

Review

Adaptive Protection and Cross-Resistance to Antibiotics of *Stenotrophomonas maltophilia* Mediated by Biocides: Molecular Mechanisms

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Abstract

Stenotrophomonas maltophilia is considered an emerging multidrug-resistant opportunistic pathogen. The prevalence of *S. maltophilia* community and healthcare-associated infections is increasing, particularly in immunocompromised individuals. The persistence of *S. maltophilia* in healthcare facilities and hospital environments, where biocides are applied intensively to control microbial contamination and infections, is one of the leading factors that enables its transmission. This bacterium has developed both intrinsic and acquired resistance mechanisms to overcome biocide toxicity. In this review, we describe the function and mechanisms of gene regulation of the different biocide resistance genes that have been reported in *S. maltophilia* to date. The expression of some biocide resistance genes is controlled by inducible transcriptional regulators, which allow adaptive resistance against biocides. Interestingly, some biocide-inducing genes, such as *smeDEF*, contribute not only to resistance against biocides but also antibiotics. This raises the concern that the overuse and misuse of biocides in healthcare settings can induce cross-resistance to multiple antibiotics.

Keywords: antimicrobial resistance, antibiotic, biocide, gene regulation, *Stenotrophomonas maltophilia*

Резюме

Stenotrophomonas maltophilia се счита за нововъзникващ мултирезистентен опортюнистичен патоген. Разпространението на инфекции, причинени от *S. maltophilia* в общността и свързаните със здравеопазването инфекции, се увеличава, особено при имунокомпрометирани лица. Персистирането на *S. maltophilia* в здравните заведения и болничната среда, където биоцидите се прилагат интензивно за контрол на микробното замърсяване и инфекции, е един от водещите фактори, които позволяват предаването му. Тази бактерия е развила както вътрешни, така и придобити механизми на резистентност, за да преодолее токсичността на биоцидите. В този преглед описваме функцията и механизмите на генна регулация на различните гени за резистентност към биоциди, които са докладвани при *S. maltophilia* до момента. Експресията на някои гени за резистентност към биоциди се контролира от индуцируеми транскрипционни регулатори, които позволяват адаптивна резистентност срещу биоциди. Интересното е, че някои гени, отговорни за експресията на биоциди, като *smeDEF*, допринасят не само за резистентност към биоциди, но и към антибиотици. Това поражда опасения, че прекомерната употреба и злоупотребата с биоциди в здравните заведения може да предизвика кръстосана резистентност към множество антибиотици.

Introduction

Stenotrophomonas maltophilia, which was initially classified as *Bacterium booker* and later designated *Pseudomonas maltophilia* and

Xanthomonas maltophilia, is an aerobic gram-negative bacillus belonging to the class Gammaproteobacteria (Looney *et al.*, 2009). *S. maltophilia*

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causes healthcare-associated infections mainly in long-term hospitalized and cystic fibrosis patients globally (Karpati *et al.*, 1994; Brooke, 2012). It is also capable of causing community-acquired infections, particularly in immunosuppressed individuals and children (Prates *et al.*, 2020; Jiang *et al.*, 2022). *S. maltophilia* is ubiquitous in soil and aquatic environments (Chauviat *et al.*, 2023). Its ability to adhere to and form biofilms on both the biotic and non-biotic surfaces of indwelling medical devices and the tubing of medical equipment as well as its capacity to survive with minimal nutrients, for example, medical-grade ultrapure water, hemodialysis water, normal saline solution, hand-washing soap, and topical antiseptics, makes this bacterium one of the major causes of hospital-acquired infections (Brooke, 2012; Adegoke *et al.*, 2017; Kanaujia *et al.*, 2022). The major concern regarding *S. maltophilia* infections is the bacterium's heavy shield of intrinsic and acquired antimicrobial resistance mechanisms, which render treating such infections challenging and costly (Crossman *et al.*, 2008; Adegoke *et al.*, 2017).

The emergence of biocide resistance in pathogenic bacteria and its potential for cross-resistance to antibiotics pose a serious health threat and have resulted in the spread of hospital-acquired infections (Gupta *et al.*, 2018). Biocides, which include antiseptics and disinfectants, are antimicrobial chemical agents that are used extensively in healthcare settings to kill or reduce the number of unwanted microorganisms in order to control their dissemination, thereby preventing or diminishing the incidence of hospital-acquired infections (Gerald *et al.*, 2023). Biocides are also heavily used as antimicrobials in domestic products and various industries to control microbial contamination. Common biocides include ethanol, hydrogen peroxide (H_2O_2), peracetic acid, quaternary ammonium compounds (QACs), biguanides, formaldehyde, glutaraldehyde, chlorhexidine, triclosan, sodium hypochlorite (Jones and Joshi, 2021). Bacteria have thus developed various strategies, both intrinsic and acquired, that allow them to adapt and survive under biocide stress conditions. The mechanisms of increased tolerance to biocides include changes in membrane permeability to reduce the influx of biocides, the extrusion of biocides through efflux pumps, and the degradation of biocides to less toxic products (Wand and Sutton, 2022).

In bacteria, alterations in the cell wall, such as changes in the lipopolysaccharide (LPS) structure or modifications to the LPS charge, contribute

to the tolerance of bacteria to the cationic biocide chlorhexidine (Tattawasart *et al.*, 2000; Clarke *et al.*, 2023). Modifications to the cell membranes, including changes in the outer membrane proteins, fatty acid and phospholipid composition, and electrochemical gradient, can increase bacterial tolerance to biocides, especially cationic surface-active substances, such as QACs (Winder *et al.*, 2000; Bisbiroulas *et al.*, 2011; Gerald *et al.*, 2023).

Efflux pumps are a major mechanism that accounts for the tolerance of several bacteria to cationic biocides (Chen *et al.*, 2021). The efflux pumps belonging to the adenosine triphosphate (ATP) binding cassette (ABC) family, the multidrug and toxic-compound extrusion (MATE) family, the major facilitator (MFS) family, the resistance-nodulation-cell-division (RND) family, and the small multidrug resistance (SMR) family (Paulsen *et al.*, 2001) have been shown to efflux a variety of biocide substrates (Wand and Sutton, 2022). The expression of efflux pumps in several bacteria can be induced in response to exposure to biocides (Grkovic *et al.*, 1998; Wand *et al.*, 2022).

QACs

QACs belong to a large class of chemicals that have a central nitrogen atom with a permanent positive charge that is bonded to alkyl and aromatic substituents. QACs are used in a variety of applications, including biocides, preservatives, surfactants, antistatic agents, fabric softeners, and dispersants (Arnold *et al.*, 2023). QACs exert antimicrobial action through interactions between the cationic quaternary nitrogen and the negatively charged acidic phospholipids of the bacterial cell membrane. This interaction destabilizes the cell membrane and leads to the leakage of intracellular biomolecules, proteins, and nucleic acids and subsequently causes cell lysis (Mohapatra *et al.*, 2023).

Some of the most well-characterized mechanisms of QAC resistance are the plasmid-encoded *qacA*, *qacB*, and *qacC* genes of *Staphylococcus aureus* (Tennent *et al.*, 1989; Paulsen *et al.*, 1995; Paulsen *et al.*, 1996). *QacA* and *QacB* belong to the drug/ H^+ antiporter 2 (DHA2) family, which is a part of the MFS family, while *QacC* is a transporter in the SMR family. The *S. maltophilia* genome contains *mfsQ* (*smlt2678*), which shares 34.2% identity with *S. aureus* *QacA* (Chittrakanwong *et al.*, 2021a). *MfsQ* is required for induced adaptation to benzalkonium chloride (BAC), and the *mfsQ* mutant has shown increased susceptibility to BAC and the bisbiguanide compound chlorhexidine gluconate (CHX) relative to its parental strain (Chittrakan-

wong *et al.*, 2021a). The expression of *mfsQ* can be induced by exposure to BAC, CHX, acriflavine, and ethidium bromide in an MfsR-dependent fashion (Chittrakanwong *et al.*, 2021a; Chittrakanwong *et al.*, 2021b). A gene encoding MfsR is located immediately downstream of *mfsQ* and forms an operon. MfsR is a TetR-type transcriptional regulator (TFTR) that regulates the expression of the *mfsQR* operon in a QAC-inducible manner (Fig. 1).

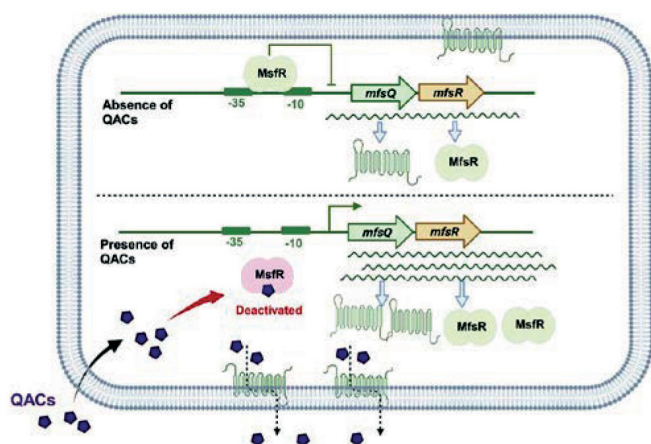


Fig. 1. Adaptive resistance of *S. maltophilia* against QACs. In the absence of QACs, MfsR binds to the operator site sequence spanning the promoter motif of the *mfsQR* promoter, which prevents the binding of RNA polymerase. In the presence of QACs, QAC-bound MfsR is released from the operator site, allowing RNA polymerase to bind and promote *mfsQR* transcription. Enhanced expression of MfsQ efflux pumps increases the bacterial ability to cope with QAC biocides.

The data from *in vivo* and *in vitro* analyses illustrate that in the absence of QACs, MfsR binds to the operator site of the *mfsQR* promoter, which contains the inverted repeat sequence AACTATA-CAGGA-TCCTGTATAGTT, located from positions +1 to -32. The binding of MfsR to the *mfsQR* promoter region impedes the subsequent binding of RNA polymerase and represses the transcription of *mfsQR* (Chittrakanwong *et al.*, 2021b). Similar to staphylococcal QacR, which is a TFTR (Grkovic *et al.*, 1998), QACs function as ligands and are capable of binding to the ligand-binding domain of MfsR, which causes its conformational change that abrogates its binding ability to the *mfsQ* operator site. Thus, once *S. maltophilia* is exposed to QACs, MfsR is released from the operator site, thereby allowing RNA polymerase to bind and initiate *mfsQR* transcription (Fig. 1). This adaptive expression of *mfsQ* enables *S. maltophilia* to survive in healthcare settings where QACs, especially BAC, are routinely used as disinfectants (Chittrakanwong *et al.*, 2021b).

The contribution of the efflux pumps SugE

and EmrE, which are members of the SMR family, to QAC tolerance has been reported in *E. coli* and other pathogenic bacteria (Yerushalmi *et al.*, 1995; Ninio *et al.*, 2001; Chung and Saier, 2002). *S. maltophilia* possesses two *sugE* homologs (*smlt1007* and *smlt4304*) and an *emrE* homolog (*smlt3363*) (Crossman *et al.*, 2008). The frequency of the *sugE* and *emrE* genes in *S. maltophilia* clinical isolates ranges from 90% to 100% (Youenou *et al.*, 2015; Patil *et al.*, 2018; Anari *et al.*, 2022). However, the presence or absence of *sugE* has shown no correlation with resistance to biocides, including chlorhexidine and ethanol (Anari *et al.*, 2022). Moreover, EmrE efflux pumps have been shown to confer resistance to antibiotics, including β -lactams and macrolides (Youenou *et al.*, 2015).

Oxidizing biocides

H_2O_2 is a common oxidizing biocide used in healthcare settings in both aqueous and vaporized forms for antiseptic purposes, to disinfect surfaces, medical devices, and equipment, and to fumigate rooms. The mechanisms of the antimicrobial activity of H_2O_2 involve the production of the most deleterious hydroxyl radicals through interactions with superoxide anions (Haber–Weiss reaction) or ferrous ions (Fenton reaction) (Linley *et al.*, 2012). The hydroxyl radicals thus formed can cause damage to biomolecules, including nucleic acids, proteins, and lipids, and lead to cell death. H_2O_2 can be degraded by bacterial enzymes, such as catalases, peroxidases, and alkyl hydroperoxide reductase (Farr and Kogoma, 1991; Imlay, 2008). Catalases and peroxidases catalyze the conversion of H_2O_2 to water and oxygen, while alkyl hydroperoxide reductases convert both alkyl hydroperoxides (ROOH) and H_2O_2 to the corresponding alcohol and water, respectively (Loewen, 1997; Seaver and Imlay, 2001; Poole, 2005). Moreover, the peroxide-scavenging enzymes catalase and alkyl hydroperoxide reductase can protect bacteria from the toxicity of sodium hypochlorite, which is a biocide frequently used as a disinfectant in healthcare settings (Charoenlap *et al.*, 2015).

The genome of *S. maltophilia* K279a contains three major groups of putative genes encoding H_2O_2 -scavenging enzymes: four catalases (*smlt0372* [*srpA*], *smlt1385* [*katA*, also named *katA2*], *smlt2537* [*katN*], and *smlt3583* [*katE*]), one alkyl hydroperoxide reductase (*smlt0841*–*smlt0842* [*ahpCF*]), and three glutathione peroxidases (*smlt3183* [*gpx1*], *smlt3228* [*gpx2*], and *smlt4676* [*gpx3*]) (Crossman *et al.*, 2008; Charoenlap *et al.*, 2019; Li *et al.*, 2020). The deletion of *S. maltophil-*

ia *katA* and *katE* reduces tolerance to H_2O_2 (Li *et al.*, 2020). The genes *smlt0372* and *smlt2537* encoding SrpA catalase-related peroxidase and manganese-containing catalase, respectively, play no role in the protection of *S. maltophilia* against H_2O_2 (Li *et al.*, 2020). Even though *S. maltophilia* carries three *gpx* genes, the deletion of only *gpx3* reduces the ability of the bacteria to cope with the toxicity of H_2O_2 (Li *et al.*, 2020). The regulation of *gpx3* expression is uncharacterized.

The exposure of *S. maltophilia* to H_2O_2 induces the elevated expression of both *ahpCF* and *katA* (Li *et al.*, 2020). KatA is a monofunctional catalase containing porphyrin heme groups that contribute to the decomposition of H_2O_2 (Charoenlap *et al.*, 2019). High expression of *katA* in *S. maltophilia* is commonly associated with increased tolerance to H_2O_2 (Charoenlap *et al.*, 2019). AhpC, a member of the two-cysteine peroxiredoxin family, protects cells from low concentrations of H_2O_2 and ROOH (Seaver and Imlay, 2001). AhpF is an NADH-dependent flavoprotein that contributes to the regeneration of active reduced AhpC (AhpC^{RED}) (Poole *et al.*, 2000).

Once *S. maltophilia* has been exposed to H_2O_2 , it can elicit adaptive protection to subsequent treatments with H_2O_2 (Fig. 2). This adaptive response renders *S. maltophilia* resistant to and able to survive oxidative stress induced by H_2O_2 . The expression of *ahpCF* and *katA* is regulated by OxyR, a peroxide-sensing regulator (Li *et al.*, 2020). OxyR

is a LysR-type transcriptional regulator commonly found in Gram-negative bacteria (Dubbs and Mongkolsuk, 2016). Unlike the majority of LysR-family regulators, which are activated by the binding of small-molecule ligands (Maddocks and Oyston, 2008), OxyR responds to direct oxidation of the protein.

E. coli OxyR is best understood in terms of its oxidant-sensing mechanism (Dubbs and Mongkolsuk, 2016). It contains two highly conserved cysteine residues: the sensing cysteine C199 and the resolving cysteine C208. OxyR is active as a tetramer protein and exists in two forms, namely, reduced (OxyR^{RED}) and oxidized (OxyR^{OX}). Both OxyR^{RED} and OxyR^{OX} regulate the target genes through binding to a conserved sequence motif (5'-ATAGntnnnnCTATnnnnnnATAGntnnnnCTAT-3') in the operator regions of the target genes (Zheng *et al.*, 2001). H_2O_2 -mediated oxidation of *E. coli* OxyR^{RED} occurs in a two-step process. Upon exposure to increased levels of H_2O_2 , C199 is oxidized to form a cysteine sulfenic acid intermediate, which undergoes a rapid condensation reaction with C208 to form an intermolecular disulfide linkage (Choi *et al.*, 2001; Lee *et al.*, 2004). Disulfide bond formation results in a conformational change of the overall OxyR structure, which changes its DNA-binding properties and promote interactions with RNA polymerase (Storz *et al.*, 1990; Lee *et al.*, 2004). In *E. coli*, OxyR functions as an activator of the peroxide stress response, but in other organisms, OxyR can function as either an activator or a repressor in both oxidized and reduced forms (Tseng *et al.*, 2003; Ieva *et al.*, 2008; Heo *et al.*, 2010). Similar to *E. coli* OxyR, *S. maltophilia* OxyR has two conserved cysteine residues at positions 199 and 208. Expression analysis suggests that OxyR in both reduced and oxidized forms acts as a positive regulator for *katA* gene expression but that OxyR^{OX} is more potent than OxyR^{RED} (Li *et al.*, 2020). OxyR regulates *ahpCF* in a different manner. Under physiological aerobic growth, OxyR acts as a repressor for *ahpCF* transcription. When OxyR is oxidized by treatment with H_2O_2 , OxyR^{OX} functions as an activator that promotes *ahpCF* transcription (Li *et al.*, 2020). Nevertheless, it is worth noting that the mutation of *ahpC* can cause a compensatory upregulation of *katA*, which makes *S. maltophilia* highly tolerant to H_2O_2 (Charoenlap *et al.*, 2019).

Triclosan

Triclosan is a widely used biocide that serves as a disinfectant, antiseptic, and preservative. It is

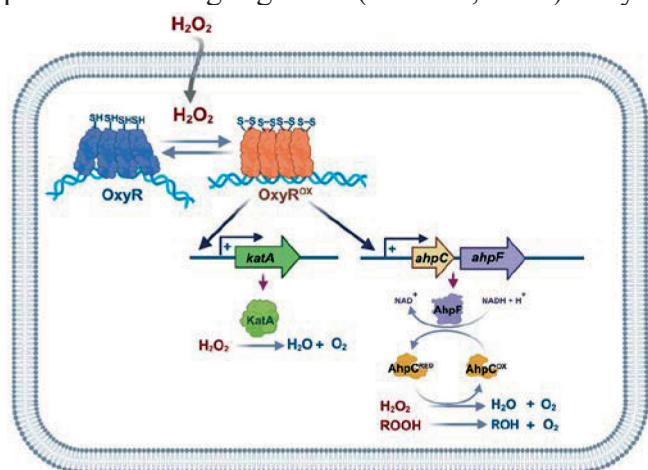


Fig. 2. Adaptive tolerance of *S. maltophilia* to H_2O_2 . When *S. maltophilia* is exposed to external H_2O_2 , the sulfhydryl group of the reactive cysteine residue in OxyR is oxidized, forming an intermolecular disulfide bond with the resolving cysteine residue. The oxidized OxyR (OxyR^{OX}) facilitates the transcription of both *katA* and *ahpCF*. KatA directly decomposes H_2O_2 , while AhpC requires a coupling reaction with the NADH-dependent AhpF to regenerate the active reduced form (AhpC^{RED}), which is capable of detoxifying both H_2O_2 and alkyl hydroperoxides (ROOH).

found in healthcare settings, personal care products, household items, and the textile industry. At low concentrations, it exerts bacteriostatic activity through the specific inhibition of an enzyme called enoyl-acyl carrier protein reductase (FabI), which is involved in bacterial fatty acid biosynthesis (Heath *et al.*, 1999; Levy *et al.*, 1999), but at high concentrations, it exhibits bactericidal activity by causing membrane destabilization and damage (Villalain *et al.*, 2001). Moreover, mutations of *fabI* render *E. coli* more resistant to triclosan (Heath *et al.*, 1999). In a study on *S. maltophilia*, some of the triclosan-resistant mutants selected in the laboratory acquired triclosan resistance through the overexpression of the RND efflux pump SmeDEF, which simultaneously increases resistance to several antibiotics, including tetracycline, chloramphenicol, ciprofloxacin, and tobramycin, all of which are known substrates for SmeDEF (Sanchez *et al.*, 2015). This evidence indicates that SmeDEF possibly confers triclosan resistance through the efflux transport of triclosan and also raises the concern that the biocide triclosan can act as a driving force for the selection of multiple antibiotic resistance in *S. maltophilia*.

The expression of *smeDEF* is regulated by SmeT, a transcriptional regulator in the TetR family, which is encoded by a gene located divergently upstream and shares a promoter with the *smeDEF* operon (Sanchez *et al.*, 2002). The crystal structure of SmeT is shown in Fig. 3 (Hernandez *et al.*, 2009). Similar to most TetR family repressors, *S. maltophilia* SmeT is a homodimer, and each subunit consists of nine α -helices ($\alpha 1$ – $\alpha 9$) divided into two domains. The N-terminal domain is composed of $\alpha 1$, $\alpha 2$, $\alpha 3$, and the beginning of $\alpha 4$. The $\alpha 2$ and $\alpha 3$ helices, which are almost perpendicular to one another, form the helix-turn-helix motif responsible for DNA binding. The C-terminal domain is involved in the ligand binding and dimerization of the protein, which is formed by the $\alpha 8$ and $\alpha 9$ helices (Hernandez *et al.*, 2009).

SmeT binds as two complexes to two pseudopalindromic regions of the 28 bp sequence, which include the -10 and -35 motifs of the *smeT* promoter and the -35 motif of the *smeDEF* promoter (Hernandez *et al.*, 2009). One half of the pseudopalindrome in the *smeDEF* promoter contains the TGTATGT sequence, which is well conserved among the operators of the TetR family members in gram-negative bacteria (Hernandez *et al.*, 2009; Hernandez *et al.*, 2011). The binding of SmeT to the *smeT* and *smeDEF* promoter sequences repress-

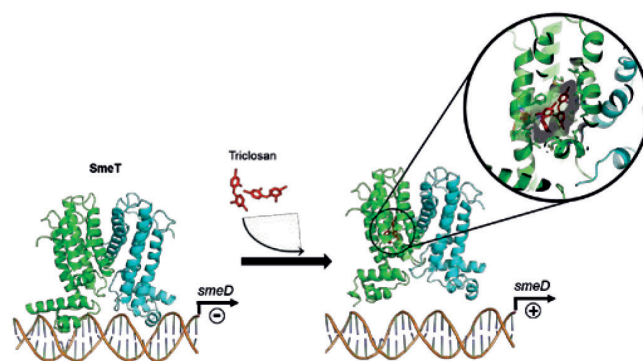


Fig. 3. The crystal structures of SmeT dimers from *S. maltophilia*. In the absence of inducer molecules, SmeT dimer binds to the operator site of the *smeD* promoter covering the -35 motif and repressing its transcription. Upon exposure to triclosan molecules (shown in red), binding of two triclosan molecules to each SmeT subunit at the ligand binding domain changes the SmeT configuration to a form unable to bind the *smeD* promoter, thus allowing *smeD* transcription. Triclosan binding are shown only in subunit B. The Swiss Protein Data Bank (PDB) identification numbers (IDs) for SmeT and triclosan-bound SmeT are 2W53 and 3P9T, respectively.

es its own and *smeDEF* expression by steric hindrance of RNA polymerase binding to the promoters (Sanchez *et al.*, 2002; Hernandez *et al.*, 2009).

Upon exposure to triclosan, two molecules of triclosan bind to each SmeT subunit. One triclosan molecule binds at the bottom of the binding pocket, parallel to helix $\alpha 6$, while the other triclosan molecule binds close to the dimer interface of the N-terminal domain. The binding of the triclosan molecules triggers the stabilization of the loop connecting helices $\alpha 6$ and $\alpha 7$ in one subunit through the formation of H-bonds with the loop connecting helices $\alpha 1$ and $\alpha 2$ of another subunit. This in turn makes the N-terminal domain, especially helix $\alpha 3$, which is associated with DNA binding, more stable (Hernandez *et al.*, 2011). The changes in the relative orientation of the N-terminal domain after triclosan binding generate a ligand bound structure that is unable to bind DNA (Fig. 3), which thereby enables the transcription of *smeT* and *smeDEF* (Hernandez *et al.*, 2011).

Thus, the exposure of *S. maltophilia* to the biocide triclosan can induce the production of SmeDEF, which makes *S. maltophilia* more tolerant to triclosan as well as antibiotics, including co-trimoxazole, the first-line antibiotic (Sanchez and Martinez, 2018). This triclosan adaptation is one mechanism that allows this bacterium to withstand triclosan stress conditions. The QAC biocides BAC and hexachlorophene are able to bind SmeT and trigger SmeDEF expression (Sanchez *et al.*, 2015); however, the contribution of the SmeDEF

efflux pump to BAC and hexachlorophene resistance is yet to be elucidated. Nevertheless, it has been shown that the deletion of *smeDEF* increases susceptibility to the QAC biocide acriflavine and other toxicants, including sodium dodecyl sulfate, crystal violet, proflavine, and ethidium bromide (Zhang *et al.*, 2001). Moreover, plant flavonoids, such as phloretin, can act as inducer molecules for SmeT and as substrates for the SmeDEF efflux pump (Garcia-Leon *et al.*, 2014).

Conclusion

S. maltophilia is one of the leading causes of nosocomial infections associated with multiple antibiotic resistance. The persistence of bacterial communities in healthcare settings, where biocides are used extensively to control microbial contamination, is a potential risk factor that facilitates healthcare-associated infections. *S. maltophilia* has developed mechanisms comprising inherent, acquired, and adaptive resistance to withstand biocide stress conditions. The three adaptive resistance mechanisms of *S. maltophilia* against biocides have been well elucidated. The MsfQ/MfsR, KatA, AhpCF/OxyR, and SmeDEF/SmeT systems are responsible for the adaptive resistance of *S. maltophilia* against QACs, H₂O₂, and triclosan, respectively. Induced adaptation not only renders *S. maltophilia* resistant to biocides but also causes cross-tolerance to other substances. For instance, the high expression of SmeDEF efflux pumps triggered by the triclosan-mediated inactivation of SmeT can increase tolerance to multiple antibiotics. This raises the concern that the improper use of biocides may facilitate the selection of bacteria that are cross-resistant to clinically used antimicrobials.

Moreover, exposure to the oxidizing biocide H₂O₂ elicits high expression of OxyR-regulated genes, including KatA and AhpCF. A study of *Pseudomonas aeruginosa* PA14 revealed that KatA monofunctional catalase was crucial for the full virulence of the bacterium in the host models *Drosophila melanogaster* and mice because oxidative stress is one of the main innate immune responses generated by a host during host–microbe interactions (Lee *et al.*, 2005). Even though the contribution of KatA to the virulence of *S. maltophilia* is unclear, it is possible that elevated levels of KatA triggered by H₂O₂ exposure may alter the interactions between *S. maltophilia* and human hosts by increasing the tolerance of *S. maltophilia* to host-generated oxidative stress and thus increase the chance of successful infection. Further investigation regarding this hypothesis is therefore warranted.

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