey and Morris 1972), there is no detailed breakdown of the chemical composition of these structures.

Although a number of major proteins have been identified as components of the *B. cereus* and *B. anthracis* spores' exosporia (Lai *et al.* 2003; Todd *et al.* 2003; Henriques and Moran 2007; Fazzini *et al.* 2010; McPherson *et al.* 2010), their exact function in the spore is unknown. It has been suggested that the adherent, hydrophobic properties of the exosporium may be involved in the pathogenicity of some spores (Koshikawa *et al.* 1989; Bowen *et al.* 2002). However, to the best of our knowledge, the exosporium has not in itself been shown to provide the spore with any significant protection from biocide attack.

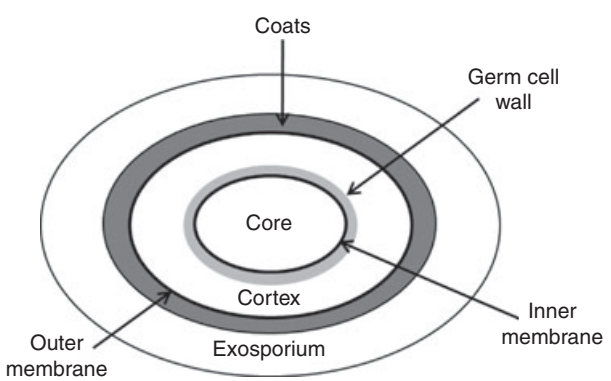

Figure 2 Spore structure. A representation of a 'typical' bacterial spore (structures are not drawn to scale). Modified from Setlow (2006).

Spore coat

The spore coat sits within the exosporium (if present) and generally comprises a series of thin, concentric layers, the numbers of which differ depending on the organism under investigation (Driks 1999). Indeed, the structure as visualized by electron microscopy and the biochemical composition of the spore coat vary between species and even within different strains of the same species (Fitz-James and Young 1959; Kondo and Foster 1967; Kornberg *et al.* 1968). *Bacillus subtilis* spores have two prominent coat layers, the inner and outer spore coats, plus a basement layer between the inner coat and the cortex and an outermost crust (Aronson *et al.* 1992; Driks 1999; Henriques and Moran 2007; McKenney *et al.* 2010). The inner coat is often described as having a lamellar appearance and is less dense when viewed by electron microscopy, whereas the thicker outer coat lacks the clear lamellar structure of the inner coat and appears darker under electron microscopy (Warth *et al.* 1963; Kay and Warren 1968; Aronson and Fitz-James 1976). There are many excellent reviews dealing specifically with the structure and molecular genetic control of coat assembly (Driks 1999; Henriques and Moran 2000, 2007; McKenney and Eichenberger 2012); in this review, we will briefly summarize some of the details of the composition of the spore coat.

The coat is made up predominantly of protein, but also contains minor (6%) carbohydrate components, most likely owing to the glycosylation of two low-molecular-weight coat polypeptides of approximately 8–9 kDa (Pandey and Aronson 1979; Jenkinson *et al.* 1981). The protein fraction of the coat represents 50–80% of the total spore protein (Aronson and Fitz-James 1976; Pandey and Aronson 1979) and can itself be divided into two separate fractions, soluble and insoluble. The soluble fraction accounts for approximately 70% of the total coat protein and may be isolated by treatment with a combination of reducing and denaturing agents at alkaline pH (Goldman and Tipper 1978; Pandey and Aronson 1979). The soluble fraction contains upwards of 40 proteins, as viewed on polyacrylamide gels, ranging from 6 to >70 kDa in size (Driks 1999; Henriques and Moran 2000). Particularly abundant within the soluble fraction is CotG, a hydrophilic protein of 24 kDa that is thought to be morphogenetic and when deleted prevents the incorporation of another protein, CotB, into the mature spore coat (Sacco *et al.* 1995). Molecular genetic manipulation in the *B. subtilis* model organism has allowed the identification and investigation of other coat proteins from the soluble fraction, including CotA, CotB, CotC and CotD (Donovan *et al.* 1987). Unlike CotG, these proteins can be deleted without any major detrimental effects to the mature spore. However, spores lacking CotD germinated more slowly than wild-type spores, and loss of CotA resulted in the loss of the wild-type brown colour (Donovan *et al.* 1987).

Approximately 30% of isolated coat proteins resisted solubilization and define the coat insoluble fraction (Pandey and Aronson 1979). This fraction is characterized by a high cysteine content, which likely contributes to its insoluble nature because of the formation of disulfide cross-links (Goldman and Tipper 1978; Pandey and Aronson 1979). Evidence for the presence and function of such disulfide cross-links in the spore coat is given by Gould and Hitchins (1963) and Gould *et al.* (1970), who showed that spores became sensitive to hydrogen peroxide and lysozyme following treatment with various chemical disruptors of disulfide bonds. Other types of cross-linking, including dityrosine (Pandey and Aronson 1979) and ε-(γ-glutamyl)lysine (Kobayashi *et al.* 1996) cross-links, have also been detected in the spore coat. The presence of heavily cross-linked material in the spore coat is likely responsible for some of the spore's chemical and mechanical resistance (Wold 1981), as is the case in other biological structures such as the sea urchin egg, which is surrounded shortly after fertilization by a rigid envelope containing dityrosine cross-links to protect the developing embryo (Shapiro 1991).

Of the 70 or so coat proteins identified in *B. subtilis* spores (Kim *et al.* 2006; Henriques and Moran 2007), at least 50 are also shared with its close relatives, *B. cereus* and *B. anthracis* (Kuwana *et al.* 2002; Lai *et al.* 2003; Liu *et al.* 2004; Giorno *et al.* 2007). Whilst little is known about the structure and composition of the clostridial spore coat, only about 20 of the *B. subtilis* coat proteins have orthologs in the clostridial genomes currently available (Henriques and Moran 2007). It would be interesting to discover whether this discrepancy in the number of known coat proteins is indicative of a simpler coat in these Clostridia, or perhaps the presence of unique coat proteins in the clostridial coat. In a recent study, it was demonstrated that there was no cross-reactivity between antispore serum from *Cl. difficile* 630 and *B. subtilis* spore coat proteins, suggesting significant differences between the coats of these two species (Permpoonpattana *et al.* 2011) and adding weight to the bioinformatic observations of Henriques and Moran (2007) outlined earlier. Such species-specific differences in coat composition could impact upon the biocidal formulation required to rapidly overcome the defence provided by the spore coat.

Functionally, the spore coat serves as an initial barrier to large molecules, such as the PG-lytic enzyme lysozyme, which would otherwise have access to the spore cortex (Nicholson *et al.* 2000). Probably for this reason, the coat is essential for spore resistance to predation by bacteriovores (Klobutcher *et al.* 2006; Laaberki and Dworkin 2008). In contrast to the coat's impermeability to lysozyme, smaller molecules such as spore germinants must presumably pass through this barrier (Driks 1999). The spore coat has also been identified as a critical resistance mechanism against many chemicals, especially oxidizing agents such as hydrogen peroxide (Riesenman and Nicholson 2000; Young and Setlow 2004b), ozone (Young and Setlow 2004a), peroxynitrite (Genest *et al.* 2002), chlorine dioxide and hypochlorite (Young and Setlow 2003; Ghosh *et al.* 2008), all of which kill spores more rapidly when the coat layer is absent. This protective role was perhaps most clearly illustrated by Ghosh *et al.* (2008), who showed that *B. subtilis* spores lacking most coat layers owing to mutations in the *cotE* and *gerE* genes (coding for a morphogenetic protein essential for formation of the outer coat, and a DNA-binding protein that itself regulates several genes coding for coat proteins (Driks 1999), respectively) became sensitive to hypochlorite to a level similar to that of vegetative cells. Despite the clear protective role of the spore coat, and an increasingly detailed understanding of the mechanisms, components and genetic controls involved in spore coat assembly (Driks 1999; Takamatsu and Watabe 2002; Henriques and Moran 2007; McKenney and Eichenberger 2012), no individual coat proteins have been identified as an essential protective component. The coat may simply be serving to detoxify these chemicals before

they penetrate the inner regions of the spore structure, such as the inner membrane and the core (Nicholson *et al.* 2000; Riesenman and Nicholson 2000; Setlow 2006). It has also been suggested that the spore coat is unable to protect spores from some toxic chemicals, for example low-molecular-weight alkylating agents (Setlow *et al.* 1998), which apparently are small enough to bypass the coat's molecular sieving effect and gain access to their target site in the spore core.

It has been suggested that superoxide dismutase (SOD), an enzyme associated with the exosporium or spore coat of *B. subtilis* and *B. anthracis* and thought to be involved in the formation of the spore coat (Henriques *et al.* 1998), may also serve to detoxify potentially damaging chemicals at the spore surface, as is the case for some vegetative cells (Nicholson *et al.* 2000; Setlow 2006). Whilst such a protective role was not found in *B. subtilis* (Casillas-Martinez and Setlow 1997), it has been shown in *B. anthracis* (Cybulski *et al.* 2009), where SODs present on the surface of the spore protect against oxidative stress and increase spore pathogenicity within the host lung. Other coat proteins have been shown to possess enzymatic activity, such as CotE of *Cl. difficile* 630. This bifunctional protein shows both peroxiredoxin and chitinase activity, which may be associated with the characteristic inflammation associated with infection by this organism (Permpoonpattana *et al.* 2011).

Outer membrane

Under the spore coat lies the outer spore membrane. Whilst this structure is essential for spore formation (Piggot and Hilbert 2004), its precise function remains unclear, reportedly having no great effect on resistance to radiation, heat or some chemicals (Nicholson *et al.* 2000; Setlow *et al.* 2000). There is some confusion as to whether the outer membrane, which is morphologically distinct during sporulation, actually serves as an intact membrane in the mature spore (Racine and Vary 1980), and there are no reports of the isolation of a purified outer membrane in the literature. It is difficult to identify the outer membrane in electron micrographs following synthesis and maturation of the coat and cortex, and dormant spores from several species are reported to have poorly defined or indistinguishable outer membranes (Freer and Levinson 1967; Fitz-James 1971; Holt *et al.* 1975; Aronson and Fitz-James 1976). Despite the lack of conclusive morphological evidence for the presence of an outer spore membrane, there is functional and biochemical evidence to support the presence of such a structure in the mature spore. For instance, there is evidence that 11 spore coat proteins are related antigenically to membrane proteins from vegetative cells (Fujita *et al.* 1989; as cited in Henriques and Moran 2000). Crafts-Lighty and Ellar (1980) identified cytochromes and enzymes of the electron transport chain in extracts of spore outer integuments (cortex, coats and any outer membrane structure), both of which imply the presence of a membranous element, and which were not because of contamination by inner-membrane fractions. Further evidence in support of an outer spore membrane was presented by Rode *et al.* (1962), who identified a sharply delineated permeability barrier between the cortex and coats of *Bacillus megaterium* spores that prevented the uptake of methacrylate. This barrier was disrupted following spore fixation using potassium permanganate ($KMnO_4$). It has also been shown that glucose will only permeate as far as the cortex of dormant spores (Gerhardt *et al.* 1982), again suggesting the presence of a functioning membrane at this point in the spore. If present, this membrane does not prevent the uptake of the small uncharged, lipophilic molecule, methylamine (Setlow and Setlow 1980; Swerdlow *et al.* 1981), and presumably does not hinder the passage of germinants, which must penetrate as far as their receptors in the inner membrane. Further study regarding the presence, functionality and in particular the permeability properties of the outer spore membrane would therefore be of interest with regard to spore resistance and susceptibility to biocides and also with regard to permeability to germinants.

Cortex and germ cell wall

The spore cortex is composed of PG that, whilst broadly similar to vegetative cell PG, has some notable spore-specific modifications, notably the complete absence of teichoic acids from the *N*-acetylmuramic acid (NAM) residues in spore PG (Atrih *et al.* 1996).

Vegetative cell PG from *B. subtilis* cell walls consists of glycan chains of alternating *N*-acetlyglucosamine (NAG) and NAM residues (Warth and Strominger 1971). Approximately 40% of the NAM residues in vegetative cell PG are cross-linked to other glycan strands via their peptide side chains (Warth and Strominger 1971; Popham and Setlow 1993), whilst around 2% are complexed with teichoic acids (Atrih *et al.* 1999a).

In spore cortex PG, approximately 50% of the NAM residues present have no peptide side chains and instead are cyclized to form the spore-specific residue, muramic-δ-lactam (M-L; Fig. 3), whilst a further 25% of NAM residues have only an L-alanine side chain (Warth and Strominger 1969, 1972). Both of these NAM modifications preclude the formation of peptide cross-links between glycan strands (Fig. 3); indeed, only around 3% of spore NAM residues contain peptide side chains that are cross-linked (Popham *et al.* 1996).

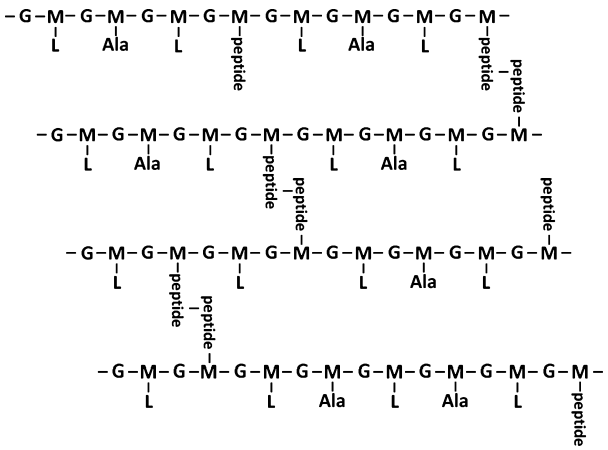

Figure 3 Schematic representations of spore peptidoglycan structure. G, *N*-acetylglucosamine; M, *N*-acetyl-muramic acid; M-L, muramic-δ-lactam; Ala, L-alanine; peptide, tri- or tetrapeptide side chains that can form cross-links between glycan strands. Modified from Popham (2002).

It has been speculated that M-L, being a structure unique to spore cortex PG, was in some way important in attaining spore dormancy and/or resistance properties (Popham 2002). However, mutants lacking a functional *cwlD* gene that encodes an autolysin of the *N*-acetylmuramoyl-L-alanine amidase class (Sekiguchi *et al.* 1995; Popham *et al.* 1996) produce cortex PG that lacks M-L, and yet *cwlD* spores maintain full spore dormancy and have normal heat resistance (Atrih *et al.* 1996; Popham *et al.* 1996), although they cannot complete germination and outgrowth. The low level of cross-linking in spore PG has also been identified as a possible mechanism responsible for attaining and maintaining maximum core dehydration, a hypothesis referred to as the contractile cortex concept (Lewis *et al.* 1960). More recent studies have demonstrated that the level of cross-linking in spore PG does not alter spore dehydration (Popham 2002).

The PG from spores of other organisms, including *B. megaterium, B. cereus, Bacillus sphaericus* (now *Lysinibacillus sphaericus*), *Bacillus stearothermophilus* (now *Geobacillus stearothermophilus*), *Clostridium botulinum* and *Clostridium sporogenes* (Warth and Strominger 1969; Atrih *et al.* 1999b; Atrih and Foster 2001), has also been analysed in some detail and was in all cases very similar to that of *B. subtilis*. The only subtle difference noted was the de-N-acetylation of an amino sugar, most likely the glucosamine, in *B. cereus*, *B. sphaericus* and *Cl. botulinum*, which was not present in *B. subtilis* (Atrih and Foster 2001).

Bacterial spores contain another PG structure, the germ cell wall (GCW), which becomes the cell wall as the spore undergoes germination and outgrowth. Structural differences between GCW and cortex PG, in particular the absence of M-L, allow the selective degradation of the spore cortex, but not the GCW during spore germination; specifically, the M-L in cortex PG is a key substrate specificity determinant for recognition by cortex-lytic enzymes during spore germination (Atrih *et al.* 1998). There is currently no indication that the GCW plays any great part in spore resistance properties.

Cortex-less mutants of spore-forming bacteria have been produced, for example *spoVD* and *spoVE* mutants in *B. subtilis*, which apparently lack any/most of the cortex (Piggot and Coote 1976; Daniel *et al.* 1994). However, the resistance properties of these mutant spores have not been studied. Imae and Strominger (1976) used a conditional cortexless mutant of *B. sphaericus* in which the amount of cortex present was alterable by changing the level of meso-diaminopimelic acid in the growth medium to show that a critical mass of cortex was required for resistance to xylene, octanol and heat. However, owing to the complex nature of spore development, they were unable to attribute resistance specifically to the cortex itself.

Inner membrane

Several studies have demonstrated that the dormant spore is remarkably impermeable, as small molecules such as the uncharged lipophilic molecule methylamine and even water permeate into the spore core only slowly (Setlow and Setlow 1980; Swerdlow *et al.* 1981; Sunde *et al.* 2009). This characteristic has led to the suggestion that the spore inner membrane must differ significantly from the vegetative cell plasma membrane, and this may be responsible for the low spore inner-membrane permeability. However, the lipid composition of the spore's inner membrane appears very similar to that of the vegetative cell plasma membrane in both *B. megaterium* where both membranes contain principally phosphatidylglycerol, diphosphatidylglycerol (cardiolipin), phosphatidylethanolamine and glucosaminylphosphatidylglycerol (Bertsch *et al.* 1969; Scandella and Kornberg 1969; Racine and Vary 1980), and *B. subtilis* where both membranes contain primarily phosphatidylglycerol, cardiolipin and phosphatidylethanolamine, although vegetative cell membranes contain much more diglucosyl diacylglycerol (Griffiths and Setlow 2009). In contrast, the vegetative cell membrane and spore inner membrane have very different protein compositions, in particular as the spore's inner membrane contains germinant receptors and SpoVA proteins not found in vegetative cells (Setlow 2003). However, the precise composition of the spore inner membrane does not provide any obvious reason for this membrane's low permeability.

Biophysical analysis of the inner membrane in intact spores (Cowan *et al.* 2004) has further suggested that it is not the lipid content of the inner membrane that confers its remarkable impermeability, but the state of these lipids in the membrane. By incorporating fluorescent lipid probes into the membranes of dormant spores of *B. megaterium* and *B. subtilis*, it was demonstrated that lipids located in the inner spore membrane were largely immobile but became mobile upon spore germination, when permeation into the spore core becomes rapid (Cowan *et al.* 2004). This led the authors to speculate that lipid immobility and the low permeability of the inner membrane may be due to compression of the inner membrane in some fashion, perhaps by the spore cortex, and thus maintaining the inner membrane at a state 1·3- to 1·6-fold more compressed than that of the plasma membrane of the germinated spore, in which the cortex has been degraded. The authors also noted other possibilities for reduced lipid mobility, such as the low water content of the spore core (potentially reducing mobility of lipids in the inner leaflet of the spore membrane), or that the membrane contains distinct lipid domains of differing lipid mobility (Cowan *et al.* 2004).

Several oxidizing agents are known to increase the permeability of the spore to external chemicals, including methylamine and various dyes and stains, as well as to potentiate the release of the core's large DPA depot (Loshon *et al.* 2001; Genest *et al.* 2002; Young and Setlow 2003; Cortezzo *et al.* 2004; Cortezzo and Setlow 2005) implying that the mechanism of spore killing by these oxidizing agents involves damage to the inner membrane. However, the exact damage to the inner membrane remains unknown.

Mutant spores having very different levels of unsaturated fatty acids in their inner membranes compared with WT spores showed essentially identical resistance profiles when treated with several different oxidizing agents, suggesting that oxidation of unsaturated fatty acids within the inner membrane was not the cause of membrane damage/spore killing by oxidizing agents (Cortezzo *et al.* 2004; Cortezzo and Setlow 2005). Similarly, the inner membrane's phospholipid composition appears not to have a great effect on either the permeability or the resistance properties (at least for NaOCl and the disinfectant product Oxone, which contains potassium peroxymonosulphate as the active component) of the inner membrane, as spores lacking some of the major membrane phospholipids showed very similar resistance profiles and also no difference in their permeability as measured by the uptake of methylamine (Griffiths and Setlow 2009). It should be noted that spores lacking cardiolipin from their membranes were sensitized to treatment with 5% liquid hydrogen peroxide (H_2O_2), an oxidizing agent that apparently does not affect the integrity of the inner membrane in wild-type spores when used at this concentration (Melly *et al.* 2002a), suggesting a specific role of this phospholipid in preventing access of H_2O_2 to the core (Griffiths and Setlow 2009). Another possible target for oxidizing agents within the inner membrane could also be the various protein constituents of the membrane, such as the germinant receptors GerA, GerB and GerK, as well as SpoVA proteins that are putative DPA channel proteins, all of which are thought to reside within the inner membrane (Setlow 2003; Vepachedu and Setlow 2005) and therefore could conceivably compromise the integrity of the membrane if altered. However, the interaction of oxidizing agents with cell proteins is complex and might differ profoundly between oxidizers (Finnegan *et al.* 2010).

The spore core

At the centre of the spore lies the core, which contains the spores' DNA, RNA, ribosomes and most of its enzymes (Setlow 2006, 2007). The core is relatively dehydrated, water making up only 28–57% of spores' wet weight, a factor that is thought to contribute to both spores' enzymatic dormancy and their characteristic resistance to heat and some chemicals (Beaman and Gerhardt 1986; Paidhungat *et al.* 2000; Cowan *et al.* 2003; Sunde *et al.* 2009). The core also contains high levels (*c.* 5–15% of core dry weight) of the small molecule DPA, which exists as a 1 : 1 chelate with divalent cations, predominantly Ca^{2+} (Huang *et al.* 2007). The conditions within the core are strongly linked to the resistance properties of the spore, many of which are in some way involved in protecting spore DNA from damage.

Core water, dipicolinic acid and mineral content

Core water content is the major determining factor of a spore's wet heat resistance. Generally, the lower the core water content, the higher the wet heat resistance (Nicholson *et al.* 2000; Setlow 2006). Whilst the precise target of wet heat within the spore is not known, it has been suggested that one or more proteins in the spore core are the likely targets (Setlow *et al.* 2000; Coleman *et al.* 2007). Melly *et al.* (2002b) suggested that core proteins in a more highly dehydrated spore core have greater resistance to wet heat, presumably as a result of reduced molecular motion (Setlow 2006). Killing of spores by liquid hydrogen peroxide is also affected by core water content, with higher core water levels being associated with greater sensitivity to peroxide, although the reason for such a relationship is unclear (Popham *et al.* 1995). The relationship between high spore water content and

decreased resistance to wet heat and some chemical treatments (nitrous acid, but apparently not hydrogen peroxide) has also been demonstrated for spores of *Clostridium perfringens* (Paredes-Sabja *et al.* 2008b,c).

Spore core water content can be varied in several ways, including by the variation of the sporulation temperature, higher temperatures generally leading to a lower core water content (Melly *et al.* 2002b; Setlow 2006), or using strains that lack the ability to produce DPA. Spores of strains lacking the ability to synthesize DPA have far higher water content in their core, although this can be lowered to near wild-type levels if DPA is present in the sporulation medium (Paidhungat *et al.* 2000). That the spore resistance profile can be altered considerably according to the conditions under which spores are prepared should be considered carefully when choosing conditions for the preparation of spores for monitoring sterilization conditions (Melly *et al.* 2002b).

Core mineralization also confers some level of wet heat resistance to the spore, and in general, higher core mineralization gives higher wet heat resistance (Nicholson *et al.* 2000). Whilst some of this resistance may be due to decreased core water content associated with higher mineralization (water content affecting resistance as outlined earlier), it has been observed that different mineral ions confer differing levels of wet heat protection, with Ca^{2+} providing greater protection than other divalent (Mg^{2+} and Mn^{2+}) or monovalent (K^+ or Na^+) cations (Slepecky and Foster 1959; Bender and Marquis 1985; Beaman and Gerhardt 1986).

DPA may itself play a role in resistance to wet heat, as suggested by Mishiro and Ochi (1966) who found that 0·05% solution of DPA served to protect human serum albumin from heat denaturation, which could therefore hint at a protective role within the spore, although this is yet to be demonstrated experimentally. DPA complexed with Mn^{2+} or Ca^{2+} has also been shown to protect proteins from ionizing radiation *in vitro* (Granger *et al.* 2011), which again may serve the same protective role *in vivo*. Conversely, DPA actually increases the sensitivity of spores to UV radiation, as demonstrated in spores that lack DPA (Setlow and Setlow 1993b; Paidhungat *et al.* 2000).

Small acid-soluble spore proteins and DNA damage/repair

There are two principal methods of minimizing the effect of DNA damage to the spore: (i) preventing DNA damage in the first place and (ii) the rapid repair of any DNA damage during spore outgrowth (Setlow 1995).

Small acid-soluble spore proteins (SASPs) are a group of very abundant small proteins found exclusively in spores. They are synthesized late in sporulation only in the developing spore and are degraded early during germination, providing a vital source of free amino acids for the outgrowing spore (Setlow 1988, 1995). SASPs are common to spores of all *Bacillus* and *Clostridium* spp. and come in two main types, the α/β-type and the γ-type SASP; the γ-type SASPs are apparently absent from the Clostridia and some members of the order *Bacillales* and play no known role in spore resistance (Setlow and Waites 1976; Granum *et al.* 1987; Raju *et al.* 2006; Vyas *et al.* 2011). The α/β-type SASPs are small proteins generally with molecular weights of 6–9 kDa, and the major α/β-type SASPs contain a large percentage of hydrophobic amino acids. The α/β-type SASPs bind directly to and saturate spore DNA, providing an important component of spore resistance against chemical and other treatments that target spore DNA.

It has been demonstrated *in vitro* that α/β-type SASPs protect DNA from chemical attack by hydrogen peroxide, most likely by directly shielding the DNA strand, in particular the DNA backbone, from hydroxyl radical attack. This is supported by additional resistance to DNase and several restriction enzymes, relative to un-protected DNA (Setlow *et al.* 1992). Spores are highly resistant to chemical attack by liquid hydrogen peroxide despite its ability to damage the DNA of vegetative cells (Imlay and Linn 1988). This appears to be due to the protective role of the α/β-type SASPs, as demonstrated in *B. subtilis* mutants lacking the α/β-type SASPs that became considerably more sensitive to this biocide (Setlow and Setlow 1993a; Setlow *et al.* 2000). Similarly, α/β-type SASPs also protect spore DNA from damage by wet heat, and spores lacking the major α/β-type SASPs are more sensitive to wet heat; this is also true with spores of *Cl. perfringens* (Setlow 1995; Setlow *et al.* 2000; Raju *et al.* 2006; Leyva-Illades *et al.* 2007). The α/β-type SASPs also protect spores of both *B. subtilis* and *Cl. perfringens* against some potentially DNA-damaging chemicals, including nitrous acid and formaldehyde (Loshon *et al.* 1999; Tennen *et al.* 2000; Paredes-Sabja *et al.* 2008a). However, the effects of other DNA-damaging chemicals, such as the alkylating agents ethyl methanesulphonate and ethylene oxide that kill spores, at least in part, by DNA damage, are not affected by the presence of α/β-type SASP in the spore core (Setlow *et al.* 1998; Loshon *et al.* 1999). DNA damage *in vitro* by these alkylating agents is also not blocked by α/β-type SASP binding (Setlow *et al.* 1992, 1998).

The α/β-type SASPs play only a minor role in gamma radiation resistance (Hackett and Setlow 1988; Moeller *et al.* 2008), but are involved in protection from UV. This appears to be due to a conformational change in DNA structure following α/β-type SASP binding that favours the formation of a specific DNA defect termed the spore

photoproduct (5-thyminyl-5,6-dihydrothymine, which is readily repaired in WT spores – see below) and suppresses formation of other photoproducts such as cyclobutane dimers and (6-4)-photoproducts (Setlow and Setlow 1993a; Setlow 1995, 2001; Lee *et al.* 2008).

Owing to the dormancy of the mature spore, any damage sustained to their DNA during this period needs to be rapidly repaired during outgrowth if the spore is to survive. Consequently, DNA repair is an important spore resistance mechanism. Spore DNA damage can be repaired by at least three mechanisms. The first, the spore photoproduct lyase, is specific to the spore photoproduct and this enzyme is made only in the developing spore. The other two are recombination and excision repair and are RecA dependent and also can require some spore-specific repair proteins (Salas-Pacheco *et al.* 2005; Setlow 2006; Moeller *et al.* 2008). Spores lacking RecA and/or spore DNA repair proteins are considerably more sensitive to DNA-damaging treatments such as UV radiation, dry heat and some chemicals (Setlow *et al.* 1998; Loshon *et al.* 1999; Tennen *et al.* 2000; Salas-Pacheco *et al.* 2005; Setlow 2006).

Conclusions and future perspectives

Spores are a unique dormant form of many types of bacteria, which develop through a remarkable series of stages to render the parent cell naturally resistant to harsh environmental conditions. Spores are also known to demonstrate the greatest resistance to various disinfection and sterilization methods compared with other micro-organisms (but excluding prions) and are widely used to develop, study and test sterilization methods in particular. Their resistance is clearly due to the cumulative effects of structural, chemical and biochemical features. Even those structures such as the spore cortex that at first glance may appear unimportant to spore resistance can play a functional role. For example, the cortex exerts its influence on the inner layers of the spore, apparently affecting spore resistance indirectly, for example by assisting in the establishment and maintenance of core dehydration and possibly also by influencing the permeability of the inner membrane.

Although there has been considerable work investigating the mechanisms of action of many sporicidal biocides on *B. subtilis* spores, there is far less information available for other species and particularly for various Clostridia (Setlow 2006). This paucity of information represents a major gap in our knowledge given the importance of the Clostridia as human pathogens (Lyerly *et al.* 1988; Hatheway 1990; Samore 1999; Wilcox and Fawley 2000). A useful starting point in investigating the resistance mechanisms of clostridial spores is to employ comparative genomic techniques to identify orthologous genes encoding spore structures known to be related to resistance in *B. subtilis* spores, as has been done previously for comparisons of spore coat proteins (Henriques and Moran 2007) and germination proteins (Paredes-Sabja *et al.* 2011). Following their identification, these genes and the protective effect of their protein products can be investigated by mutagenesis of the host organism, as successfully shown for various spore structures in *B. subtilis*. Historically, genetic manipulation of the Clostridia has been difficult, although various methods are now available, including random transposon-mediated mutagenesis (Hussain *et al.* 2005, 2010) and the site-specific ClosTron mutagenesis system (Heap *et al.* 2007, 2010). In addition to such genetic manipulations, it is also possible to alter various spore structures and/or conditions by changing the conditions in which sporulation takes place (Melly *et al.* 2002b) or by removing the outer spore structures by chemical treatment (Russell 1990). Employing a combination of these techniques will help advance our understanding of the mechanisms of action of sporicidal chemicals against *Clostridium* spores and assist in the rational design and development of novel sporicidal chemistries with activity against clostridial pathogens such as *Cl. difficile*.

More generally, despite the accumulation of knowledge on spore structure and chemical composition, it is remarkable that to date, there is still no effective chemical biocide formulation that is able to destroy the spore within a minute without also affecting drastically the surface intended to be disinfected or without significant toxicity (Maillard 2011). A greater understanding of the structure and resistance factors in various spore-forming bacteria is thus necessary for the development of optimized methods (chemical and/or physical) to inactivate these unique structures.

Conflict of interest

None to declare.

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