Bacterial outer membrane and cell wall penetration and cell destruction by polluting chemical agents and physical conditions

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In the environment, bacteria and other microorganisms are subjected to a variety of constantly changing chemical and physical agencies. Chemical ones include antimicrobial compounds (both biocides and antibiotics), pollutants, drugs, cosmetic and pharmaceutical ingredients and pesticides. The physical agents include desiccation and drying, osmotic pressure, hydrostatic pressure, temperature and pH changes and radiations (ultraviolet, sunlight, ionizing). Bacteria must thus adapt to survive these inimicable conditions. Organisms such as bacterial spores usually survive, whereas other types of microorganisms may be much more susceptible.

Depending on the type of organism, the bacterial cell wall, outer membrane or the spore outer layers may act as permeability barriers to the intracellular uptake of antibiotics and biocides. Some antibacterial agents interact with, and damage or modify, the outer components. Physical agencies are known to damage the cytoplasmic membrane or to produce alterations in DNA or proteins or enzymes. Nevertheless, significant damage to the cell wall or outer membrane may also occur.

Four types of organisms are considered: cocci, mycobactria, Gram-negative bacteria and bacterial spores. The nature of the damage inflicted on, or in some cases prevented by, their outer cell layers is discussed for each type of organism.

Keywords: biocides, chemical pollutants, physical processes, outer cell damage

Introduction

Several chemical and physical agents are known to inhibit the growth of, or to inactivate, microorganisms. Chemical agents include bio-

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cides (antiseptics, disinfectants and preservatives) and antibiotics (Fig.1), whereas important physical agencies include high and low temperatures, desiccation, radiations (including sunlight) and some gaseous environments.[1]

The nature of the microbial responses to these agents depends on the environmental conditions and the type of microorganism itself. Such aspects have been described for thermal injury[2] and biocides[3,4] and will be explored further during the course of this paper.

A constantly changing environment in terms of temperature, humidity, sunlight and the degree of pollution by chemical agents can be envisaged.[5,6] 'Pollutants' are usually thought of as being pesticides, chemicals with toxic and/or carcinogenic properties, or industrial chemicals that persist for varying periods of time in the atmosphere, rivers or lakes.[7] Other bioactive materials that can act as potential environmental pollutants include antibiotics,[8] pharmaceuticals and the ingredients of personal care products (PPCPs).[9] All of these could have effects on bacteria and other microorganisms thereby altering the 'normal' microbial flora. Furthermore, there is a possible association between the ingredients of pharmaceutical products and antibiotic resistance [10] and the enhancement of antibiotic resistance development by residual levels of pesticides.[11]

Some organisms are capable of surviving for long periods in the environment.[12-15] Some are associated with droplet nuclei and may be carried for long distances. In many cases, aerial transmission of infection is possible. The outer cell layers may be damaged by some harmful agencies, but in other cases they may serve to protect the underlying cellular structures from significant damage.

The role of these outer bacterial layers in relation to inactivation by, or insusceptibility to, chemical pollutants and physical conditions will be discussed. It is clearly impossible to consider all types of microbes and thus only four major groups of bacteria will be examined, namely bacterial spores, mycobacteria, other Gram-positive bacteria (predominantly staphylococci) and Gram-negative bacteria.

The Bacterial Surface

The surface of a bacterial cell is not a chemical constant. It differs not only between organisms of different types but also within a species when subjected to different environmental stresses. The surface components of the different types of bacteria considered in this paper are summarised in Table 1.

Table 1 *Surface structures of bacterial cells*

Cellular type	Component*	Composition
Cocci	Capsule	Polysaccharides
	Cell wall	Peptidoglycan, teichoic acids
Mycobacteria	Cell wall	Lipids, mycolic acid, arabinogalactan, peptidoglycan
Gram-negative	Outer membrane/cell wall	LPS, lipoprotein, protein, phospolipid, peptidoglycan
Bacterial spores	Outer spore coat	Proteins (alkali-resistant)
	Inner spore coat	Proteins (alkali-soluble)
	Cortex	Spore-specific peptidoglycan

Cell wall of staphylococci and other Gram-positive bacteria

The cell wall of staphylococci and other Gram-positive bacteria has been widely studied.[16-18] It consists essentially of highly cross-linked peptidoglycan, which can provide about 90% of the wall structure, together with 'secondary' wall polymers (teichoic acids, polysaccharides and proteins), which are covalently linked to peptidoglycan. The teichoic acids are major cell wall components of most Gram-positive bacteria.[18] Mostly, they are polymers of ribitol or glycerol-phosphates attached to glycosyl and D-alanine ester residues.

The peptidoglycan is made up of amino sugars (*N*-acetylglucosamine and *N*-acetylmuramic acid) and various amino acids, some of which are in the unnatural D-form. The peptidoglycan and associated anionic polymers permit the entry of large molecular weight polymers.[16] Under normal circumstances, therefore, it is unlikely that the staphylococcal cell wall acts as a barrier to the uptake of antibiotics and biocides. Unlike Gram-negative cells, there is no periplasm.

Capsular polysaccharides (serotype 5 and 8) predominate among clinical isolates of *S. aureus.*[19]

Cell wall of mycobacteria

The cell wall of mycobacteria is a highly complex structure and differs considerably from that of other Gram-positive bacteria.[20] It is made up essentially of a basal inner layer of peptidoglycan covalently linked to arabinogalactan (a polysaccharide copolymer of arabinose and galactose) which is esterified with mycolic acids, and lipids, to present a highly hydrophobic structure. Free lipids account for some 25–30% of the weight of the cell walls. There are, however, porin protein channels through which nutrients can diffuse.[21-23]

The very nature of the cell wall means that it can act as a very efficient permeability barrier to the intracellular uptake of many biocides and antibiotics,[24,25] as discussed below.

Outer membrane of Gram-negative bacteria

In Gram-negative bacteria, the periplasm is located between the inner membrane and the outer membrane (OM). It communicates with the external environment through the OM proteins. The OM differs markedly from the cell walls of staphylococci. Not only does it provide a permeability barrier to the entry of hydrophobic compounds and higher molecular weight hydrophilic ones, but it also has other uses. The OM surrounds the peptidoglycan, which makes up only about 10% of the cell wall and which is less extensively cross-linked than in staphylococci. It consists essentially of lipopolysaccharide (LPS), proteins and phospolipids. The latter is made up of phosphatidylethanolamine, phosphatidylglycerol and diphosphatidylglycerol. Proteins in *E. coli* are found in four distinct locations, namely the outer and inner membranes and the aqueous environments, cytoplasm and periplasm.[26] The β-barrel proteins are synthesized in the cytoplasm and after translocation they probably pass through the periplasm in soluble form before localizing in the OM.[26] Many of them, such as LamB and OmpF, act as porins through which solutes can diffuse into the cell.[27] Periplasmic and OM proteins are considered to be the first targets of potentially harmful changes that affect membrane integrity and periplasmic function.[28] In the periplasm, these proteins fold, thus creating a high demand for protein-folding catalysts and chaperones in this compartment.[28,29] Misfolding or unfolding results from environmental stress or spontaneous mutation. Chaperones and proteases are specialized proteins that repair or remove unfolded polypepeptides.,[26,29] and some of the most prominent ones are heat-shock proteins that are overexpressed at high temperatures or other stress conditions.[29]

The LPS consists of lipid A, a core polysaccharide in which the sugars are linked to lipid A by 2-ketodeoxyoctanoate (KDO), and a non-specific side-chain.[30] The LPS molecules are linked together by divalent cations, especially Mg^{2+} and Ca^{2+}, to form a stable surface on the OM. Alterations in LPS, from smooth to rough or deep rough, can effect the susceptibility of Gram-negative bacteria to many antibiotics and biocides. The LPS is of importance for two reasons: first, it participates in the physiological membrane functions, contributing to low membrane permeability; second, it is a target for polycationic antibacterial agents.[30]

The most abundant protein is lipoprotein, which is involved in the attachment of the OM to peptidoglycan. Other major OM proteins are the specific and non-specific porins, which have been comprehensively studied in *Pseudomonas aeruginosa,* an organism that is particularly resistant to many antibiotics and biocides. These porin proteins include OprB, OprC, OprD. OprE and OprF (the major porin and structural protein) expressed by *oprB, oprC, oprD, oprE* and *oprF*, respectively. OprF exhibits homology with the OmpA protein from *Escherichia coli*, but whereas OprF permits the diffusion of much larger molecules, the actual rate of diffusion is two orders of magnitude less. In essence, the overall exclusion limit in *P. aeruginosa* is >400 daltons as opposed to >about 600–650 in *E. coli.* Other important proteins in *P. aeruginosa* are the efflux proteins OprJ, OprM and OprN, expressed by *oprJ, oprM* and *oprN*, respectively. These OM proteins constitute the OM factor of the multidrug efflux pumps MexAB-OprM, MexCD-OprJ and MexEF-OprN, which are involved in the removal of many different types of harmful molecules from the cells, including antibiotics, biocides and organic solvents.[31–35]

Surface layers of bacterial spores

A 'typical' bacterial spore consists of an outer (OSC) and inner spore coat (ISC), cortex, germ cell wall and core.[36–39] An outer (OM) and inner membrane (IM) are also present, the former between the ISC and the cortex, and the latter between the germ wall and the core. An exosporium may be present external to the OSC. The OSC contains the alkali-resistant alkali fraction, and contains disulphide-rich (–S–S–) bonds, whereas the ISC contains the alkali-soluble protein fraction and consists predominantly of acidic polypeptides.

The OSC and ISC are considered to present significant barriers to the entry of many antibacterial agents. The OM may not be a true membrane and may not have a significant role in (im)permeability, whereas the IM may be a major permeability barrier.[40]

Lethal environmental chemical agencies

A variety of chemical agents are likely to be found in the environment (Fig. 1). They may include biocides and residues, antibiotics, antibiotic degradation products, detergents, pesticides, and pharmaceuticals and active ingredients in personal care products (PPCPs). All of these would be expected to have some effect on the environmental microbial flora. In general terms, the activity of such 'pollutants' will be influenced by their concentration, the temperature at which they are acting, the presence of organic or other interfering

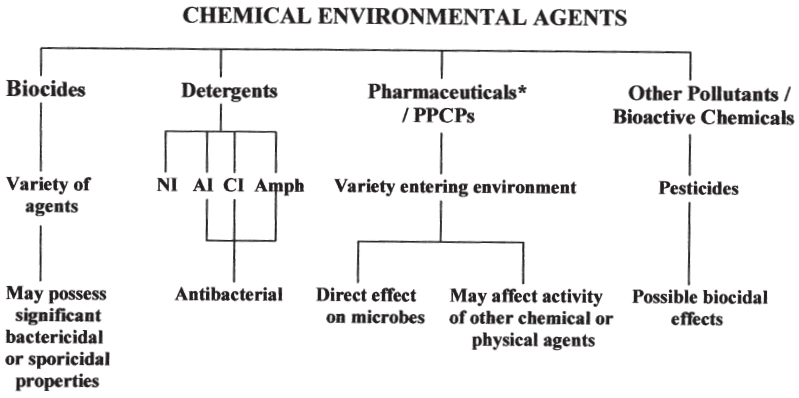

Fig. 1. *Chemical environmental agents that are likely to have harmful effects on bacteria and other microorganisms. NI, non-ionic surfactants; AI, anionic surfactants; CI, cationic surfactants; Amph, Amphoteric surfactants. *Including antibiotics*

matter and the type, nature and condition of the microbes with which they come into contact. For example, bacteria or other microorganisms present within biofilms will be less susceptible to most antibiotics and biocides than planktonic cells.[41, 42] Furthermore, bacterial spores and mycobacteria are much less readily inactivated than are other types of bacteria.[43,44]

Bacterial cell surfaces and antibacterial agents

(1) General considerations

In the laboratory, the effect of a biocide on bacterial and other microbial cells is conveniently measured by adsorption isotherm studies.[45–47] Such experimental approaches are not without problems since they do not necessarily describe the uptake of that biocide into cells but rather binding to cell surface components. Furthermore, with non-radioactive biocides, dense bacterial suspensions and a suitable, accurate and sensitive chemical assay are required. Wherever possible, radiolabelled biocides should be employed[48] since fewer cells are needed, the accuracy may be greater and it is possible to fractionate cells to determine the different sites at which the biocide is bound.

Reaction with a bacterial cell surface may be the initial effect of a biocide or other agent on a bacterial cell. Several different classes of adsorption have been described.[49] Different types of adsorption isotherm are known, which can shed light on the possible type of interaction with the cell surface. The main types are summarised as follows

(i) C (constant partition) pattern, in which the solute penetrates more readily into the adsorbate than does the solvent. This pattern occur with the adsorption of phenols by bacteria containing a high lipid content in their cell walls
(ii) L (Langmuir) pattern, in which, as more sites are filled, it becomes increasingly difficult for a solute to find a vacant site.
(iii) S (S-shaped) pattern, which occurs when the solute molecule is monofunctional and orientates vertically, meeting strong opposition for substrate sites from molecules of the solvent
(iv) H (high affinity) pattern obtained when the solute is almost completely adsorbed
(v) Z pattern, in which there is a sharp break in the isotherm, followed by an increased uptake, which is believed to occur at that concentration of adsorbed species that promotes a breakdown in the structure of the adsorbing species with the generation of new adsorbing sites. This has been found to occur with 2-phenoxyethanol and also with triclosan.[50]

Full details of these can be found elsewhere.[47] Resistant cells may take up less of a biocide than sensitive cells, but this is not invariably so. Some biocides interact strongly with cell wall or outer membrane components. However, another aspect must also be considered is that the cell surface might itself prevent intracellular uptake of a chemical agent. Both aspects are considered below (see also Fig. 2).

(2) Interaction of chemical agents with bacterial cell surfaces

Ethylenediamine tetraacetic acid (EDTA) is not an antibacterial agent in its own right, but it does increase the permeability of the OM of Gram-negative bacteria, in particular *P. aeruginosa.*[51] EDTA chelates divalent cations and causes the release of some 30–50% of the OM LPS, thereby rendering the cells more susceptible to a range of chemical inhibitors. There are, however, marked differences in the synergy obtained, depending on the nature of the inhibitor.[52] Other chemicals that act in a similar manner include sodium hexametaphosphate, and citric, malic and gluconic acids.[53] Polyethyleneimine (PEI) displaces Mg^{2+}, thereby also opening up the OM.[54,55]

The monoaldehyde, formaldehyde has long been known to possess potent microbicidal activity. It reacts rapidly with proteins, including the non-protonated groups of amino acids.[56] Unlike gramicidin, which produces pores in the membrane of *E. coli*, thereby rendering the cells more permeable to ions, formaldehyde probably interacts with the OM.,[57]

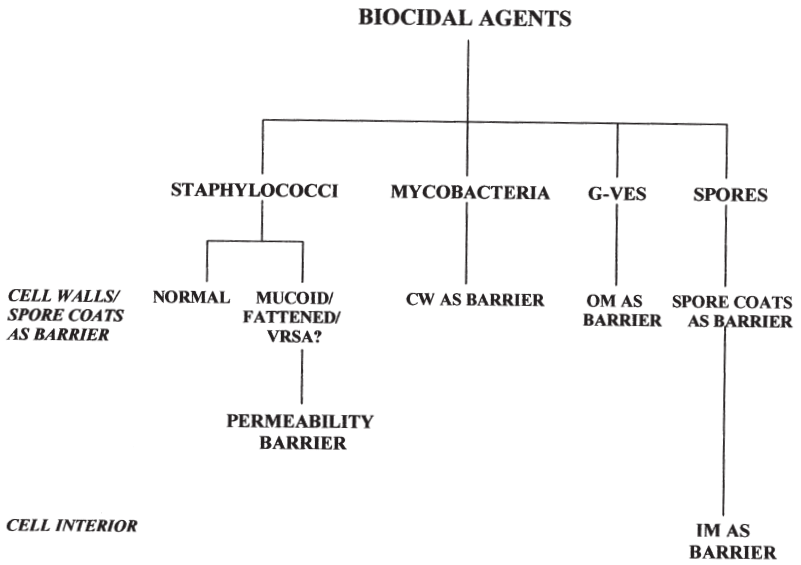

Fig.2*. Biocides and outer cell components in staphylococci, mycobacteria, Gram-negative bacteria and bacterial spores. CW, cell wall; IM, inner membrane; G-ves, Gram-negative bacteria; VRSA, vancomycin-resistant* Staphylococcus aureus*.*

The dialdehyde, glutaraldehyde (GTA; pentanedial) agglutinates bacterial cells and cross-links peptidoglycan in staphylococcal cell walls and in the Gram-negative OM.[58-60] In *Bacillus subtilis*, adsorption or uptake of alkaline or acid GTA is greatest to vegetative cell forms, followed by germinating and then by resting spores. *E. coli* cells take up more, and *S. aureus* cells, less GTA than *B.subtilis* vegetative cells.[61] *Ortho*-phthalaldehyde (OPA) is an aromatic dialdehyde. It has been shown to be less sporicidal than GTA but a more potent mycobactericidal agent.[62,63] OPA is a less effective cross-linking agent than GTA.[64]

Cationic biocides such as chlorhexidine salts (CHX), diamidines and quaternary ammonium compounds (QACs) and cationic antibiotics such as the polymyxins are believed to damage the OM of Gram-negative bacteria thereby promoting their own uptake into the cells.[53] *B. cenocepacia* K56-2 lacks the self-promoted uptake pathway and consequently is resistant to cationic antibiotics (polymyxins, aminoglycosides-aminocyclitols) and cationic biocides (CHX, QACs).[65]

Antimicrobial peptides are increasingly being studied as potential antimicrobial agents.[66-68] They interact with LPS, displacing cations, and thereby self-promote their own uptake.

(3) Permeability barriers and reduced uptake of antibacterial agents
The plasticity of the bacterial cell envelope in relation to its environment is a well-known phenomenon.[69,70] Growth rate and growth-limiting nutrients affect the physiological state. of bacterial cells. The growth of Gram-negative bacteria under conditions of nutrient limitation produces changes in cell envelope composition that help define the responses to biocidal agents.[71]

(a) Gram-positive non-sporulating bacteria
Biocides and antibiotics can probably diffuse freely across the staphylococcal wall. Inhibitory and lethal concentrations of many of these antibacterial agents are usually considerably less than for Gram-negative bacteria, especially highly resistant organisms such as *P. aeruginosa, Providencia stuartii* and *Burkholderia cepacia.* As such, staphylococcal cells are unlikely to contain a permeability barrier to the free uptake of either biocides or antibiotics. There are, however, circumstances in which reduced susceptibility of staphylococci may be found. Staphylococcal cells subcultured repeatedly in media containing high concentrations of glycerol have a greatly increased concentration of cell wall lipid and are less sensitive to several, but not all, biocides and antibiotics.[72,73] In this particular instance, the likely reason is reduced wall permeability to these agents. Mucoid strains of *S. aureus* are often foumd in nature, in which the cells are surrounded by a slime layer. Non-mucoid cells are inactivated more readily than mucoid cells by chloroxylenol, the QAC, cetrimide and CHX, although there is little difference with phenols or chlorinated phenols.[74] Interestingly, removal of slime from the mucoid cell increases their susceptibility. It is conceivable that the slime plays a protective role, either as a physical barrier to uptake or as a loose layer that interacts with, or absorbs, the biocide molecules. A capsule does not act as a permeability barrier to antibiotic or biocide uptake.[75a]

Methicillin-resistant *S. aureus* (MRSA) strains frequently show a reduced sensitivity in terms of mimimal inhibitory concentrations (MICs) to cationic biocides,[75b] but not necessarily to triclosan,[76] when compared to methicillin-sensitive *S. aureus* (MSSA) ones. Whereas a possible reason for this is an altered wall in MRSA cells, the reduced uptake to produce elevated MICs is associated with efflux rather than decreased permeability.[77]

In Gram-positive bacteria, the thickness and degree of cross-linking of peptidoglycan occurs under specified conditions producing altered responses to CHX and phenoxyethanol, possibly as a result of their decreased intracellular uptake.[78] Thickened cell walls in

vancomycin-resistant staphylococci are also claimed to be responsible for reduced sensitivity to phenols.[79]

Listeria monocytogenes is less sensitive to biocides than staphylococci. The reasons for this have yet to be fully elucidated but reduced uptake may be linked to both cell wall modification[80] and efflux systems.[81]

Mycobacteria contain highly hydrophobic cell walls and are generally much more resistant to many biocides and antibiotics than are other non-sporulating Gram-positive bacteria and Gram-negative organisms.[12,13,44] It was suggested many years ago that the relative resistance of various species of mycobacteria was directly related to the content of waxy material in the cell wall.[82] CHX and QACs are not mycobactericidal, but are mycobacteriostatic at low concentrations that approach their MICs against staphylococci.[83] CHX causes lysis of spheroplasts.[64] However, neither CHX nor QACs can be considered as being mycobactericidal, from which it may be inferred that the concentration that diffuses through the wall is sufficient to reach the primary target, the cytoplasmic membrane, but insufficient to cause significant damage to the cell interior. The activity of CHX and QACs against mycobacteria can be enhanced by using them in combination with a permeabilizing agent that increases the permeability of the mycobacterial cell wall.[83] This would seem to be a promising issue for further study both in the laboratory and in the environment.

Mycobactericidal agents include alcohols, phenols, GTA, OPA and peroxygens.[44] These might generally be able to penetrate into mycobacterial cells. OPA is a less effective cross-linking agent than GTA, but its high activity against mycobacteria is believed to result from its lipophilic nature that enables it to penetrate more readily the complex cell wall.[64] This may apply particularly to GTA-resistant strains of *Mycobacterium chelonae* isolated from endoscope washers. It has been suggested that this results from an altered cell wall polysaccharide.[84]

Generally, few studies have been undertaken on the uptake of biocides into mycobacterial cells. Consequently, too little is known about this important issue. Efflux pumps in mycobacteria may be associated with antibiotic resistance,[85] but there is no evidence to date that they are a factor in the comparatively high resistance of mycobacteria to biocides. An additional issue in nature is the interaction of environmental mycobacteria with protozoans.[12] The latter can survive phagocytosis and thus provide a considerable advantage to water-borne bacilli. It is known that various mycobacteria can invade and multiply within *Acanthamoeba* or other protozoans. In

fact, they can use protozoan cysts as carriers to survive starvation stresses and are also less amenable to inactivation by antibacterial agents.[86]

(b) Bacterial spores

Bacterial spores have been well documented as being resistant to environmental extremes both on Earth and during postulated interplanetary transfer through space.[87] The mechanisms of their high resistance to inimical chemical and physical agencies have been widely studied. It is particularly important to understand these mechanisms in the light of recent environmental incidents involving anthrax spores in the United States. Various components of the spore structure (Table 1) have been associated with the reduced susceptibility to biocides.

Several procedures have been devised for studying the underlying mechanisms.[36,53] These include (i) the removal of the OSC and ISC, (ii) the additional (but partial?) removal of the cortex, (iii) the use of mutants that produce spores with defective coats, (iv) the utilisation of spore mutants (Spo^-) that will develop only to certain stages during sporulation, (v) the effects of biocides in preventing sporulation, (vi) 'step-down' procedures that enable synchronously developing spores to be produced, (vii) $\alpha^-\beta^-$ spores that enable the role of DNA in inactivation to be elucidated. From a comprehensive examination of these, it has been possible to obtain important information not only about the mechanisms of spore insusceptibility, including reduced uptake into the spore, but also about the mechanisms of spore inactivation.

The OSC and ISC act as a barrier to many biocides, including chlorine-releasing agents (CRAs), GTA, OPA, iodine, hydrogen peroxide, alcohols, phenols, CHX and QACs. Nevertheless, many of these are important sporicides albeit at concentrations that are much higher than those that are effective bactericidal agents. Phenols, alcohols, QACs and CHX are not sporicidal even at elevated concentrations over prolonged periods of time, which suggests that the spore coats present a significant barrier to their intracellular uptake. The onset of reduced sensitivity during sporulation occurs with cortex development and is fully functional when the coats are synthesized.[88,89] Onset is latest with GTA and lysozyme.[88] The effect of the spore coats varies with the type of spore. Thus, in *Clostridium bifermentans*, the coats offer a protective barrier against hydrogen peroxide but are less effective in *B. cereus*.[90] Hydrogen peroxide, in fact, causes degradation of the outer spore layers of *B. subtilis*, including the spore coats and cortex.[91] Ozone is believed to cause disruption

of the OM of Gram-negative bacteria and of the outer coat of spores[92] thereby, in the lattere case, exposing the spore core.[93] An additional protective barrier is presented by the inner spore membrane, which has a very low permeability to small hydrophilic molecules.[94]

Germination is defined as an irreversible process in which there is a change in an activated spore from a dormant to a metabolically active state within a short period of time.[95] The initiation of germination is followed by various degradative changes leading, within a short period, to outgrowth. Spore germination (time scale about 5 minutes) involves the loss of heat resistance, excretion of calcium and dipicolinic acid, a refractility loss, a loss of resistance to stains, a release of fragments of hydrolysed peptidoglycan and a decrease in the optical density of cell suspensions. Outgrowth takes place in a synchronous and orderly manner, with synthesis (in this order) of RNA, protein, cell wall and DNA. Germinated and outgrowing cells become more sensitive to biocides. One reason for this altered susceptibility probably resides in the fact that greater amounts of a biocide are taken up following the loss of the spore coat permeability barrier. Such an event has been found with GTA[96] and halogens[97] but generally speaking too little information is available.

(c) Gram-negative bacilli

Although less susceptible than Gram-positive non-sporulating bacteria (other than mycobacteria), there is a wide variation in response of Gram-negative bacteria to biocides. The most resistant organisms include *P. aeruginosa, Providencia stuartii, Proteus* spp. and *Burkholderia cepacia*, especially to cationic biocides such as CHX and QACs, whereas *E. coli* strains are much more susceptible.

There are several possible reasons for this. They include reduced uptake via OM impermeability and the presence of active efflux systems, enzymatic degradation or mutation at a primary target site or sites. Efflux-mediated resistance is a major mechanism at low biocide concentrations but is clearly less effective at high concentrations, degradation has not been been found to be of importance at in-use biocide levels and mutation to high-level resistance remains unproven.[98] Intrinsic susceptibility as a consequence of reduced uptake related to OM impermeability is a factor of considerable importance.

In Gram-negative bacteria, the OM acts as a permeability barrier that limits the entry of chemically unrelated compounds, both biocides and antibiotics.[70,99–105] This conclusion is based on several pieces of evidence, notably (i) the relative sensitivities of Gram-

negative and Gram-positive bacteria other than mycobacteria, (ii) results of studies with OM mutants of organisms such as *E. coli, P. aeruginosa* and *Salmonella typhimurium*, (iii) the binding of a fluorescent probe to membranes or to nucleic acids, and (iv) the use of permeabilizing agents such as EDTA and PEI, both referred to above.

The antibacterial activity of the parabens (the methyl, ethyl, propyl and butyl esters of *para*(4)-hydroxybenzoic acid) increases as the homologous series is ascended, but this is accompanied by a corresponding decrease in solubility. Wild-type strains of *E. coli* and *S. typhimurium* are intrinsically less sensitive to the four esters than are rough and especially deep rough strains, with the methyl ester the least active and the butyl ester the most active against any one strain. Increased sensitivity to the parabens is likely to arise from the defective LPS with the appearance of phospholipid patches in the outer leaflet of the OM. These aid the penetration of an ester and especially the most hydrophobic (butyl) one across the OM to the presumed target, the inner membrane.[106,107]

QACs and amidines are considerably less active against wild-type cells than deep rough OM mutants of *E. coli* and *S.typhimurium*, whereas with CHX the OM of wild-type *S. typhimurium*, but not of *E. coli*, confers intrinsic resistance to this biocide. This suggests that CHX readily damages and penetrates the OM of the latter, but not of the former, and that QACs and diamidines have difficulty in traversing, and possibly damaging, the wild-type cells.[108-110] In support of this contention is the roughly equivalent susceptibity of *S. aureus* and *E. coli* to CHX but not to QACs or diamidines.

Proteus spp. are highly resistant to cationic biocides, and strains highly resistant to CHX, QACs, EDTA and diamidines have been isolated from clinical sources. The OM presents an efficient barrier to the uptake of these agents. This is believed to arise from the presence of a less acidic type of LPS, so that adsorption to the cell surface is reduced.[111] In addition, there is a reduced cationic content, which would account for reduced sensitivity to EDTA. Interestingly, *P.mirabilis* is highly sensitive to triclosan,[112] which suggests that the phenylether is readily taken up into the cell.

P. aeruginosa displays above-average intrinsic insusceptibility to many antibiotics and biocides. Whilst there may be several reasons (including efflux) that contribute to this property, the OM permeability is of considerable importance in limiting the uptake of antibacterial agents. The OM contains strong LPS-LPS cross-links related to the high Mg^{2+} concentration. The organism is thus particularly sensitive to EDTA.[53]

In *B. cepacia*, the OM contains high concentrations of phosphate-linked arabinose that decreases the affinity of the OM for cationic antibiotics and biocides.[113] The high intrinsic insusceptibility of this organism is related, at least in part, to this OM impermeability.

Aromatic alcohols such as phenylethanol (PEA) inhibit the growth of Gram-negative bacteria, including *B. cepacia*, but not *Ps. aeruginosa*, as well as some mycobacteria, but *S. aureus* is less susceptible. The more hydrophobic alcohol, 5-phenyl-1-pentanol (PP), is more potent than PEA,[114] possibly because it is taken up to a greater extent by the cells.

Several multidrug-resistant Gram-negative bacteria have been implicated in hospital-acquired infections. Non-fermenting Gram-negative bacteria (NFGNB) have emerged as being significant causes of nosocomial infections, especially in immunocompromised patients. They include *Acinetobacter* spp. and *Strenotrophomonas maltophilia.* These appear to be readily inactivated by in-use concentrations of biocides,[115] but little is known about the effects of lower concentrations and their uptake into the cells.

The presence of broadly specific efflux systems can exclude a range of chemically unrelated compounds. These include antibiotics, biocides, detergents, dyes and organic solvents. They are particularly important as a mechanism of antibiotic resistance.[116–119] Biocides also may be extruded by bacterial cells,[120] but this is unlikely to be a major factor in insusceptibility at in-use concentrations.

(d) Biofilm cells

Sessile bacterial cells within a biofilm are much less susceptible to antibiotics and biocides than cells in planktonic culture. This has been described for many types of Gram-positive and Gram-negative bacteria and for a range of antibacterial agents.[41,42] There are many reasons for this, one of which is relevant to the present discussion. Access of a biocide or antibiotic to the underlying cells may, depending on the chemical nature of the biocide, be prevented by the glycocalyx; modulation of the micro-environment, including reduced oxygen tension and nutrient limitation, may produce changes in the chemical composition of the cell envelope, thereby reducing drug susceptibility.[98]

Lethal environmental physical agencies

Bacteria show a wide response to lethal environmental agencies. In their widest context, such agencies represent thermal, radiation, photodynamic (light) and desiccation. Each of these has been widely studied

and the mechanisms of bacterial evaluation evaluated, although some issues remain in contention.

It is logical to sub-divide some of these different physical agents. Thus, temperature can be construed as being low (cold and cold shock), freezing (or freezing and thawing) and elevated. Radiation is normally considered as encompassing ionizing, ultraviolet (UV) and infrared (although with the latter it is the heating effect that is likely to achieve bacterial inactivation), whereas light refers to visible light or sunlight. Some of these, for example, ionizing and UV radiations and the effects of low temperatures are discussed at length elsewhere in this volume and will thus be alluded to here only briefly. A summary of the effects of lethal agencies is provided in Fig. 3.

Of the four types of bacteria considered in this paper, bacterial spores are undoubtedly the most resistant to some, but not necessarily all, of these processes. Thus, they are the least susceptible to moist and dry heat, but not necessarily the most insensitive to ionizing or UV radiations. Although the bacterial cell surface is the first to come into contact with the environment, it is considered unlikely that this forms any barrier to the physical agency in question, although it may suffer some structural damage.

(1) High temperatures: moist heat

Bacteria vary considerably in their response to temperature. For every type of organism, there is a minimum, optimum and maximum growth temperature, depending on whether the organism is psychrophilic, mesophilic or thermophilic. Organisms (archaea) that produce extremoenzymes are extremophilic.[2]

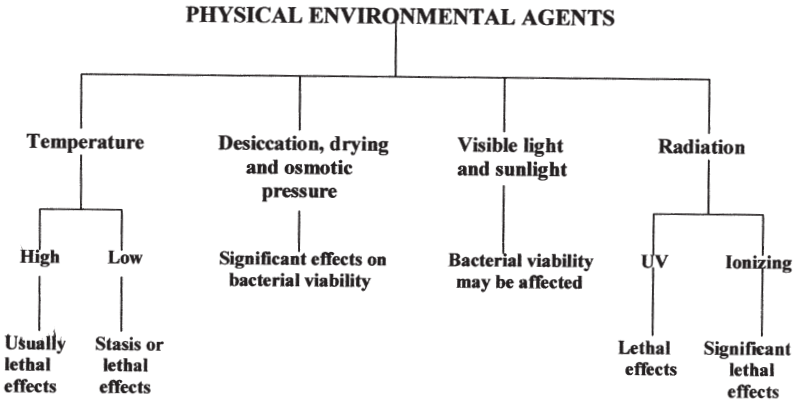

Fig. 3. *Physical environmental agents that are likely to have harmful effects on bacteria and other microorganisms.*

Thermal inactivation by moist heat of non-sporulating bacteria involves every cellular component (outer layers, cytoplasmic membrane disruption, DNA strand breakage, RNA degradation, enzyme inactivation and protein denaturation or coagulation).[2] In a Gram-positive coccus, damage to the cell wall is likely to be less than to the OM of a Gram-negative cell.[121] Whilst this implies that the extensively cross-linked staphylococcal cell wall could confer some protection against damage, in actual fact staphylococci are no less sensitive than Gram-negative bacteria. Thermoduric enterococci[122] are less susceptible to moist heat than staphylococci, but the reasons are unlikely to result frrom differences in cell wall composition or structure.

Damage to the OM of Gram-negative bacteria such as *E. coli* is brought about when the cells are exposed to mild heat shock. Increased permeability to antibiotics, entry of otherwise impermeable fluorescent dyes, release of periplasmic protein, loss of OM LPS, bleb formation and transient increase in nisin susceptibility have all been demonstrated.[123-128]

In addition to these direct effects of high temperatures on non-sporulating bacteria, stress responses occur in the form of extracellular alarmones[129] and inducible intracellular heat-shock proteins (HSPs).[130,131]

A 'typical' bacterial spore was described above (Table 1). There are several potential target sites for spore inactivation by moist heat. These are the spore membranes, proteins and enzymes and core DNA.[2,132]

(2) High temperatures: dry heat

Dry heat (high temperatures in the absence of moisture) is a less effective inactivating process than moist heat.[1] As with moist heat, spores are the most resistant form of bacteria. However, dry heat requires much higher temperatures to inactivate bacteria and other microorganisms than moist heat.[1]

The lethal mechanism involved in dry heat is considered to be essentially one of oxidation, although other mechanisms must be involved. In spores, sublethal temperatures may induce mutants.[133] The water content of spores, controlled by the cortex, is a key factor in determining inactivation by dry heat, since only a relatively small amount of water is claimed to protect the heat-sensitive site(s).[134] There is no evidence that the outer cell layers of spores or non-sporing bacterial cells are involved in either inactivation or resistance.

(3) Low temperatures

Microbial growth is retarded and eventually ceases at low temperatures. Psychrophiles can grow at temperature approaching 0°C. A range of environmental factors can alter the minimum growth temperature; these include nutrient status, salt concentration and water activity (A_w).

Cold shock, a process in which organisms are chilled without freezing, may inactivate non-sporulating bacteria.[135,136] Increased membrane permeability, caused by a phase transition in membrane lipids,[136,137] results in leakage of low molecular weight intracellular materials. The age of the culture is an important factor in cellular response, with exponential phase cells being much more susceptible than stationary phase ones. Divalent cations can pretect cells against chilling. Cold osmotic shock, in which bacteria are held in hypertonic sucrose containing EDTA and then transferred to ice-cold magnesium chloride solution induces the release of periplasmic enzymes from Gram-negative bacteria.[138]

Freeze-drying, widely used as a means of preserving microbial cultures, may produce single-strand breaks in DNA and an increase in the frequency of mutation.[139] Damage to the outer and inner membranes in *E. coli* results from freezing and thawing.[125,140]

(4) Desiccation, drying, osmotic pressure and hydrostatic pressure

Drying and desiccation have an important effect on microbial survival and dissemination in the environment. Desiccation, the removal of the majority of water, is essentially a time-honoured method for preserving different types of items from microbial attack.[141]

Microbes require water in which to grow, but they differ markedly in their moisture requirements.[142-144] The most osmotolerant micrococci require A_w levels in excess of 0.82 for growth, whereas few common fungi or yeasts will grow below A_w values of 0.65. Limiting A_w values are generally Gram-negative rods 0.95; staphylococci, micrococci and lactobacilli 0.99; most yeasts 0.88. However, syrup-fermenting osmotolerant yeasts may cause spoilage in products with A_w levels a s low as 0.73, and some filamentous fungi, such as *Aspergillus glaucus*, may grow at A_w values as low as 0.61.[145]

Although the A_w value gives a good indication for growth potential, the nature of the solute exerts an addition al effect.[142] Organisms that live in high concentrations of sugar are osmophiles, whereas those that grow optimally at the A_w of seawater are halophiles,[146]

with degrees of response to sodium chloride concentrations in media varying between non-halophilic (*E. coli*), halotolerant (*S. aureus*), halophile (*Vibrio fischeri*) and extreme halophile (*Halobacterium salinarium*).

Organisms that grow under conditions of low water activity obtain environmental water by adjusting their internal solute concentration by (1) synthesizing or concentrating an internal organic solute, or (2) pumping inorganic ions into the cell. In *S. aureus*, the amino acid proline is synthesized as a compatible solvent. A more effective osmoprotectant is betaine.[147] Changes in membrane lipids occur during growth at low A_w values, with increases in anionic lipids relative to other lipids.[148]

Osmotic stress effects have been widely studied. [149,150] *E. coli* is capable of adjusting to a wide range of environments, from very dilute to much more concentrated, with a difference of at least 100-fold in osmolarities. The organism thereby adjusts a wide range of cytoplasmic solution variables that include water and charged and uncharged molecules. In *E. coli*, the cell wall (consisting of the OM and attached peptidoglycan) is porous and elastic and the peptidoglycan stretches without bursting in response to a modest, outwardly directed osmotic pressure difference.[149,150] This is found when the osmolarity of the cell is greater than its environment.

Desiccation is an unusual state of biological organisation wherein bacterial cells cease metabolism but remain viable.[149,150] The removal of water is a severe process that may be lethal. Desiccation tolerance is then considered as a state of suspended metabolism or stasis induced by the removal of cell water.[149,150]

The effects of hydrostatic pressure on bacteria have been studied for many years. Recently, it has been demonstrated that structural changes occur in *Leuconostoc mesenteroides,* with changes to the external surface that include dose-related blistering and internal structures. Inactivation was considered to result from ribosomal denaturation.[153,154]

(5) Visible light and sunlight

The survival of microorganisms in the environment has been comprehensively discussed.[155] The discussion included a consideration of the effects of sunlight. However, this evaluation was based on degree of survival and dormancy rather than on cellular changes. The photodynamic effects on bacteria have been known for many years[156] and include membrane damage and the induction of mutations.

(6) Ionizing radiation

Ionizing radiations strip electrons from the atoms of the material through which the radiations pass. They are best exemplified by X-rays, γ-rays and β-rays (high-speed electrons). Their effects are essentially due to the stripped-off electrons that initiate a chain of chemical reactions. Ionizations occur principally in water resulting in the formation of short-lived but highly reactive hydroxyl ($OH^{\cdot}$) radicals and protons (H^{+}). Single strand breaks (SSB) and double strand breaks (DSB), depending upon the severity of the radiation dose, are produced in DNA.[157]

Bacterial spores are generally more resistant than non-sporulating bacteria, but *Deinococcus radiodurans* is the most highly resistant organism known. *Enterococcus faecalis* is highly resistant under some artifial conditions. Low radiation doses might cause cell lysis, but there is no evidence to support an earlier contention that disulphide bond-containing proteins in the spore core were acting in a radioprotective manner.[158]

(7) UV radiation

The effects of UV radiation on sporulating and non-sporulating bacteria have been well documented, with comprehensive studies on the production of photoproducts, the role of small, acid-soluble proteins (SASPs) in spores and the various repair processes.[159,160] It is, nevertheless, considered that much remains to be discovered about radiation resistance processes.[161] Damage to bacterial cell walls, outer membranes or spore coats is unlikely to be a contributory factor to cell inactivation.

Overall comments

Chemical agents that are present in a constantly changing environment can be considered as being representative of biocides and residues therefrom,[162–165] pharmacologically active drugs,[10,166] antibiotics (or degradation products from them),[8] as well as pesticides, pharmaceuticals, cosmetics and other products.[7,11] All of these might have some effects on bacteria and other microorganisms. Biocidal agents have been in use in one form or another for many years and so some degree of adaptation and resistance has been known.[167] The outer cell layers of sporulating and non-sporulating bacteria provide an important means of defence to many chemical and at least some physical agents. The latter include desiccation, radiations, high or low temperatures, osmotic pressure and hydrostatic pressure.

It would, nevertheless, be erroneous to imply that impermeability is the sole means of conferring insusceptibility, or of reducing sensitivity, to chemical agents in the environment or elsewhere[168] This represents one, albeit very important, facet in the continuous fight of bacteria for survival. Other factors that limit activity, including efflux, stress responses, the possibility of degradative enzymes (especially within biofilm communities) and the other factors involved in the recalcitrance of cells within biofilms, must form part of the overall consideration.

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