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Review

Biocide resistance in *Acinetobacter baumannii*: appraising the mechanisms

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SUMMARY

A global upsurge in antibiotic-resistant *Acinetobacter baumannii* requires supervised selection of biocides and disinfectants to avert nosocomial infections by reducing its spread. Moreover, inadequate and improper biocides have been reported as a contributing factor in antimicrobial resistance. Regardless of the manner of administration, a biocidal concentration that does not kill the target bacteria creates a stress response, propagating the resistance mechanisms. This is an essential aspect of the disinfection programme and the overall bio-contamination management plan. Knowing the mechanisms of action of biocides and resistance modalities may open new avenues to discover novel agents. This review describes the mechanisms of action of some biocides, resistance mechanisms, and approaches to study susceptibility/resistance to these agents.

Introduction

Acinetobacter baumannii, also known as 'Iraqibacter', is famed for its implication in causing severe infections among soldiers in US military treatment facilities. Progressively, *A. baumannii* quickly positioned itself as one of the most troublesome pathogens in healthcare facilities throughout the world [1,2]. *A. baumannii* is part of the *Acinetobacter calcoaceticus–baumannii* complex, which includes *A. baumannii*, *Acinetobacter pittii*, *Acinetobacter nosocomialis* and *Acinetobacter calcoaceticus*. The first three are associated with infections, while the fourth is rarely established as a pathogen. *A. calcoaceticus–baumannii* complex grows at temperatures between 35°C and 37°C; however, specific environmental isolates thrive at temperatures between 20°C and 30°C. The only bacterium in the family able to survive at 44°C is *A. baumannii* [3,4].

A. baumannii accounts for more than 12% of hospital-acquired bloodstream infections in intensive care units (ICUs), with broad regional variations: it is common in Southern Europe,

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the Middle East, Asia and South America, but uncommon in Northern Europe and Australia [5]. *A. baumannii* has been found in the nose, ears, throat, forehead, trachea, conjunctiva, vagina and perineum, axillae, groin, hands and toe webs, among other places, in healthy people [4,6]. In healthcare centres, *A. baumannii* can be found on tables, furniture, roofs, medical equipment and supplies, as well as medical personnel's possessions, tap water sinks, telephones, door handles, hand sanitizers, dispensers, trolleys, cabinets and even computers [4,7,8]. Reservoirs of *A. baumannii* are found in the hospital environment, and the bacterium's ability to survive for up to 1 month on wet or dry surfaces has been allied with outbreaks of hospital-acquired infection in the form of ventilator-associated pneumonia, meningitis, bacteraemia, urinary tract infection, bone infection and wound infection [9,10]. Invasive procedures or usage of medical devices, extended ICU stay, mechanical ventilation, enteral feeding, burns, and recent use of broad-spectrum antibiotics (especially cephalosporins or fluoroquinolones) are risk factors for acquisition of *A. baumannii* [11]. In hospital facilities, a mortality rate of 26% has been reported, with rates rising to 40–50% in ICUs [4,9]. *A. baumannii* is the most common cause of ventilator-associated pneumonia in the hospital setting, accounting for 15% of all nosocomial infections, and has the highest morbidity and mortality rates in medical wards, especially ICUs [4,12]. Of all the antibiotics prescribed elsewhere in the hospital setting, more than half are recommended for *A. baumannii* for patients admitted to ICUs [4,13]. While *A. baumannii* is not considered a community pathogen, it can populate tracheostomy sites in immunocompromised adults and children, causing community-acquired bronchiolitis and trachea bronchitis. It has also been linked to community-acquired pneumonia caused by underlying disorders such as obesity, alcohol abuse, diabetes mellitus and chronic obstructive pulmonary disease in tropical regions [4].

A. baumannii belongs to the group of ESKAPE pathogens comprising *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* spp. — because of their capacity to develop antibiotic resistance [9]. Prolonged environmental persistence allows *A. baumannii* to spread quickly and gain resistance to both traditional antimicrobials and certain biocides [14]. According to the US Centers for Disease Control and Prevention, almost 40% of *A. baumannii* are imipenem-resistant and multi-drug-resistant [15]. In recent years, carbapenem-resistant *A. baumannii* (CRAB) has been listed as a 'highest priority pathogen' by the World Health Organization for discovering novel antimicrobials [9]. Awareness of antimicrobial resistance mechanisms will support this effort [16,17].

In 1970, ampicillin, cephalosporins, carbapenems and various antibiotic groups were effective against *A. baumannii* [11]. Resistance to ampicillin, carbenicillin, gentamicin and nalidixic acid was first reported after 1975, and over time, this has increased, with the first record of CRAB appearing in the early 1990s [8]. *A. baumannii* has innate (chromosomal) antimicrobial resistance pathways, but may also develop novel resistance determinants by mobile genetic elements, including transposons, integrons, plasmids, insertion sequences and resistance islands [11]. Various antibiotic resistance mechanisms explored in *A. baumannii* include: influence of efflux pumps and beta-lactamases; presence of aminoglycoside-modifying enzyme; loss of lipopolysaccharide (LPS); a point mutation in the *pmrAB* gene implicated in colistin resistance; modification of outer membrane porins (OMP) and penicillin-binding protein; mutation in DNA gyrase and topoisomerase IV; modification of the ribosomal binding protein; and biofilm formation [4,16,18–20]. Biofilm formation by *A. baumannii* underlies its contribution in many hospital-acquired infections. The disproportionate presence of *A. baumannii* infections in various wards in the same hospital raises serious concerns about biocide usage and efficacy [21]. Several biocides are used in healthcare settings, but the crucial point is to use appropriate biocides to prevent and control the spread of infectious diseases in hospitals and other health facilities. The emergence of antibiotic-resistant pathogens in the hospital setting has increased the debate on their usage; understanding modes of action and efficacy may prevent indiscriminate usage. This review discusses certain biocides and their modes of action, as well as their resistance mechanisms. Four search engines were used in this review: Google Scholar, PubMed, Science Direct and Scopus. 'Name of the biocide', 'action', 'efficacy', 'reduced susceptibility', 'susceptibility', 'tolerance' and 'minimum inhibitory concentration (MIC)' were used for each search.

Biocides and their mechanism in control of *A. baumannii*

Biocides is a broad term for chemical agents utilized to achieve hygiene. Their classification within particular groups, such as antiseptics and disinfectants, is determined mainly by their practical usage [15]. They are widely used to prevent or eradicate pathogens in hospitals, laboratories, factories and homes. As such, they play an essential role in reducing the spread of pathogenic micro-organisms, especially in the hospital setting [22,23]. Two young boys were among the first patients to benefit from biocides. In 1867, Joseph Lister explained how he used carbolic acid to save their wounded limbs from being infected, and prevented amputation. In the face of potentially untreatable infections caused by multi-drug-resistant (MDR) pathogens, our reliance on biocides has resurfaced as prevention (through the use of biocides) is once again preferable to treatment (use of failing antibiotics) [24].

According to the reports, typical disinfectants such as 70% ethanol, chlorhexidine, sodium hypochlorite and quaternary ammonium compounds (QACs) are totally effective against *A. baumannii* isolates if used at the manufacturers' recommended concentrations [25,26]. These biocides and others can be divided into four categories depending on their target of action (Figure 1): those that act on proteins (alcohols, phenols, phenyl ethers, aldehydes, heavy metal derivatives, isothiazolones, acids or parabens, peroxygens, chlorine compounds, biguanides and vapour-phase disinfectants), membranes (QACs, biguanides, phenols, phenyl ethers, acids, terpenes, alcohols, anilides, peroxygens, parabens, isothiazolones and anionic surfactant), nucleic acids (alcohols, acids or parabens, antimicrobial dyes, acridines, biguanides, aldehydes, diamidines, chlorine-releasing compounds, heavy metal derivatives, peroxygens, halogens and vapour-phase disinfectants) and cell walls (alcohols, phenols, aldehydes, chlorine-releasing compounds and heavy metal products) [27]. In general, biocides are thought to have many target sites within the bacterial cell, and they cause overall damage to these targets [13]. The following subsections provide a brief overview of common biocides used to combat *A. baumannii* and other micro-organisms.

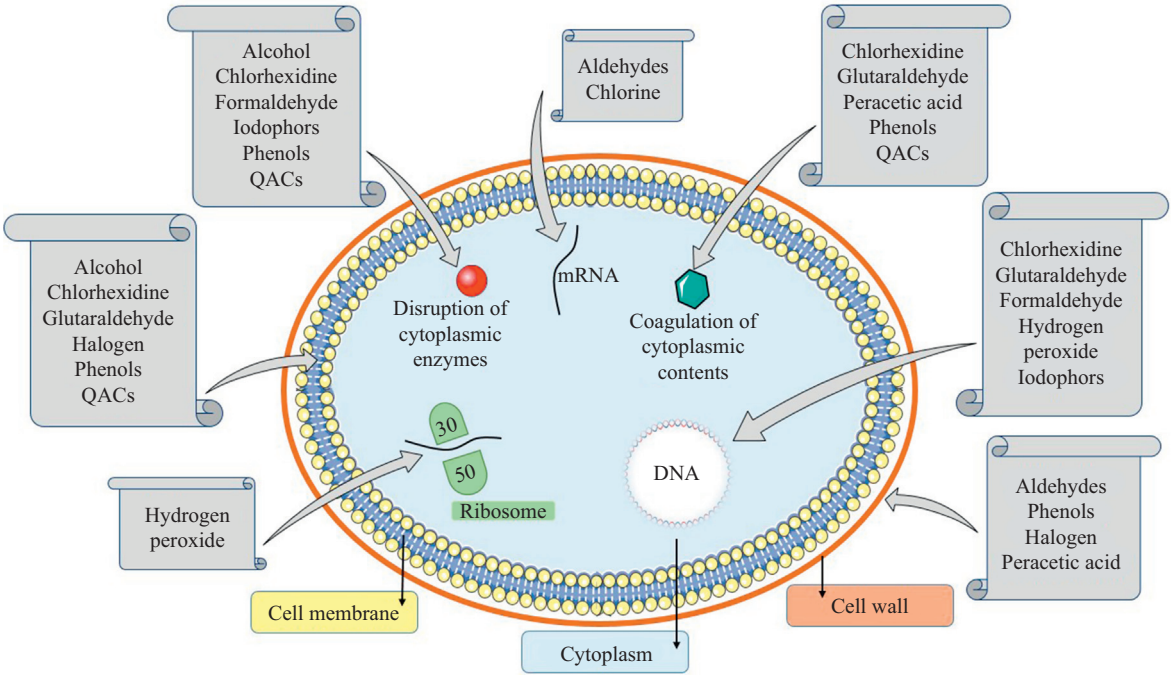

Figure 1. Cellular targets of biocidal agents. QACs, quaternary ammonium compounds.

Quaternary ammonium compounds

QACs are cationic detergents (surfactants or surface-active agents) that reduce surface tension and shape micelles, allowing liquid dispersion, and prevent pathogenic bacteria from spreading [28,29]. QACs are algistatic, bacteriostatic, tuberculostatic, sporistatic and fungistatic at low concentrations (0.5–5 mg/L). They are microbicidal for these same groups at 10–50 mg/L concentrations, depending on the organism and formulation [28]. QAC activity has been proposed to reach beyond the surface to intracellular targets, despite their well-known membrane-damaging properties [24]. Their positively charged head group absorbs acidic components of the bacterial cell envelope, while the long alkyl chains solubilize the membrane causing cell death. Benzalkonium chloride disrupts membranes; however, it causes widespread protein aggregation at low concentrations [29].

Chlorhexidine

Chlorhexidine is a divalent cationic biguanide molecule available in various forms, such as chlorhexidine gluconate which is water soluble [30]. To minimize healthcare-associated infections, the agent has risen in popularity when chlorhexidine bathing is used with intranasal mupirocin in patients in ICUs [31]. Chlorhexidine is most often utilized at a concentration ranging from 0.5% to 4%, depending on the clinical indication. Hand disinfectants, for example, usually contain between 0.5% and 4% chlorhexidine [30]. In contrast to other biocides, chlorhexidine is a broad-spectrum biocide with long-lasting residual activity [32]. It is most effective against Gram-positive bacteria, but it can also suppress Gram-negative bacteria, enveloped viruses and fungi [33]. The positively charged chlorhexidine binds to the negatively charged bacterial cell membrane and cell surface, and thus causes loss of osmoregulation and metabolic energy at low concentrations, as well as a loss of cytosolic potassium ions, thereby inhibiting cellular respiration. At higher concentrations, chlorhexidine causes a complete lack of membrane integrity, resulting in the leakage of cellular contents from the cell and, eventually, cell lysis and death [13,30]. However, chlorhexidine is unable to penetrate biofilm [34].

Hydrogen peroxide

Hydrogen peroxide is a commonly used antiseptic that exists in both gas and liquid forms. In liquid form, it is used as an antiseptic on the skin at concentrations ranging from 3% to 6% (v/v) [35]. It is also widely found as a dental disinfectant at concentrations varying from 0.4% to 1%. It is a typical active ingredient of contact lens solution, where it is usually used at a concentration of 3% [30]. Hydrogen peroxide is a broad-spectrum antimicrobial that is effective against bacteria, viruses and protozoa [36]. Although the exact mechanism of action of hydrogen peroxide is not known, it is postulated that it is mainly related to its oxidative activity. The inclusion of trace metals, such as iron, catalyses the formation of strongly reactive hydroxyl radicals after hydrogen peroxide has passed through the cell membrane, which results in the cleavage of nucleic acid and protein backbones, leading to cell membrane damage. Many cellular processes, including RNA, DNA and protein synthesis pathways, are impaired due to oxidative damage [30,37].

Povidone-iodine

Povidone-iodine (PVP-I) is a potent, broad-spectrum antimicrobial that has been used for over 60 years in infection control and prevention [38]. In the early days of its discovery, low solubility, instability and toxicity made it unsuitable for

use; however, to overcome some of these problems, free iodine was mixed with potassium iodide salts and alcohol, which increased its solubility significantly [30]. It is available in various formulations for use as a skin, hand and mucosal surface disinfectant, as well as wound care and eye applications [38]. While 10% PVP-I solutions are often used for pre-operative skin disinfection, lower concentrations, such as 5%, are used for ophthalmic procedures. Furthermore, more dilute solutions (<2%) are used to prevent and treat childhood conjunctivitis [39,40]. PVP-I has been shown to have antimicrobial activity against Gram-positive, Gram-negative and certain spore-forming bacteria (*Clostridia* spp., *Bacillus* spp.) as well as mycobacteria [38]. In the mechanism of action, near to the cell membranes of target micro-organisms, PVP releases free iodine, which destabilizes membrane integrity, denatures nucleic acids, and can quickly destroy micro-organisms by inhibiting critical cellular processes such as electron transfer, cellular respiration and protein synthesis non-specifically while within the cell [30].

Triclosan

Triclosan is a bisphenol class of compounds that has been used mainly in meticillin-resistant *Staphylococcus aureus* decolonization protocols. Triclosan is also used in various materials and plastics, including surgical drapes, toothbrush handles, wound sutures, mop handles and even children's toys [27,30]. The lack of effectiveness of triclosan in household soap products was later confirmed, leading to a ban on its usage along with another 18 biocidal chemicals by the US Food and Drug Administration in September 2017 [41]. Triclosan mainly targets bacteria. Like certain other biocides, it is believed to attack the cell membrane in a non-specific way. On the other hand, it has been shown to act on a specific target within the bacterial fatty acid biosynthetic pathway known as FabI in many studies. However, it has been proposed that at higher doses, such as those used in topical antiseptics, triclosan may have a non-specific action, causing cell lysis by effects on RNA and protein synthesis, resulting in detrimental effects on membrane integrity [30].

Alcohol

n-propanol is the most widely used alcohol compound in biocides; its optimum bactericidal effectiveness is attained at concentrations varying from 60% to 90% [30,42]. Pure alcohol, or alcohol that contains <1% water, is less bactericidal than alcohol at intermediate concentrations. Thus, water is crucial in the denaturation of proteins [42]. The exact mechanism of alcohol's antimicrobial activity is not known with certainty; however, it may be linked to membrane disruption, inhibition, or uncoupling of mRNA and protein synthesis through effects on ribosomes and RNA polymerase or associated with protein denaturation [30,43].

Despite the effectiveness of the described biocides, when bacteria are exposed to a biocide or biocidal product, they go through a stress response that involves the expression of global gene regulators and, eventually, the expression of non-specific mechanisms that may enable them to survive [44].

Mechanism of resistance to biocides

Bacterial resistance to biocides has been classified as either intrinsic, a natural property of the bacterium, or acquired, resulting from the acquisition of resistance genes in the form of transposons or plasmids [22]. These definitions remain valid, although the concept of transient resistance recognizes that the effect of a biocide on a bacterium may be more complex and short-lived after the expression of a mechanism(s) in response to direct selective pressure [44]. Among the mechanisms involved, phenotypic alterations (such as altering the cell membrane charge), alteration of the antimicrobial target, and inactivation of the disinfectant are some well-known resistance mechanisms to antimicrobials [22,27,44]. Since the mechanism of biocide resistance and antibiotic resistance are almost the same, cross-resistance to biocides and antibiotics is expected to occur simultaneously in bacteria. The efflux pumps and genetic causes of disinfectant resistance in *A. baumannii* are shown in Figure 2.

Biofilm formation

Biofilm is a term used to describe a complex group of micro-organisms in which the cells are coated in an extracellular polymeric material, a self-produced matrix, which facilitates bacteria to prevent the penetration of antimicrobial agent [17]. Enzyme-mediated resistance, the composition of the outer membrane, efflux pumps and genetic tolerance can also play a role in the antimicrobial resistance of biofilm [6]. Formation of biofilm is important for the bacteria's survival in the presence of antibiotics, host immune defence and adverse environmental conditions, causing increased tolerance to acid exposure and dehydration in *A. baumannii* cells, resulting in colonization, which is a significant cause of device-related infection [21,45]. Biofilm-forming bacteria are estimated to be associated with 65–80% of human infections [21]. Factors such as surface hydrophobicity, temperature and oxygen concentration are documented to influence the biofilm formation of *A. baumannii* and other bacteria [1]. The biofilm-related gene, *csuE*, is a member of the usher-chaperone assembly system, which mediates attachment and biofilm formation. Type IV pili (T4P) is another crucial component in the early stages of biofilm formation [9,46]. *A. baumannii* biofilm formation is also influenced by an auto-inducing quorum-sensing molecule (acyl-homoserine lactone), which is generated by the *abaI*-encoded autoinducer synthase [3]. Several causes, including the existence of Bap (biofilm-associated protein), some novel proteins (e.g. CarO, OmpA, OprD-like, DcaP-like, PstS, LysM and Omp33) and histidine metabolism (e.g. urocanase) were linked to the capacity of *A. baumannii* to form mature biofilms on polypropylene, polystyrene, titanium and other medical-device-related materials [1,47]. Bacterial biofilm production along the catheter surface is thought to be the most critical step in the development of bacteriuria [21]. *A. baumannii* at the air–liquid interface also produces pellicles. In clinical isolates, the production of these floating biofilms is a rare occurrence, linked to motility on the surface [48]. Motility and pellicles, or surface-attached biofilms, have a complicated interaction. Although motility appears to be the exact opposite of sedentary lifestyle in biofilms, it may be necessary for the formation of microcolonies during the early

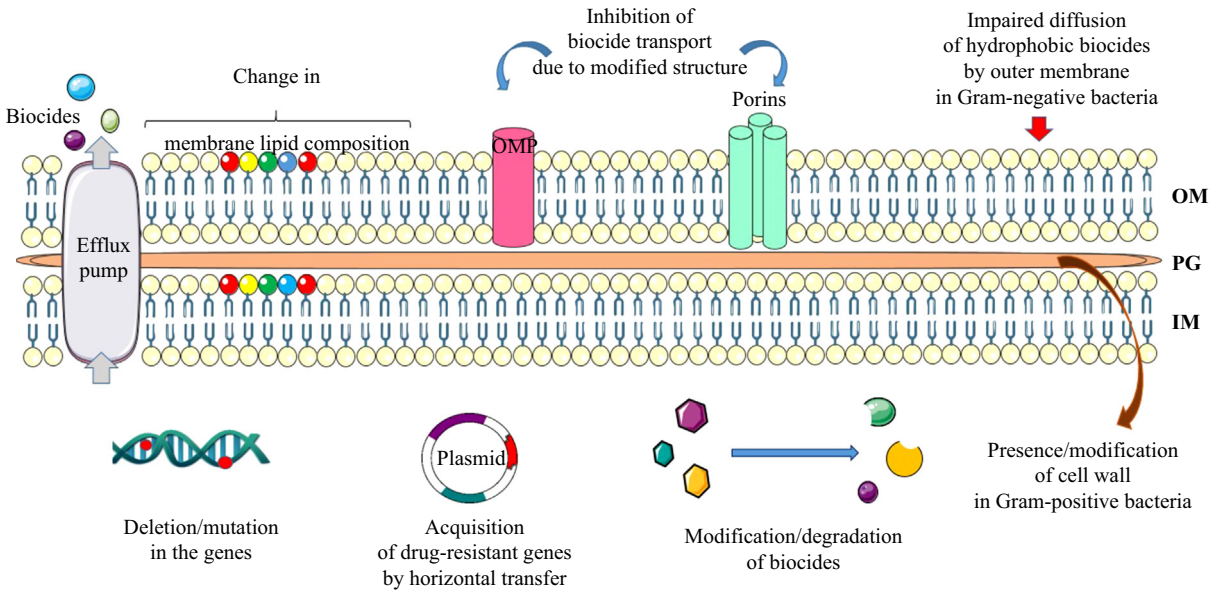

Figure 2. Various mechanisms of biocide resistance in bacteria. Bacteria are either naturally resistant to biocides (intrinsic resistance) or can acquire resistance to biocides through multiple mechanisms (acquired resistance). Intrinsic resistance is achieved by having a cell wall, an efflux mechanism, etc. Resistance can also be developed by altering genes involved in synthesizing cell walls, membrane lipids, porins or outer membrane porins (OMP). Horizontal transfer of mobile genetic elements such as plasmids is another mechanism through which bacteria acquire resistance. Specific genes that encode for proteins that can modify or degrade the biocide can be produced either by modifying existing genes or horizontally transferring genes. OM, outer membrane; PG, peptidoglycan; IM, inner membrane.

phases of biofilm development, and the re-organization of mature three-dimensional biofilm structures [9] (Figure 3).

Efflux pumps

Antimicrobial concentrations that permeate bacterial cells are reduced by efflux pumps, which are found widely in bacteria [44]. The action of MDR efflux pumps, such as AdeIJK and AdeABC of *A. baumannii*, is one of the most well-known biocide and antibiotic cross-resistance mechanisms [15]. Following antimicrobial exposure, the expression of efflux pumps can rise, not necessarily through stimulation of the efflux pumps, but by modulating global gene regulators, particularly *marA* and *soxS* [44]. The major facilitator superfamily, the ATP-binding cassette superfamily, the resistance nodulation-division family, the small multi-drug-resistance family, and the multi-drug and toxic chemical extrusion family have been identified as five main classes of efflux pumps [44] (Figure 4). The QAC genes (*qac*), which can be horizontally transferred to other bacteria through plasmids, encode these multi-drug efflux pumps named Qac proteins [23,30]. QacA and QacB belong to the major facilitator superfamily, whereas QacC (also known as Smr), QacE, QacE1, QacF, QacG, QacH, QacJ and QacZ belong to the other families [49]. The *qacE* gene (and its attenuated type *qacEΔ1*) is commonly present in Gram-negative bacteria, mediates resistance through a proton pump, and confers resistance to QAC disinfectants (e.g. benzalkonium chloride), biguanide compounds (such as chlorhexidine) and hydrazones [23].

The capacity of efflux pumps to provide resistance to biocides is debatable; efflux pumps are most likely one of several mechanisms used by bacteria to survive biocide/biocidal product exposure [50,51].

Mobile genetic elements

In response to selection pressure, mobile genetic elements are amplified [52]. These elements are frequently responsible for the transmission, accumulation and widespread predominance of resistance genes, resulting in MDR strains. Transposons, insertion sequences, integrons and gene cassettes are examples of DNA elements that can transfer molecules within or across membranes. Others, such as integrative and conjugative elements (ICEs) and plasmids, can migrate from one bacteria to another [53]. Plasmids, ICEs and bacteriophages enable intercellular mobility. Furthermore, interactions between these mobile genetic elements are critical for the rapid evolution of antimicrobial resistance and may cause disinfectant resistance [52].

Insertion sequences and composite transposons

Insertion sequences and transposons are two types of mobile DNA fragments which were previously thought of as 'passenger' genes carrying resistance genes, but later proved that they translocate resistance genes to new locations on the same or different DNA molecules almost randomly [53]. Insertion sequences, by containing a strong promoter upstream of their location or translocating upstream of an essential chromosomal gene, can influence the expression of their passenger genes. Insertion sequences carrying resistance genes have also been shown to have a 35-hexamer sequence region that forms a hybrid promoter with an adjacent 10-like sequence, increasing expression [54]. Insertion sequences usually contain one or two transposase (*tnp*) genes, and two copies of identical or related insertion sequences flank one or more genes to form a composite transposon. This entire region moves simultaneously [52]. It has been reported that a type of insertion sequence is associated with the resistance gene *qacC*, which confers resistance to disinfectants and antiseptics [55].

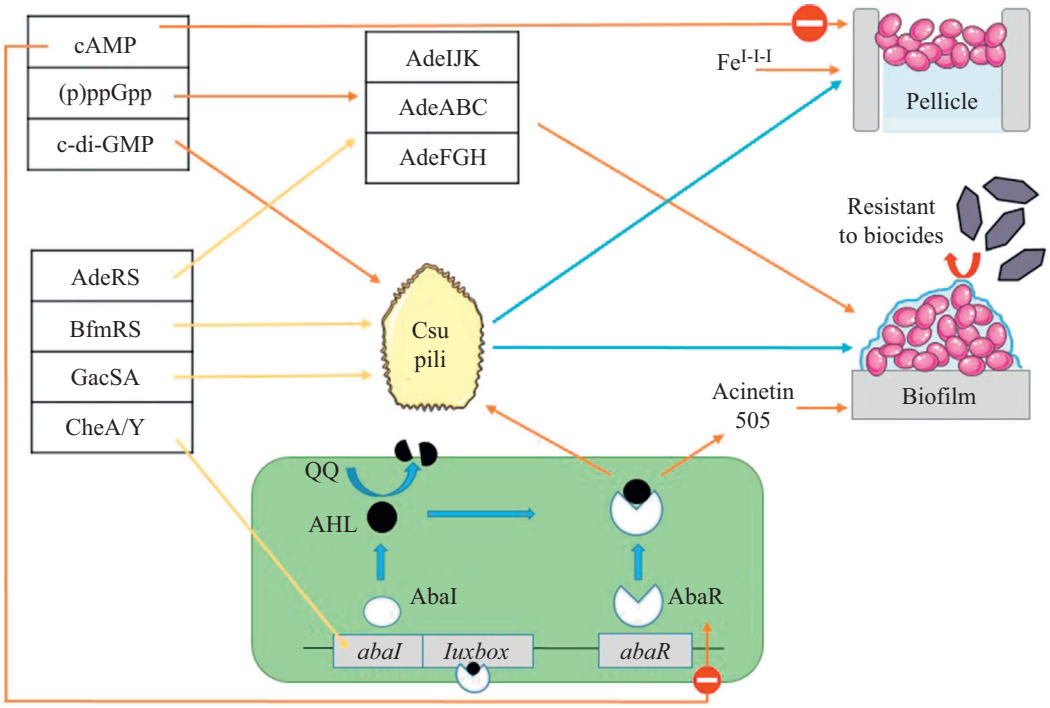

Figure 3. Pellicles formed by *Acinetobacter baumannii* at the air–liquid interface and biofilms on solid surfaces. The nucleotide second messengers, two-component signal transduction systems and quorum sensing (QS) regulate the formation of the *A. baumannii* biofilm and pellicle, while cAMP suppresses pellicle production [9,48]. Cyclic di-GMP (c-di-GMP), BfmRS and GacSA are required for Csu pili synthesis [87,88]. CheA/Y, a two-component hybrid regulator, controls Csu pili and acinetin-505 expression via QS [89,90]. Acinetin-505 is a lipopeptide with a molecular weight of 505 Da that may function as a surfactant to promote surface-associated motility, biofilm formation and virulence [90,91]. An AbaI inducer and its associated receptor AbaR constitute the QS system of *A. baumannii*. AbaR binds N-acyl homoserine lactone (AHL) molecules produced by AbaI, an autoinducer synthase. The AbaR–AHL complexes trigger the production of acinetin-505 and Csu pili by activating the synthesis of AbaI and the activation of QS-dependent genes [90,92]. Biofilm formation can be prevented by quorum-quenching enzymes, which degrade AHLs [9], as well as high concentrations of Fe^{III} ions that bind AHLs [93]. On the other hand, Fe^{III} ions are essential for pellicle formation [94,95]. The AdeABC and AdeFGH efflux pumps controlled by the AdeRS two-component signal transduction system are involved in biofilm formation [96,97]. ppGpp suppresses the synthesis of AbaR and acinetin-505 through regulating the expression of genes encoding efflux pump components [91,98].

Unit transposons

Unit transposons are usually larger than insertion sequences, include inversion repeats instead of two insertion sequences flanking them, and may contain passenger and transposase genes. The Tn3 and Tn7-like superfamilies are mainly linked to antimicrobial resistance [53]. Tn2053-like transposons confer disinfectant resistance [52] (Figure 5). When separate resistance genes were translocated on to the same transposon backbone, this led to several resistance islands evolving in *A. baumannii*. As a result, larger structures with several resistance genes were formed [53]. Parts of these resistance islands are linked to the R1215 plasmid in some bacteria such as *Serratia marcescens*. However, this plasmid has not been proven to be stable in *A. baumannii* [56].

Integrons and gene cassettes

Gene cassettes are short strands of DNA usually found integrated into integrons or in free circular structures. Gene cassettes typically contain one or two genes with no recombination site (*attC*) or promoter [52]. A few components, including an *intI* gene, a promoter and an *attI* recombination site, can be used to identify integrons [53]. Integrons allow resistance genes to move between specified locations and undergo site-specific recombination. As these mobile genetic components are frequently present in many copies across the genome, homologous recombination can be facilitated [52]. When several gene cassettes are placed into the same integron, a cassette array is created. Depending on which passenger genes they carry, cassette arrays can confer antibiotic and disinfectant resistance upon bacteria [27]. As a result, co-resistance to disinfectants and antibiotics has been found in highly-resistant strains [57,58]. Many integrons have their origins in simpler transposons [52]. Both antibiotic and disinfectant resistance are frequently linked to class 1 integrons [53].

Genomic islands and integrative and conjugative elements

A genomic island is a non-native section of a bacterial chromosome introduced by horizontal gene transfer (not native) [52,53]. Integrative and mobilizable elements and genomic elements transported horizontally by phage-mediated conjugation are all terms used to describe genomic islands [59].

ICEs are mobile genetic elements integrated into the host chromosome. These elements are removed from the chromosome after stimulation of ICE gene expression, and can self-

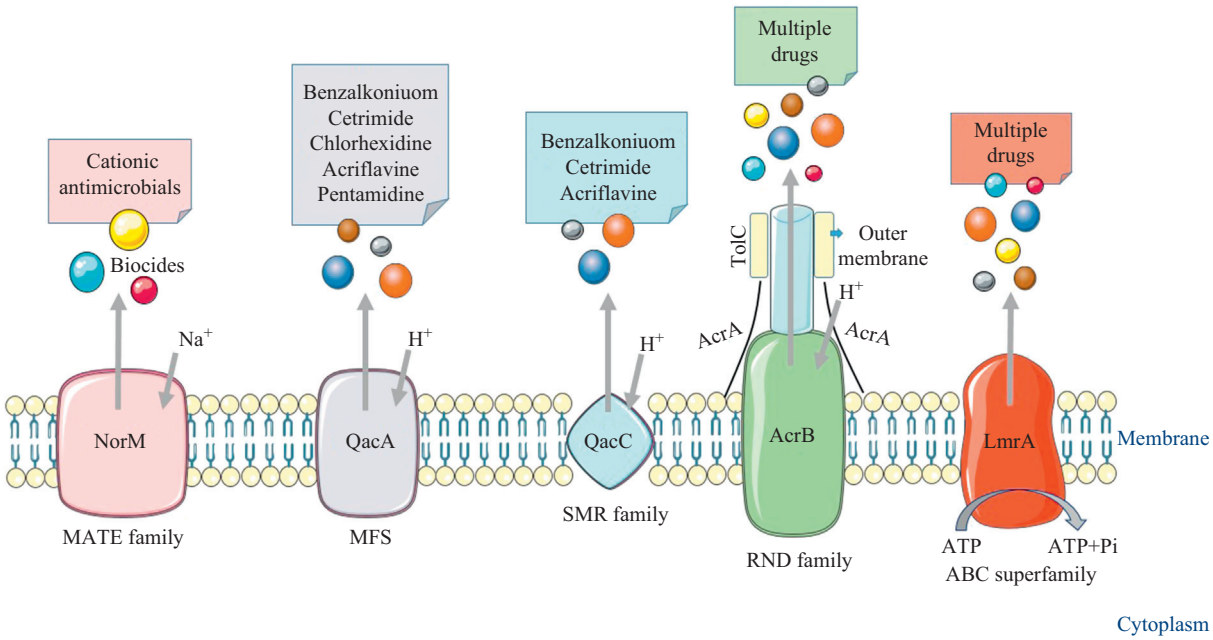

Figure 4. Comparison of the five families of efflux pumps involved in biocide efflux. MATE, multi-drug and toxic chemical extrusion family; MFS, major facilitator superfamily; SMR, small multi-drug resistance family; RND, resistance nodulation-division family; ABC, ATP-binding cassette superfamily.

transmit via conjugation [60]. Cargo genes that are unrelated to the ICE life cycle but impart beneficial phenotypes on host cells, such as resistance genes, are commonly found in ICEs [60]. Even if the ICE does not include resistance gene(s), it is possible that it is responsible for the mobilization of neighbouring resistance islands with an *oriT* site [59]. It has been reported that ICEs can play an essential role in the mobilization of disinfectant-resistant class 1 integrons/transposons [61].

A bacterium's sequenced genome can be used to detect horizontal gene transfer. Transformation is more likely to be the cause of the presence of genes that are found only in distantly related organisms. A cluster of genes with codon bias or a guanine/cytosine content considerably different from the rest of the bacterial genome is another signal [62]. By identifying resistance genes and assessing the effects of their existence, next-generation sequencing and bioinformatic methods provide insight into resistance mechanisms [52].

Laboratory techniques to study biocide resistance/susceptibility in bacteria

Standardization of parameters such as culture medium, inoculum density, incubation temperature and time is required for phenotypic susceptibility testing. As a result, standardized testing methods are essential for obtaining consistent and comparable data [63]. Organizations such as the Clinical and Laboratory Standards Institute and the European Committee on Antimicrobial Susceptibility Testing have described established and matched procedures for antimicrobial susceptibility testing, such as Kirby–Bauer disc diffusion, and micro broth and macro broth dilutions [23,63]. However, no harmonized and approved biocide susceptibility testing method has been developed to date [63]. There have been no documented minimum inhibitory concentration (MIC) breakpoints for specific biocides related to reduce susceptibility until recently. Furthermore, based on a statistical analysis of MIC values in different test samples, various papers have asserted different epidemiological cut-off values [64]. On the other hand, MICs also serve as a helpful reference to biocides once utilized as preservatives, where preventing microbial growth and reducing viability to predefined levels are preferable to inactivation [23]. Microbiologists will be better able to choose appropriate disinfectants if they know the susceptibility breakpoints of biocides. This will aid in monitoring the success of the disinfection programme. However, little progress has been achieved in this approach, and guidelines have not been developed to provide MIC breakpoints.

Accurate determination of resistance/susceptibility is difficult in the absence of valid clinical breakpoints. Nonetheless, phenotypic and genotypic assessments of biocide resistance/susceptibility have been attempted in some studies [30].

Cell membrane permeability changes

Biocides such as chlorhexidine and QAC change the permeability of a bacteria's outer membrane [13,30]. This altered membrane permeability can be measured using a tetraphenylphosphonium ion (TPP+) electrode. The absorption of TPP+ and the efflux of potassium are measured in the test. The presence of ions shows that the membrane has been permeabilized. Due to the existence of charged LPS residues, TPP+ penetration is often blocked by the outer membrane.

On the other hand, the action of biocides causes the outer membrane to permeabilize, allowing TPP+ to diffuse into the cell, followed by the efflux of K+ ions. This assay can be used to check for the development of resistance in bacteria against membrane-acting (permeabilizing) agents [65]. To evaluate the outer membrane's structural organization, energy-

dispersive X-ray analysis (EDAX) has proven to be a valuable resource. In this way, the structure of the membrane is studied using the X-ray diffraction pattern [27].

Uptake/exclusion studies

Drug exclusion experiments can also be used to examine the changed expression of exporter porins. For this type of analysis, compounds such as ethidium bromide, Hoechst dyes and acriflavine are utilized. The absorption of biocides such as chlorhexidine has also been investigated using EDAX. Here, an electron beam is used to irradiate the sample, and the X-ray pattern is evaluated. Since each element emits X-rays with distinct energies, EDAX may be used to determine the elemental composition of a sample, and hence the presence of a biocide such as chlorhexidine [27].

Fatty acid profile of outer membrane

Analysing the lipid or fatty acid profile is used to assess changes in membrane characteristics. For this, lipids from the bacterial surface are collected and studied using gas chromatography or thin layer chromatography. The profile of altered lipids can also be analysed using mass spectrometric techniques, such as matrix-assisted laser desorption/ionization and electrospray ionization. The percentage of each class of lipids, such as saturated vs unsaturated, phospholipids vs amino/glycol-lipids, polar and neutral lipids, and anionic phospholipids, is revealed by lipid analysis, indicating whether the change is beneficial in the development of resistance to biocides or antibiotics. For example, an increase in unsaturated fatty acids improves fluidity, making the organism resistant to antimicrobials. Reduced net negative charge and resistance to cationic antimicrobials can be achieved by reducing anionic phospholipids and increasing cationic phospholipids. Several bacteria change lipid moieties/teichoic acid polymers to get cationic antimicrobial resistance. The addition of groups that cause a positive charge on the lipid surface prevents cationic antimicrobials from interacting with the bacterial surface. In addition, reducing the phosphorous group in lipids decreases the net negative charge on the bacterial surface, making it more resistant to cationic antimicrobials. Increased resistance to cationic biocides can be achieved by altering the structure of LPS [27].

Surface hydrophobicity changes

Changes in cell surface hydrophobicity may limit the biocide's uptake or permeability. The changed hydrophobicity can be determined using the microbial adherence to hydrocarbon method, which involves mixing the bacterial suspension with the appropriate hydrocarbon and estimating the partitioning of bacteria in the hydrocarbon vs aqueous phase [27,66].

Use of microscopic techniques

The ultrastructural variations between susceptible and resistant bacterium isolates have been studied using transmission and scanning electron microscopy. The resistant cells are intensely stained with negative staining (as determined by transmission electron microscopy). They have a rough surface with amorphous material outside (analysed by transmission and scanning electron microscopy). Exopolysaccharide has been stated to be present in the amorphous substance. The susceptibility of biofilms to biocides may be determined using confocal laser scanning microscopy (CLSM), which visualizes the spatiotemporal pattern of the biofilm. Bacteria in the biofilms are dyed with a fluorogenic dye, and disruption to the

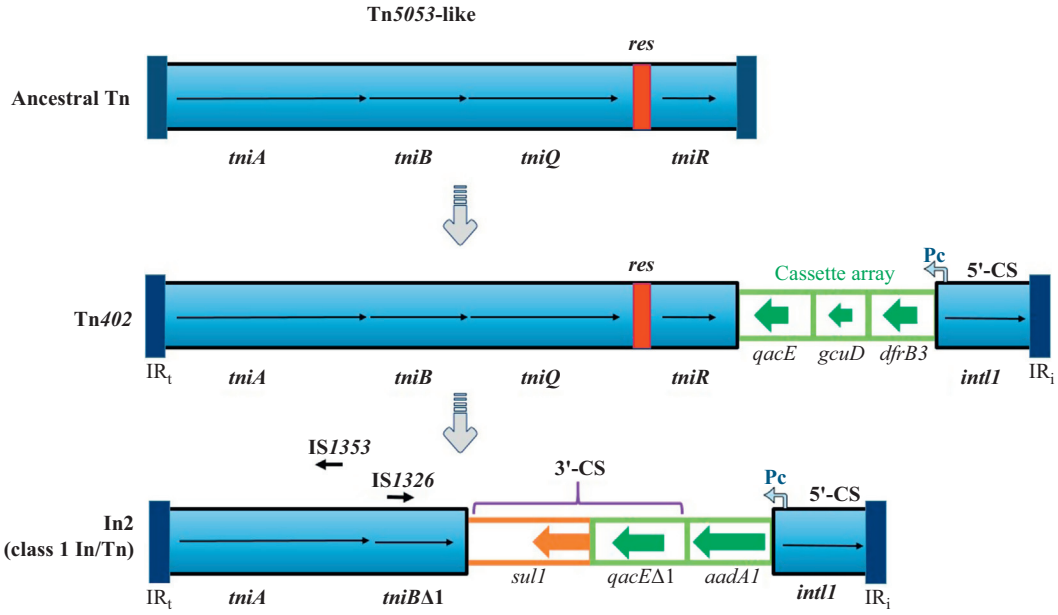

Figure 5. Evolution of Tn5053-like mobile genetic elements. Transposon (Tn) 402, which contains a cassette array of disinfectant resistance genes, integrates and develops a class 1 integron (In)/Tn, allowing disinfectant resistance genes to disseminate more widely. The quaternary ammonium compound resistance gene *qacE* cassette and its derivative *qacEΔ1* are carried by this class 1 In developed from Tn402 [52,53].

bacterial membrane (due to biocides) causes dye leakage, which CLSM monitors in real-time. The patterns of fluorescence loss can help estimate the biocide's selectivity and, if any, its limitations in killing bacteria in biofilms [27,67].

Expression analysis

Polymerase chain reaction (PCR), micro-array, SDS-polyacrylamide gel, and two-dimensional (2D) gel analysis followed by mass spectrometry can be used to identify changes in OMP expression. 2D differential fluorescence gel electrophoresis was recently utilized to detect differentially expressed genes in resistant vs susceptible bacterial strains, as well as differentially expressed proteins found by mass spectrometry. The change in the LPS profile was also analysed using an SDS-polyacrylamide gel. The effective detection of overexpression of efflux genes in biocide-resistant bacteria has been achieved using PCR (real-time or reverse transcription). In *A. baumannii*, *qac* genes and *ade* (multi-drug efflux pumps) are examples of such genes. Micro-array, real-time PCR and proteome analysis can all be used to assess whether the biocide causes any changes in the expression of genes implicated in resistance that are not OMPs. The development of cross-resistance can be tested by PCR-restriction fragment length polymorphism analysis of the known genetic markers imparting antibiotic resistance [22,27].

Nucleotide sequencing

Although several studies have employed nucleotide sequencing and whole-genome sequencing to find antibiotic resistance mechanisms, few studies have utilized similar techniques to find biocide resistance mechanisms [68]. According to nucleotide sequencing results, mutation in the *sdeS* gene enhances production of the SdeAB efflux pump genes, resulting in multi-drug resistance in *S. marcescens* [69]. To identify the bacteria that are resistant to the biocide, flow-cytometric-based tests can be employed [70]. Using either of the approaches mentioned above, fluorescent-activated cell sorting may be used to sort these resistant cells and subsequently analyse the resistance event [27].

Also, it has been reported that in some prokaryotes, genome engineering techniques such as the CRISPR-Cas system can be used to identify disinfection resistance genes, and even reverse resistance [52]. Regarding use of the CRISPR-Cas system, it has been evaluated that 2-aminoimidazole molecules can resensitize bacteria to antibiotics [52,71].

Novel therapeutic approaches for antibiotic-resistant *A. baumannii*

Biocides used in community or hospital settings are either antiseptics or disinfectants. When these compounds remain on any surface at subinhibitory concentrations, even in very minute amounts, it could lead to reduced susceptibility, or even the development of MDR epidemic clones [72]. Common strategies to develop biocide resistance include cellular changes on biocide accumulation, changes in permeability of the cell envelope, modifications in features of the cell surface, and bypass of metabolic blockage [73]. The consequence of biocide resistance is the emergence of antibiotic resistance clones [72,73]. Among these, efflux pumps contribute towards the high level of intrinsic resistance to structurally unrelated agents, and help the evolution of acquired resistance [22].

The ever-increasing antibiotic resistance strains with limited treatment options has prompted researchers to consider new approaches to combat *A. baumannii*-associated infections [74]. It is impossible to ignore previously overlooked modalities with potential therapeutic activity against MDR bacteria [4]. Bacteriophages are one of the best examples of one such modality that has been ignored for many years. Bacteriophages and their encoded products, such as lysins, are being studied extensively as biocide and antibiotic alternatives [75–77]. Wild-type bacteriophages and their enzymatic products destroy target bacteria in the same way as antibiotics. In 2010, the first report of phage isolation and specificity against *A. baumannii* was published [4]. Against *A. baumannii*, the phages AB1 and AB2 have been exhibited to possess lytic behaviour [78]. Since then, many lytic phages have been discovered, described and sequenced. The majority of in-vitro investigations and characterization of phages against *A. baumannii* indicated that they must be tested *in vivo* for effectiveness and pharmacodynamics to combat infectious diseases [78,79].

Usage of monoclonal antibodies is another therapeutic modality; these antibodies bind to pathogen virulence factors and deactivate them [80]. Due to their well-studied phenomena and clinical effects, it appears sensible to utilize them as an alternative. However, their production is too expensive to be used for the routine treatment of infections [81].

Probiotics, as another choice, are living bacteria that have a positive impact on human health. They compete for nutrition and colonization space with the pathogen; however, their specific modes of action are still being researched [4].

Various eukaryotic and prokaryotic organisms produce antimicrobial peptides (AMPs) or short AMPs as part of their innate host immune response. As they have the ability to destroy bacteria, interest in AMPs is increasing. They are broad spectrum in nature, have low resistance and low immunogenicity, and carry a solution of antibiotic and biocide resistance for Gram-negative and Gram-positive bacteria [82]. Several peptides with activity against *A. baumannii* have been discovered *in vitro* and *in vivo*. In a pandrug-resistant strain of *A. baumannii*, a hybrid of cecropin A and melittin showed action in peritoneal sepsis in an animal infection model [83]. Antibacterial activity has also been established for brevinin 2, alyteserin 2 and catonic α-helical peptides against *A. baumannii*. In a mouse model, the proline-rich peptide A3-APO was more effective than imipenem at controlling *A. baumannii* bacteraemia [4]. A *Caenorhabditis elegans* model was protected from lethal infection by *A. baumannii* by a short d-enantiomeric peptide named D-RR4 [84]. Many successful studies on the potential of AMPs against such a resistant organism exist in the literature; however, issues such as cytotoxicity, moderate activity, enzymatic degradation and high production costs must be evaluated [81,84].

Another method to eliminate such bacteria is to use a gene-editing technology that uses the clustered, regularly interspaced short palindromic repeat (Cas) mechanism to knock out the resistance gene, and make it labile to antimicrobial therapy [4].

Chelators for metals, such as iron, zinc and manganese, are essential in producing bacterial virulence factors and may be a

viable target for developing novel antimicrobial therapies. Liposomes, which are artificial nanoparticles composed of lipids that closely resemble the membrane of host cells, can function as decoys for bacterial toxins, causing them to be sequestered and neutralized [85,86].

Conclusion

The development of effective biocides that target antimicrobial-resistant pathogens surviving in the hospital setting is a pressing problem. Intriguingly, a number of biocides have been explored and used; however, precise information about the MIC breakpoints has not been recommended. Regardless of the manner of administration, a biocidal product at a concentration insufficient to kill the target bacteria will create a stress response, resulting in bacterial survival. All environmental isolates must be monitored regularly, and their sensitivity to disinfectants must be assessed. This is a crucial aspect of the disinfection programme and the overall biocontamination management plan. It is expected that comprehensive data collected from healthcare settings may help in the development of strategies for correct biocide usage in the future. These various evaluations will improve understanding of biocide-resistant processes and contextualize the possible threats that certain organisms provide to the environment. To address this, insight into antimicrobial action, resistance mechanisms and the approach to testing biocides will aid the selection of correct or novel antimicrobials.

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