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Resistance of Bacteria to Biocides

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ABSTRACT Biocides and formulated biocides are used worldwide for an increasing number of applications despite tightening regulations in Europe and in the United States. One concern is that such intense usage of biocides could lead to increased bacterial resistance to a product and cross-resistance to unrelated antimicrobials including chemotherapeutic antibiotics. Evidence to justify such a concern comes mostly from the use of health care-relevant bacterial isolates, although the number of studies of the resistance characteristics of veterinary isolates to biocides have increased the past few years. One problem remains the definition of "resistance" and how to measure resistance to a biocide. This has yet to be addressed globally, although the measurement of resistance is becoming more pressing, with regulators both in Europe and in the United States demanding that manufacturers provide evidence that their biocidal products will not impact on bacterial resistance. Alongside *in vitro* evidence of potential antimicrobial cross-resistance following biocide exposure, our understanding of the mechanisms of bacterial resistance and, more recently, our understanding of the effect of biocides to induce a mechanism(s) of resistance in bacteria has improved. This article aims to provide an understanding of the development of antimicrobial resistance in bacteria following a biocide exposure. The sections provide evidence of the occurrence of bacterial resistance and its mechanisms of action and debate how to measure bacterial resistance to biocides. Examples pertinent to the veterinary field are used where appropriate.

BIOCIDE USAGE

Chemical biocides have been used for centuries for making water and foodstuff safe to consume, for treating wounds, and for preserving materials since well before the discovery of microorganisms. Today chemical biocides are heavily used in a wide range of applications and environments including the consumer product, water, wastewater, and food industries; goods manufacturing; the pharmaceutical industry; the health care and veterinary sectors; and the oil and gas industries (1). This wide range of applications reflects the versatility of biocide products for environmental disinfection, product preservation, and antisepsis (2). In Europe it is difficult to estimate the quantity of chemical biocides that are used in products or imported (1), although in 2006 the market for biocides was estimated to be €10 billion to €11 billion (1). It is, however, clear that the usage of chemical biocides is continuing to increase, particularly in consumer products. This increased usage may be partly due to consumers' increased awareness of microbial contamination and infection. The rise in antibiotic resistance in bacteria might also have impacted on the usage of biocides, at least in the health care and veterinary settings (3). Widespread media coverage of issues of hospital cleanliness and "superbugs" have also contributed to better-informed customers, providing better marketing arguments for manufacturers and distributors of biocidal products (3). Alongside a better-informed public, the global increase in antimicrobial resistance in bacteria is forcing decision makers to tackle

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this growing issue. One of the recommended interventions is better hygiene and control of bacteria on surfaces in health care settings but also in animal husbandry (4).

In health care settings biocides are heavily used for the disinfection of environmental surfaces and medical devices and for antisepsis. The growing number of studies highlighting the presence, and at times persistence, of bacterial pathogens, including multidrug-resistant ones, on surfaces despite the use of decontamination (5–14) acknowledges that microorganisms can survive on surfaces and be transmitted to patients, staff, and inanimate objects (15), thus finally emphasizing the importance of controlling the microbial burden on surfaces. This newly found appreciation for controlling microbial pathogens on surfaces has led to an explosion of surface disinfection products and their marketing (16–18), contributing to a higher concentration of biocides eventually released in the environment.

The ability of biocides or biocidal products to decrease the microbial bioburden on surfaces is also highly relevant in animal husbandry, farm buildings, barns, equipment, and vehicles, where their use should contribute to reducing the spread of pathogens. This also includes their use to prevent infectious outbreaks from spreading from farms; for example, large quantities of biocides are being sprayed in the environment and on vehicles in an attempt to decrease the spread of animal viral diseases (19). The heavy use of biocidal products where heavy soiling is present, in particular, their use on vehicle wheels and undercarriages, deserves better scrutiny of its efficacy in preventing potential outbreaks.

The use of biocidal products also includes the disinfection of various environmental surfaces, antibiofouling, the preservation of building materials, and water and wastewater treatment. Biocides play an important role as food preservatives and for controlling microbial contaminants that may enter the food chain during food production. As mentioned previously, one growing area for biocide manufacturers is consumer products, including the preservation of cosmetics, but more recently, personal care products, household products, and textiles.

In Europe, the incorporation of biocides in products and the use of chemical biocides in general is heavily regulated (20), with the consequence that fewer biocides are available for manufacturers to use. This restriction on the number and type of chemical biocides available for manufacturers has, however, not reduced the number of biocidal products and biocide applications. On the contrary, awareness of the role of microorganisms in contamination, infection, or the production of odors, together with the growing threat of bacterial resistance to chemotherapeutic antibiotics, has resulted in the biocidal product market expanding. In Europe, the amounts of chemical biocides used per application is difficult to measure (1). Chemical biocides used in diverse applications eventually find their way to the environment (1). For example, high concentrations of triclosan have been found in river and wastewater effluents (1.4 to 40,000 ng/liter in surface water, up to 85,000 ng/liter in wastewater, and up to 133,000 μg/kg in biosolids from wastewater treatment plants) (20–23). There should be little doubt that chemical biocides even at a low concentration (i.e., sub-MIC level) will exert a selective pressure on microorganisms (18, 24–26), which should be monitored where biocidal products are heavily used (18, 27). The increase in the use of biocides and biocidal products might aggravate the possible link between biocide usage and emergence of antimicrobial resistance in bacteria (1, 3, 18, 28), although there is no doubt that overall biocide usage has brought immense benefit to human and animal health (1–3, 29).

This article explores reports of bacterial resistance to biocides and our current knowledge of the mechanisms of bacterial resistance. It also reflects on the effect of biocides' interactions with bacteria that may lead to a change in susceptibility to antimicrobials. This article does not cover bacterial biofilms.

BIOCIDE RESISTANCE: A QUESTION OF DEFINITIONS

One of the main issues when dealing with bacterial resistance is the definition of "resistance." This definition is linked with the test protocols to measure resistance, and these protocols are described later. There are many definitions of resistance to biocides, some of which describe only a small decrease in susceptibility (18, 28, 31–34). This contrasts with the definition of bacterial resistance to chemotherapeutic antibiotics, which reflects clinical resistance. With biocides the terms "resistance," "tolerance," "decreased susceptibility," "reduced susceptibility," "insusceptibility," and "acquired reduced susceptibility" are used. Such diversity in terms reflects a lack of consensus within the scientific community and is contributing to a degree of confusion in our understanding of bacterial resistance to biocides. From a practical point of view, a bacterium surviving in a biocidal product is resistant to that product, whatever the concentration of biocide is in the product.

Many papers have used the term "reduced susceptibility," which is based on the measurement of the MIC

or the minimum bactericidal concentration. A biocide or biocide product at its in-use concentration may, however, still be effective (18, 35). One of the main difficulties is to determine what fold-difference in MIC or minimum bactericidal concentration reflects a change that will be significant in practice, i.e., a decrease in biocide effectiveness. This is likely to be biocide/biocidal product dependent.

From an academic perspective, other definitions of bacterial resistance have been used: (i) a bacterial strain that is not killed by a biocide concentration to which the majority of the bacterial species are susceptible and (ii) bacterial cells in a culture that survive biocide exposure that kills the majority of the bacterial population in that culture. This latest definition has been used mainly to identify specific mechanisms of biocide resistance in bacteria following stepwise exposure to a specific biocide.

Empirically, bacterial resistance to biocides has been labeled as intrinsic, a natural property of the bacterium, or acquired, following the acquisition of resistance genes or following mutations (36). These definitions still hold true, although the concept of transient resistance, following the expression of a mechanism(s) in response to a direct selective pressure, recognizes that the effect of a biocide on a bacterium may be more complex and short-lived as long as the biocide, exerting a selective pressure, is present (24, 25).

What appears to be more of a concern is the ability of a bacterium to become clinically resistant to an antibiotic(s) following exposure to a biocide/biocidal product. Such cross-resistance has been raised by the European Commission following reports from the Scientific Committee on Emerging and Newly Identified Health Risks (1, 37) and the Scientific Committee on Consumer Safety (38). The Biocidal Product Regulation (20), which regulates the commercialization of biocidal products on the European market, now mentions the potential issue of bacterial resistance and cross-resistance following biocide application. In the United States, the Federal Drug Administration (FDA) recently proposed several rules based on the concern about bacterial resistance linked to the use of certain chemical biocides (39). Demonstrating that a chemical biocide or a biocidal product will not give rise to resistance in bacteria is a question not only of definition but also of methodology.

OCCURRENCE OF BACTERIAL RESISTANCE TO BIOCIDES

Bacterial resistance to biocides and biocidal products has now been well documented in the literature, although examples are often anecdotal where a specific product was investigated. Biocides are a very diverse group of chemicals (1). Surprisingly, bacterial resistance has been studied with only a few biocides. For biocidal products, the formulation will help and hopefully optimize the delivery of the biocide(s) and/or negate some undesirable effects such as corrosiveness of surfaces, pungent smell, poor stability, or toxicity. Components of the formulations may also have a profound effect on biocide efficacy, either increasing or, on occasion, decreasing efficacy. In the peer-reviewed literature, formulations have rarely been studied in the past, although recently, several studies concerned the effect of formulated biocides on bactericidal efficacy (3, 18, 40, 41). Bacterial resistance has been investigated *in vitro* against several chemical classes, including phenolics (e.g., triclosan) (42–49), cationic biocides (e.g., chlorhexidine, quaternary ammonium compounds, particularly cetylpyridinium chloride, and benzalkonium chloride) (50–56), isothiazolinones (57), and more reactive biocides such as iodophors (58), alkylating agents (e.g., glutaraldehyde) (59–64), and several oxidizing compounds (65–68). Studies often differed in their methodology, rendering the comparison of results difficult (1, 18). Using realistic *in vitro* protocols to generate bacteria resistant to a specific biocide is not straightforward either (69). Investigations can generally be divided into four categories:

1. *In vitro* testing of bacterial resistance to a specific biocide, often involving training the bacteria to survive increasing concentrations of a biocide (46–48, 57, 69–73)
2. Studies reporting the isolation of environmental isolates resistant to specific biocides. These investigations principally concern environmental bacterial isolates from, for example, health care settings, manufacturing, and slaughterhouses and include biocides such as glutaraldehyde (59–62, 74), chlorine dioxide (65), chlorhexidine (75–79), triclosan (48, 80), quaternary ammonium compounds (79, 81–83), alcohol, and iodine (75)
3. Studies reporting the contamination of biocidal products principally used in health care settings and possible associations with infection outbreaks and pseudo-outbreaks (74, 84–89)
4. *In situ* studies reporting the impact on bacterial resistance of using specific biocidal products (90–93)

One criticism of *in vitro* studies is that they might not reflect the way bacteria encounter a biocide/biocidal

product in practice (3, 69). For example, the use of stepwise training, i.e., the passaging of bacteria in increasing concentrations of a biocide, does not reflect conditions *in situ*. These studies have, however, yielded many insights on bacterial resistance mechanisms (46–48, 57, 69–71, 94). Another issue is that the development and nature of resistance to a biocide depend on the bacterial isolates investigated. Ciusa and colleagues (95) reported that bacterial strains from standard culture collection were not necessarily appropriate to study mechanisms of resistance to triclosan because they did not reflect the level and type of mutations observed with clinical isolates when exposed to bisphenol. This study also highlighted that valuable information is being learned through the study of large numbers of isolates (in this study 1,388 *Staphylococcus aureus* isolates were used) and questioned studies reporting the use of a single isolate (95).

The study of environmental isolates rather than standard culture collection strains yields important and probably more relevant information in terms of expressed mechanisms of resistance. Such investigations reiterate that bacteria can express multiple mechanisms at the same time and that some mechanisms responsible for a stable biocide-resistant phenotype are still unknown. Martin et al. (96) described a vegetative *Bacillus subtilis* endoscope washer isolate with stable resistance to the in-use concentration of chlorine dioxide and hydrogen peroxide, but also to peracetic acid (96, 97). Although this isolate is a good biofilm producer, the mechanisms responsible for the observed level of resistance to these oxidizing agents have not all been identified (96). Other studies that have isolated bacteria from environments where antimicrobials are heavily used identified a decrease in biocide susceptibility (81, 82, 95, 98) in some but not all isolates when compared to counterpart bacteria from standard culture collection (95, 98).

Studies reporting bacterial growth in biocidal products and subsequent infections or pseudo-infections have been very helpful in identifying the risks associated with some products and practices (89). Reported incidents often result from the inappropriate application of a product or the inappropriate preparation of a product, including the use of contaminated tap water, topping up of stock solutions, use of diluted products or inappropriate dilution, and inappropriate storage conditions. Some microorganisms, notably *Pseudomonas* spp., *Burkholderia* spp., and atypical mycobacteria can, however, contaminate the stock solution of a product because of their intrinsic resistance to the product (89). The preconceived idea that bacterial resistance occurs more readily in less reactive biocides such as phenolics (e.g., triclosan) and cationic biocides (e.g., chlorhexidine) rather than reactive ones such as alkylating and oxidizing agents does not hold true. For example, there have been many studies on atypical mycobacterial (*Mycobacterium chelonae*) resistance to 2% glutaraldehyde, which is used for the high-level disinfection of medical devices (59–64). It was speculated that these bacteria arose from a decrease in the effective concentration of glutaraldehyde (i.e., <2%) (60). Fisher et al. (99) reported the presence of glutaraldehyde-resistant atypical mycobacteria associated with endoscope reprocessing systems. Outbreaks of *M. chelonae* linked to endoscope reprocessing using glutaraldehyde have been described since 1991 (100). The more recent nosocomial outbreaks of *Mycobacterium abscessus* subsp. *massiliense* in Brazil, however, identified an isolate that was resistant to both 2% glutaraldehyde and first-line antimycobacterial antibiotics, highlighting the existence of cross-resistance mechanisms that remain to be identified (74).

Studies of the effect of biocidal product applications on emerging bacterial resistance in the community or health care settings remain scarce. These studies usually highlight the difficulty in data interpretation, notably in relation to the definition of "bacterial resistance." The few *in situ* studies nevertheless provide interesting insight on the long-term usage of selected biocidal products. Two studies from Cole and colleagues failed to show any cross-resistance between antibiotics and antibacterial wash products (91, 92). Likewise, Aiello et al. (90) failed to show any statistically significant correlation between the use of triclosan-containing product and reduced susceptibility to antibiotics. A study of benzalkonium chloride-containing product usage in households, however, found a correlation between elevated QAC MIC and bacterial resistance to antibiotics (93).

There should be no doubt that bacteria have a great ability to survive biocide exposure and that the inappropriate use or preparation of biocidal products can result in bacterial resistance. The reporting of cross-resistance between biocides and unrelated chemicals such as chemotherapeutic antibiotics is increasing as scientists focus more on this possibility.

MECHANISMS OF BACTERIAL RESISTANCE

Biocides and biocidal products induce stress on the bacterial cell. In response, a bacterium expresses several mechanisms to prevent the detrimental effect caused by

TABLE 1 Levels of biocide interactions with a bacterial cell

Exposure	Interactions	Types of damage	Events
Short exposure	Disruption of the transmembrane PMF leading to an uncoupling of oxidative phosphorylation and inhibition of active transport across the membrane		Reversible
	Inhibition of respiration or catabolic/anabolic reactions		
Prolonged exposure	Disruption of metabolic processes		Reversible
	Disruption of replication		
	Loss of membrane integrity resulting in leakage of essential intracellular constituents (K^+, inorganic phosphate, pentoses, nucleotides and nucleosides, proteins)	Imbalance of pHi	Irreversible
	Coagulation of intracellular materials	Commitment to cell death (autocidal pathway)	
	Lysis	Cell death	

a biocide. These mechanisms aim to decrease the biocide concentration sufficiently that it is no longer damaging to the bacterial cells and include the ability of the bacterium to repair damages. If damage cannot be repaired efficiently or worsens, for example, because of high metabolic activity, the bacterial cell will be committed to a lethal pathway (Table 1). By some accounts that the maintenance of the cytoplasmic pH is key in that pathway (101, 102). Overall, our understanding of the bacterial mechanisms in place to decrease the susceptibility of a bacterium to biocides has improved, but they remain poorly studied. There is no doubt that bacteria have a plethora of mechanisms at their disposal and that often several mechanisms contribute together to the observed resistance phenotype. Our understanding of the effect of biocide interaction with bacteria and especially the stress response effect on gene expression remains poor. Examples given in the literature are often anecdotal. Understanding and measuring the expression of mechanisms following a biocide or biocidal product interaction with a bacterium has become important because it underlies the principle of the observed transient phenotypic changes in bacteria and the cross-resistance mechanisms between antimicrobials.

Mechanisms that Decrease the Concentration of Antimicrobials in Bacteria

Bacteria can use several mechanisms to decrease the lethal or inhibitory concentration of a biocide. Biocides have multiple target sites against the bacterial structure and as such they are often regarded as nonspecific. The sum of the damage caused to multiple target sites and the importance of the target sites defines whether the interaction will lead to a lethal or inhibitory effect (Table 1) (3, 101–105). Decreasing a damaging concentration of a biocide/biocidal product will enable the target bacteria to survive. It should be recognized that a low concentration (sub- MIC) of a biocide will affect the bacteria and, notably, trigger mechanisms to further decrease the biocide concentration. It is now well established in *in vitro* laboratory experiments but also in practice that a low concentration of a biocide will give rise to bacteria that are less susceptible to the biocide, enabling at times the survival of the bacteria in products (60, 89, 96).

Furthermore, biocides are used in complex formulations (i.e., the biocidal product) in practice, yet the effect of a biocidal product on bacterial resistance is not often tested (3, 18, 41, 76, 81). Excipients such as surfactants, chelators, and wetting agents may have a direct effect on the bacterial cell structure and increase the efficacy of a biocide. Arguably, there is sometimes incompatibility between a biocide and an excipient, effectively reducing the bactericidal activity of the product.

Reducing biocide penetration

The effect of bacterial cell structure to prevent or reduce the penetration of antimicrobials has been well established, notably with bacterial endospores (106), Gram-negative bacteria, and mycobacteria (103, 104). The presence of the lipopolysaccharide layer in Gram-negative bacteria has been well documented for its role in decreasing the activity of several membrane active agents such as quaternary ammonium compounds and biguanides. Evidence of the role of lipopolysaccharide in decreasing the activity of a membrane active agent has often been indirect with the use of permeabilizing agents such as chelators and the use of bacterial protoplasts (103, 105, 107, 108). Genetic alterations of the bacterial membrane with, for example, transposon mutagenesis have also provided some important information on

biocide/bacterial cell interactions (109). In mycobacteria, in the presence of mycolic acid associated with the arabinogalactan/arabinomannan cell wall, the lipid-rich outer cell wall is responsible for the lack of penetration of many antimicrobials (61, 104, 110–113). Likewise, porins have been shown to play an important role in the activity of glutaraldehyde and *ortho*-phthalaldehyde in mycobacteria (114). Reducing the expression of porins has been associated with reduced biocide and antibiotic efficacy (115, 116). Changes in bacterial cell membrane and cell wall composition following biocide exposure have been associated with a reduction in biocide activity (94, 115–120). Membrane alterations include membrane protein composition (57, 115, 121, 122), fatty acids (115, 123–127), and phospholipid content (128). A change in membrane potential has also been associated with a decrease in biocide susceptibility in *Pseudomonas aeruginosa* (129).

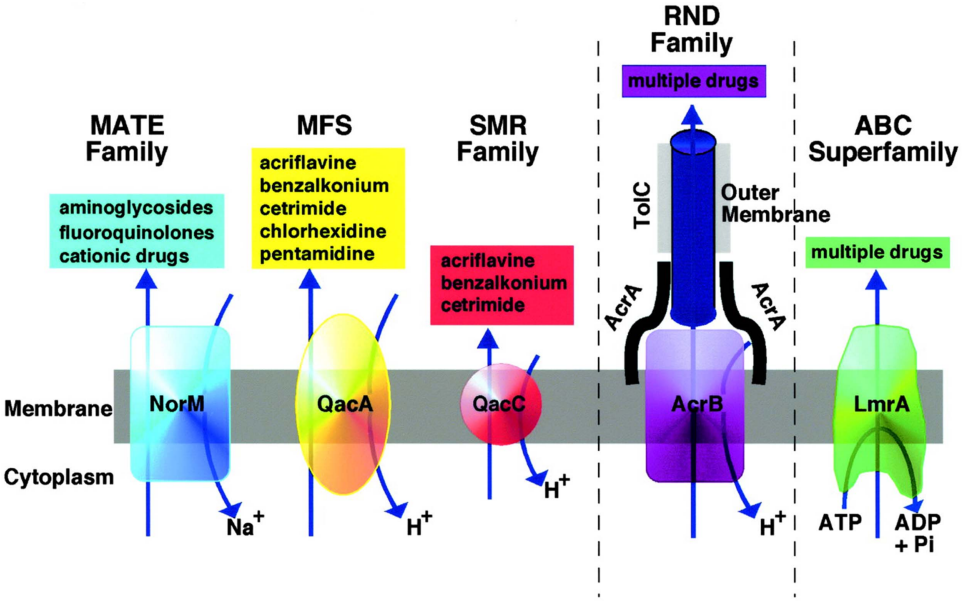
Efflux pumps

Efflux pumps, which are widespread in bacteria, contribute to decreasing the concentration of antimicrobials that penetrate the bacterial cells. The effect of active efflux on antimicrobial activity has been particularly well documented in *S. aureus* (130–139), *P. aeruginosa* (140–145), *Escherichia coli* (46, 82, 94, 146–149), *Salmonella enterica* serovar Typhimurium (150, 151), and *Acinetobacter baumannii* (116, 152). Five main classes of efflux pumps have been reported (Fig. 1) (153; 160): the drug/metabolite transporter superfamily, the major facilitator superfamily, the ATP-binding cassette family, the resistance-nodulation-division family, and the multidrug and toxic compound extrusion family.

The ability of efflux pumps alone to confer resistance to biocides/biocidal products is questionable, and it is likely that efflux pumps are part of several mechanisms used by a bacterium to survive biocide/biocidal product exposure (3, 83, 155). Some studies investigating triclosan claimed, however, that efflux was responsible for high-level resistance to the bisphenol (142, 144). Studies of bacterial isolates from environments where antimicrobials are heavily used, notably biguanides and QAC, have identified a high prevalence of efflux genes (e.g., *qacA/B*, *norA*, *nor B*, *smr*) in isolates that showed a decreased susceptibility to biocides (77–79, 82, 135).

Efflux can be induced by some antimicrobials (153, 156, 161). The expression of an efflux pump can increase following antimicrobial exposure, not necessarily by inducing the efflux pumps but by affecting global gene regulators, notably *marA* and *soxS* (46, 162). The effect of triclosan on bacteria has been particularly well studied with regard to efflux (46, 49, 140–142, 163, 164). In *S. enterica* serovar Typhimurium, overexpression of efflux results in decreased antimicrobial

FIGURE 1 Diagrammatic comparison of the five families of efflux pumps (reproduced from reference 153). MATE, multidrug and toxic compound extrusion; MFS, major facilitator superfamily; SMR, •••; RND, resistance-nodulation-division; ABC, ATP-binding cassette.



susceptibility (162–165). Overexpression of efflux pumps resulting in decreased biocide efficacy has also been described in *Stenotrophomonas maltophilia* with the overexpression of SmedEF (166); in *E. coli* with the overexpression of *acrAB*, *marA*, or *soxS* (46, 49, 162); and in *Campylobacter jejuni* overexpressing CmeB (167). The extent of efflux pumps and their role in bacteria are continuously evolving in the literature. Triggering overexpression of efflux in bacteria following biocide exposure is a concern that is debated later in this article.

Enzymatic degradation

Some bacteria can produce enzymes that degrade biocides. The presence of catalase and superoxide dismutase, for example, has been shown to decrease bacterial susceptibility to oxidizing agents (66, 168). The production of enzymes alone conferring resistance to a biocide is, however, doubtful. This would suggest that enzymatic activity is high and that enzymes are not themselves affected by the biocide. It is more likely that the production of detoxifying enzymes contributes to the battery of mechanisms available to the bacteria to survive biocide injuries (96).

Other examples of enzymatic activity conferring decreased susceptibility to a biocide include the parabens (169, 170), aldehydes (171), and metallic ions. In the latter case the ions are reduced to the inactive metal (34).

Physiological and Metabolic Changes

Bacterial metabolism can be associated with antimicrobial efficacy in that bacteria with a high metabolism are more susceptible to antimicrobials than those with no metabolic activity (172). Exposure of a bacteria to a physical or chemical process, such as a biocide/biocidal product, results in a mixed population of dead, injured, and uninjured bacteria. In the food industry, the recovery of injured bacteria is considered essential (173). This is not so when the efficacy of a biocide treatment is measured. Standard efficacy tests do not consider the effect of the recovery media and incubation conditions post-biocide treatment. The impact of resuscitated injured bacteria following treatment has been exemplified by the dual use of traditional plate counting on a rich nonselective recovery media such as tryptone soy agar and the use of the Bioscreen microbial growth analyzer, which measures bacterial growth in liquid (174). The ability of a bacterium to repair injuries is likely to play an important role when resistance is considered. As shown in Table 1, initial damage caused by a biocide is reversible. In practice, where incubation conditions posttreatment favor recovery from injury, repairs can be visualized with an extended lag phase (173). Biocide exposure has, however, been linked to a decreased growth rate and extended lag phase in bacteria (172, 175–177) because of a direct action of the biocide on the bacterial cells, although in many studies the ability of bacteria to repair injuries was not considered. Change in metabolic pathways has been particularly well exemplified with *S. enterica* exposure to triclosan. The bisphenol at a low concentration has been shown to target specifically the enoyl acyl carrier reductase in bacteria, which affects fatty acid lipid synthesis in the target bacteria (47, 177–179). Webber et al. (180) showed that *S. enterica* could alter its metabolic pathway to produce pyruvate and fatty acids following triclosan exposure. This change was part of a "triclosan resistance network" involving the expression of distinct mechanisms (180). Curiao and colleagues reported similar findings, evoking multiple pathways in the adaptation of *S. enterica* to triclosan and other biocides such as chlorhexidine and benzalkonium chloride (181).

Codling and colleagues (109) showed that in *Serratia marcescens*, the disruption of biosynthetic and metabolic pathways of the bacterium increased bacterial susceptibility to a QAC. A change in metabolic processes following exposure to biocides has also been observed in other bacteria, including *S. aureus* (182) and *P. aeruginosa* (183). The full impact of a change in metabolic pathways on decreasing biocide/biocidal product efficacy has not been assessed, nor has the reproducibility of such a change when exposed to specific antimicrobials. At present, such observations have been bacteria/biocide specific.

Mutations

Mutations in bacteria are by nature random but can be driven by the continuous presence of a selective pressure, notably the presence of antimicrobials. Although it is widely recognized that the presence of chemotherapeutic antibiotics will drive target site mutations, there are far fewer examples with biocides. Mutations resulting from a biocide exposure have been mainly described with triclosan in several bacteria (43, 178, 180, 184–190). Mutations are linked to the use of triclosan at a low concentration and concern the enoyl-acyl reductase carrier protein (47, 179, 186, 191–193). In Salmonella biocide exposure resulted in mutations included derepression of multidrug efflux pump AcrAB-TolC, and *rpoA*, which controls the RNA polymerase α-subunit (190). Interestingly, the investigation of 1,388 *S. aureus* clinical isolates' response to triclosan exposure identified

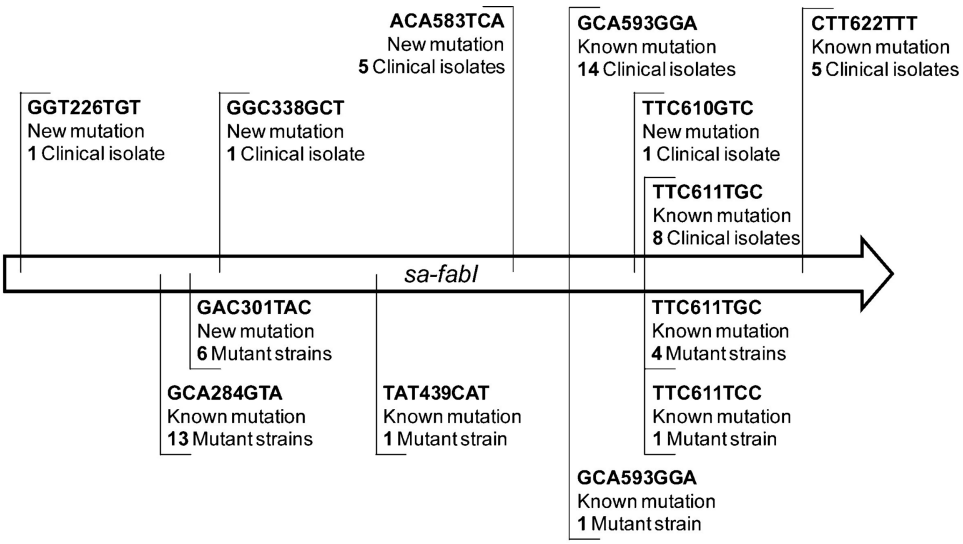


FIGURE 2 Schematic map of mutations in the *Staphylococcus aureus fabI* (*sa-fabI*) and *Staphylococcus haemolyticus fabI* (*sh-fabI*) genes. Mutations in *sa-fabI* are reported on a schematic map. Mutations detected in clinical isolates are mapped above the sequence, while mutations selected *in vitro* are shown below the sequence. (Reproduced from reference 95.)

several mutations that were similar among the isolates (Fig. 2) but not comparable to those observed with the standard culture collection strain (95). Recent observations questioned the choice of a culture collection strain to study biocide resistance. Standard strains have been widely used in biocide resistance studies with the comparability of results between studies in mind. From Ciusa et al. (95), it appears that standard strains and clinical isolates do not behave the same way. Stepwise training protocols that rely on passaging bacteria in increasing concentrations of biocides have yielded bacteria with decreased susceptibility or resistance to a given biocide but may be criticized for not reflecting real-world conditions (3). Many *in vitro* studies have investigated genetic changes in standard culture collection strains following biocide exposure. The work of Ciusa et al. (95) addresses the appropriateness of this approach and would favor the use of environmental isolates that have been exposed to a biocide/biocidal product.

INDUCTION OF GENE EXPRESSION CONFERRING BACTERIAL RESISTANCE

Biocide exposure even at a low concentration produces a stress on the target bacteria, even if the bacteria are intrinsically resistant to the biocide. The bacterial response to the stress will lead to a change in gene expression (24, 109, 116, 155, 162–167, 181, 194–196), particularly that of regulatory genes (46, 49, 68, 149, 162, 180, 197). The concentration of a biocide that is available to interact with the target bacteria is thus paramount (2, 3, 105, 198, 199), since a low, nonlethal concentration will not kill the bacterium but will undoubtedly produce a stress response. An indication of stress response is given by investigating the bacterial growth curve in the presence of a biocide at different concentrations. Increased lag phase or decreases in bacterial doubling time are indicators of a bacteria/biocide interaction and may reflect the induction/expression of mechanisms enabling the bacteria to decrease the toxicity of the biocide (28, 72, 172) and, as mentioned, allow earlier repair of injuries. Some bacterial mechanisms that play a role in decreasing the susceptibility to biocides are controlled by global regulators such as *soxS* and *marA* (46, 49, 146, 162). Antibiotic resistance mechanisms are also controlled by the same regulators (168, 200), which leads to the concern that biocide exposure can trigger antibiotic resistance. The induction of gene expression of global regulators leading to the expression of several mechanisms in bacterial resistance might not, however, be particularly problematic for the use of biocidal products since such expression might be transient. Some studies have shown that a decrease in bacterial susceptibility to biocides and sometimes to antibiotics was transient and only observed in the presence of the biocide (24, 25, 155).

As mentioned earlier, the efficient repair of sublethal injuries may play an important role in bacterial survival

of biocide exposure. However, bacteria's ability to repair damage following biocide exposure has received little attention (195, 201, 202). In *E. coli* polyhexamethylene biguanide alters the expression of several genes, notably *rhs*, involved in repairing nucleic acid (202). The involvement of effective DNA repair mechanisms has been proposed to explain the high-level resistance of an environmental isolate of *B. subtilis* to several oxidizing agents (96). Efficient DNA repair mechanisms enable *Deinococcus radiodurans* to survive ionizing and UV radiation and exposure to chemicals that damage nucleic acid (203). In *Lactobacillus pentosus*, strains that have adapted to sublethal concentrations of antimicrobials overexpressed ribosomal proteins and glutamyl tRNA synthetase, which was interpreted as a response to damaged proteins directly caused by the antimicrobial exposure (195).

CROSS-RESISTANCE

Exposure to a biocide/biocidal product can lead to a stress response involving the expression of global gene regulators and ultimately the expression of nonspecific mechanisms enabling bacterial survival (116, 155, 156, 162, 181, 190, 200, 204–211). The link between biocide usage and antibiotic resistance has led to many discussions with conflicting evidence; some studies support a link, while others fail to identify any cross-resistance (1, 24, 25, 48, 73, 78, 79, 81, 82, 90–93, 98, 196, 212–220). Where cross-resistance between biocide exposure and antibiotic resistance was identified, suggested common resistance mechanisms included overexpression of efflux (18, 82, 153, 156, 161), changes in bacterial cell wall permeability (115, 117), and changes in bacterial metabolism (180). Differences in protocols to (i) grow test bacteria, (ii) expose test bacteria to the biocide/biocidal product, and (iii) measure resistance to biocides and antibiotics contribute to differences in reported observations of the biocide's effect on antibiotic resistance (18). Although the evidence is mainly *in vitro* based, the few *in situ* studies conducted also reported conflicting information about the association between the usage of biocidal products at home and an increase in antibiotic resistance among environmental isolates (89–93). It is worth noting, however, the study from Duarte and colleagues (74) reporting a postsurgical outbreak of a *M. abscessus* subsp. *massiliense*-resistant clone resistant to 2% glutaraldehyde and resistant to frontline antimycobacterial antibiotics.

There should be no doubt that bacteria have the capacity to express mechanisms that will lead to decreased susceptibility to both biocides/biocidal products and chemotherapeutic antibiotics. The question remains as to how commonly cross- resistance occurs in practice and what triggers emerging resistance in the first place. For example, efflux can easily be triggered in bacteria, not only by biocides but by a wide range of stimuli, such as spices and essential oil. (221).

MEASURING BACTERIAL RESISTANCE

One of the most important aspects of biocide/biocidal product resistance is how to measure bacterial resistance and cross-resistance. This has become even more pressing with the publication in Europe of the Biocidal Product Regulation (20), which asks manufacturers to demonstrate that their biocidal product will not cause emerging bacterial resistance. Likewise, in North America the FDA (39) issued a final rule on the safety and effectiveness of antibacterial soaps, effectively banning the use of certain biocides for that application. Rules concerning benzalkonium chloride, benzethonium chloride, and chloroxylenol, biocides that are commonly used in several products, deferred. One major issue for manufacturers is that neither the Biocidal Product Regulation nor the FDA indicate what appropriate tests should be conducted to demonstrate the safety of biocidal products where bacterial resistance is concerned.

MIC determination has often been used as a marker for resistance (18, 28, 69–71, 183, 219), although the validity of MIC to measure bacterial resistance has been questioned (1, 3, 18) mainly since in practice, biocides are often used at concentrations exceeding the MIC (120) and biocides are used as part of a formulation whose ingredients will impact on product efficacy (3, 18, 76). MIC could, however, be used as a trend indicator (3, 18, 25, 28, 76, 198, 199, 222, 223). It is thus unfortunate that some studies measure an increase in bacterial resistance in terms of MIC (28, 224). Other studies have used a prevalue biocide concentration above which the environmental isolates were considered to be resistant. For example, Lavilla Lerma and colleagues used a threshold biocide concentration of 0.025 μg/ml at which any bacterial growth was considered bacterial resistance to the biocide (81). Furthermore, resistance has sometimes been defined as a small increase (e.g., 2-fold) in MIC. This definition remains questionable, especially when using a standard protocol such as a broth microdilution method (225) because a 2-fold change might only reflect a 1-dilution difference. The determination of changes in minimum biocidal concentration might be more appropriate, because this indicates a

change in the lethal effect of the biocide (18, 223). Some studies have looked at a change in inactivation kinetics. Such protocols, although very useful because they determine the ability of a biocide/biocidal product to kill target bacteria over time, are very cumbersome and time-consuming and would not be able to be used routinely (3, 18).

The determination of a change in the susceptibility profile to chemotherapeutic antibiosis is somewhat easier to perform because the protocols used can follow well-established standards that provide clear guidance but also breakpoints for selected bacteria/antibiotics (226, 227). It is, however, clear that the clinical significance should be reported rather than reporting a mere change in the antibiotic zone of inhibition.

Measuring a change in the susceptibility profile to determine a prediction of the risk associated with biocidal product usage is acceptable if the exposure of the target bacterium to the biocidal product is realistic, i.e., if it reflects *in situ* exposure of bacteria with the biocidal products, encompassing dilution of the product upon usage if necessary, extended contact time for residual activity, etc. (18, 25). The test bacterial inoculum preparation needs to be strictly controlled to ensure reproducibility of the assay. When a significant change (here significant means a ≥10-fold change) in susceptibility profile is recorded (25, 222), the nature of this change, whether transient or permanent, needs to be established (18, 25, 223).

A protocol to predict the change in susceptibility profile of target bacteria following exposure to a biocide/biocidal product has been proposed (18). The use of such a protocol established the effect of various biocidal products on *S. enterica* (223), *E. coli*, and *S. aureus* (25). In these studies, triclosan was used as a positive control (25, 223). Triclosan is the most studied biocide in terms of interaction with bacteria. Studies have repeatedly show amended bacterial susceptibility profile to triclosan and antibiotics following exposure to the bisphenol. (23, 25, 228).

CONCLUSIONS

Biocidal products are useful compounds to control microbial contamination and kill pathogens. A biocidal concentration that will not kill the target bacteria will, regardless of the method of application, cause a stress response, which will lead to the expression of mechanisms that enable bacterial survival (3, 18, 28, 173). The concentration of biocide available to interact with the target bacteria is thus paramount. In the veterinary field, the presence of organic matter at the point of the biocidal product application, contributes to reduce the efficacy of the product, and therefore, cleaning the animate or inanimate surface prior to use of the biocidal product should be indicated but might not be practical.

Biocidal products are heavily used for veterinary applications, notably in animal husbandry, disinfection of udders in dairy animals, and in fish farming (1). Despite an increasing use of biocidal products, information related to the occurrence of bacterial resistance in these environments remains scarce (55, 73, 150, 229, 230). Nevertheless, with the growing knowledge and evidence of bacterial resistance in environments where biocides/biocidal products are heavily used, environmental surveillance has been timidly proposed to study the potential spread and occurrence of resistant bacteria (27, 73, 77).

With an increase in biocidal product usage, emerging bacterial resistance is possible, but to date, the risk associated with biocidal product usage has not been measured, mainly because of the lack of standard protocols. The only protocol to date has not yet been widely used against a small number of bacteria (18, 25, 223). The reproducibility of the data obtained may also depend on the bacterial species investigated (24). Such a predictive protocol also relies on using appropriate test parameters that reflect the biocidal product usage in practice (25, 223). Overall, investigating the biocidal effect on bacterial resistance should be welcome because it provides a better understanding of the biocide-bacteria interactions and should contribute to the development of more performant and safer biocidal products. This is particularly pertinent with the increased usage of biocidal products and the usage conditions in animal husbandry.

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