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The Importance of RND-type Efflux Pumps in the Interactions of *Pseudomonas aeruginosa* with 4-Methoxybenzaldehyde

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by

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Abstract

Pseudomonas aeruginosa is a common hospital-acquired pathogen and is often associated with high mortality rates due to the development of multidrug-resistance. Antimicrobial plant-derived (phyto-) aldehydes present a promising alternative to antibiotics due to their broad-spectrum antimicrobial activity and low propensity to trigger resistance. However, two main problems preclude the widespread application of these compounds: chemical instability and low antimicrobial efficacy. The ongoing collaboration between the Mavrodi and Patton labs at USM has recently addressed the problem of chemical instability by incorporating plant aldehydes into polymer materials called Pro-Antimicrobial Networks via Degradable Acetals (PANDAs). Thus, this study aimed to address the low antimicrobial efficacy of phytoaldehydes. The first step involved the characterization of cellular pathways targeted by 4-methoxybenzaldehyde, which is a metabolite from star anise and a key component of PANDAs. *Pseudomonas aeruginosa* PAO1 *was* subjected to transposon mutagenesis and 10,000 mutants were screened for hypersensitivity to 4-methoxybenzaldehyde. Results of the screening revealed that inactivation of the MexAB-OprM multidrug efflux pump sensitizes *P. aeruginosa* to phytoaldehydes. Based onthis finding, several plant-derived efflux pump inhibitors (EPIs) were tested for the capacity to improve the antimicrobial efficacy of 4-methoxybenzaldehyde. Results of this testing revealed that epigallocatechin gallate (EGCG) from green tea acted synergistically with 4-methoxybenzaldehyde and significantly reduced its minimal inhibitory concentration. Finally, the response of four medically important *P. aeruginosa* efflux pump genes to 4-methoxybenzaldehyde and EGCG was analyzed using RT-qPCR. The result demonstrated that the phytoaldehyde significantly upregulated MexCD-OprJ and MexEF-OprN efflux pumps and the addition of EGCG reversed this effect.

Collectively, our results highlighted the importance of active efflux in the resistance of *P. aeruginosa* to phytoaldehydes and the potential of EPIs in improving the efficacy of these natural antimicrobials.

Keywords: *Pseudomonas aeruginosa*, multidrug efflux pumps, 4-methoxybenzaldehyde, essential oil, efflux pump inhibitor, minimal inhibitory concentration

Dedication

To my beloved family and friends who have always been by my side and believed in me.

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List of Abbreviations

ABC	ATP-binding cassette
CCCP	Carbonyl Cyanide-m-Chlorophenyl Hydrazone
CDC	Centers for Disease Control and Prevention
EPI	Efflux Pump Inhibitor
EGCG	Epigallocatechin Gallate
EO	Essential Oil
LB	Luria Bertani
MH	Mueller-Hilton
MIC	Minimal Inhibitory Concentration
PANDA	Pro-Antimicrobial Network of Degradable Acetal
RND	Resistance Nodulation Division
RT-qPCR	Reverse Transcription Quantitative Polymerase Chain Reaction

1. Introduction

The rapid emergence of antibiotic resistance in pathogenic bacteria has become a major global concern over the past few decades. The increase in antibiotic-resistant clinical isolates coupled with the lag in the development of effective antimicrobials poses a serious threat to human medicine in the upcoming years (Fernández & Hancock, 2012). The report published by the Centers for Disease Control and Prevention (CDC) (2013) states that antibiotic-resistant bacteria infect at least 2 million people and as a result, at least 23,000 people die annually in the United States.

The Gram-negative bacterium *Pseudomonas aeruginosa* is an opportunistic human pathogen that is increasingly becoming multidrug-resistant and causes nosocomial pneumonia, as well as bloodstream, urinary tract and surgical site infections (Hidron et al., 2008). According to the CDC report in 2013, this pathogen was implicated in 8% of nosocomial infections in the US, with multidrug-resistant isolates responsible for 13% of cases of severe infections. The 2015 report by European Centre for Disease Prevention and Control states that approximately 14% of isolates reported between 2005 and 2013 in Europe were not responsive to multiple antibiotic treatments.

Plant-derived essential oil (EO) contains multiple constituents that exhibit a broad range of antimicrobial activity and can potentially substitute antibiotics due to the low propensity to trigger resistance (Bassole and Juliani, 2012). Besides, many essential oils are harmless to humans and are classified by the Food and Drug Administration as Generally Recognized as Safe substances (Topuz et al., 2016). EOs could be potentially used as food preservatives and disinfectants, but poor water solubility, volatility, and chemical instability preclude the widespread practical application of these compounds (Fernandez-Lopez &

Viuda-Martos, 2018). The collaborative research by the Patton lab in the School of Polymer Science and Engineering and the Mavrodi lab in the School of Biological, Environmental and Earth Sciences at The University of Southern Mississippi has addressed these drawbacks by incorporating plant aldehydes into Pro-Antimicrobial Networks via Degradable Acetals (PANDAs), which are polymeric materials that release antimicrobials in response to changes in pH and humidity in the environment (Amato et al., 2017a). PANDAs provide a sustained release of the volatile EO constituents resulting in the prolonged exposure of bacteria to plant antimicrobials and inhibition of their growth (Amato et al., 2017b).

Recently, the antimicrobial activity of several EO-derived aldehydes in *P. aeruginosa* PAO1 has been characterized (Amato et al., 2018). The results revealed that the compound with the highest antimicrobial activity was 4-methoxybenzaldehyde (*p*-anisaldehyde), a natural antimicrobial found in the seeds of star anise (*Pimpinella anisum*) (Amato et al., 2018). 4-methoxybenzaldehyde was also used as a key component of PANDAs that kill various fungal and bacterial pathogens, including multidrug-resistant *P. aeruginosa* (Amato et al., 2017b). Since the mode of action and cellular targets of plant-derived antimicrobial aldehydes are poorly understood, Amato et al., (2018) generated 3,000 transposon mutants of *P. aeruginosa* PAO1 and screened them for hypersensitivity to 4-methoxybenzaldehyde and other EO-derived aldehydes. The screen revealed that mutations in genes involved in various transport and biosynthetic pathways, including those encoding the MexA membrane fusion protein of the MexAB-OprM efflux pump and OprF porin, resulted in a decrease in minimal inhibitory concentration (MIC) of EO-derived aldehydes (Amato et al., 2018). The first goal of my project was to generate 7,000 additional mutants, which helped to increase the overall coverage of *P. aeruginosa* PAO1 genes by transposon insertions to approximately 83%.

Results of my experiments identified the cell envelope as a major target of 4-methoxybezaldehyde and revealed other cellular pathways that contribute to the resistance of *P. aeruginosa* to this plant-derived antimicrobial.

Although plant-derived antimicrobial aldehydes present many advantages, they have lower antimicrobial efficacy compared to antibiotics and require larger amounts to eradicate microbial populations effectively. Given the relevance of efflux pumps for the susceptibility of *P. aeruginosa* to 4-methoxybezaldehyde, I hypothesized that efflux pump inhibitors (EPIs) could be used to improve the antimicrobial efficacy of phytoaldehydes. Several types of synthetic EPIs were demonstrated to lower MICs and potentiate the activity of clinical antibiotics. However, the cytotoxicity of these compounds precludes their use in antibiotic therapy, and there is a need for non-toxic, effective and broad-spectrum EPIs (Puzari & Chetia, 2017). Accordingly, the second goal of my project was to test several natural EPIs, including geraniol, berberine, epigallocatechin gallate (EGCG), daidzein and curcumin, for their ability to potentiate the activity of 4-methoxybenzaldehyde. Results of these tests revealed that epigallocatechin gallate, a compound found in green tea, acted synergistically and reduced the MIC of 4-methoxybenzaldehyde by 60%.

Numerous studies have addressed the contribution of multidrug efflux pump systems to the resistance of bacterial pathogens to certain classes of antibiotics (Puzari & Chetia, 2017). The genome of *P. aeruginosa* encodes multiple RND-type efflux pumps that contribute to the intrinsic antibiotic tolerance of this pathogen. However, the role of RND-type efflux pumps in contributing to the resistance to natural plant-derived antimicrobials is less well understood. To address this deficiency, I investigated the response of four *P. aeruginosa* efflux pumps to 4-methoxybenzaldehyde, EGCG, and their combination using

RT-qPCR assay. My results revealed that 4-methoxybenzaldehyde significantly upregulated genes for MexCD-OprJ and MexEF-OprN efflux pumps. Treatment with EGCG reversed the inducing effect of 4-methoxybenzaldehyde highlighting the importance of active efflux in the resistance of *P. aeruginosa* to phytoaldehydes, and the potential of EPIs for improving the efficacy of these natural antimicrobials.

2. Literature Review

2.1. Pseudomonas aeruginosa

Pseudomonas aeruginosa is a ubiquitous Gram-negative bacterium that inhabits various environments such as soil and surfaces in aqueous habitats (Gellatly & Hancock, 2013). It is highly adaptable and possesses intrinsic resistance to antimicrobials, which allows it to survive in both natural and artificial environments, including surfaces in medical facilities (Gellatly & Hancock, 2013). *Pseudomonas aeruginosa* can transform into an opportunistic pathogen and cause both acute or chronic infections in humans and animals (Chugani and Greenberg 2007; Rutherford and Bassler, 2012). The infections caused by *P. aeruginosa* are often hospital-acquired and involve neutropenia, severe burns, or pneumonia in cystic fibrosis patients (Lyczak et al., 2000; Gellatly & Hancock, 2013). Multidrug-resistant *P. aeruginosa* is classified as a severe threat, with 6,700 infections and 440 deaths per year in the U.S. (CDC, 2013).

Pseudomonas aeruginosa possesses a variety of mechanisms that contribute to its intrinsic and acquired resistance to antimicrobials. It secretes β-lactamases that mediate the resistance to β-lactam antibiotics, such as penicillin, cephalosporins, carbapenems, and monobactams (Poole, 2011). All four described molecular classes of β-lactamases (i.e., class

B metal-dependent Zn^{2+}-requiring and class A, C, and D metal-independent) have been found in *P. aeruginosa* (Zhao and Hu, 2010; Poole, 2011). In addition to β-lactamases, efflux systems, especially those from the resistance-nodulation-division (RND) family, play an essential role in the antimicrobial resistance of this pathogen (Poole, 2007; Poole, 2011). Last but not least, changes in the permeability of the outer membrane through mutations together with biofilms further increase resistance to some antimicrobials (Poole, 2011). As antibiotics are becoming less efficient by ever-increasing resistance, the search for alternative natural antimicrobial compounds is on the rise.

2.2. Essential oils (EOs)

Essential oils (EOs) are complex mixtures of volatile secondary metabolites produced by aromatic plants. The first evidence of humans using EO-producing plants dates back to 10,000 BC and is based on pollen analyses of Stone Age settlements (Baser and Buchbauer, 2015). The aromatic odor and healing properties of EOs have been mainly utilized through the usage of aromatic plants as spices and remedies for the treatment of illnesses (Baser and Buchbauer, 2015; Guenther, 1948). The long history of EO application in medicine is evidenced by Papyrus Ebers, a medical document from ancient Egypt from 1,550 BC, containing around 700 formulas and remedies made with aromatic plants and their products (Baser and Buchbauer, 2015).

The first development of the distillation of EOs is credited to the Catalan physician Arnaldus de Villanova (1235-1311), and his approach made a significant contribution to medieval European therapy (Guenther, 1948). Later, the Swiss medical reformer Bombastus Paracelsus von Hohenheim (1493-1541) proposed the theory that distillation released the very last and desirable extractive, the *Quinta essentia* (quintessence), which is the most

critical part of every drug (Guenther, 1948). His theory inspired further research into the properties of EOs, and the term “essential oil” was derived from the concept of *Quinta essentia* (Guenther, 1948). Over the course of the last half-century, EO constituents have been studied extensively, and our knowledge in this field has rapidly expanded.

In terms of composition, EOs represent complex natural mixtures containing 20–60 chemically distinct components at varying concentrations (Bakkali et al., 2008). Two or three of these components constitute 20–70% of an EO, while the remaining constituents are present in trace amounts (Bakkali et al., 2008). Most essential oils contain terpenes (oxygenated or not) that are composed of isoprene units and exist in acyclic, monocyclic, bicyclic and tricyclic forms (Swamy et al., 2016). The terpenes are classified into several groups such as monoterpenes ($C_{10}H_{16}$), sesquiterpenes ($C_{15}H_{24}$), diterpenes ($C_{20}H_{32}$), and triterpenes ($C_{30}H_{40}$), with monoterpenes and sesquiterpenes prevailing (~90%) in bioactive EOs (Swamy et al., 2016; Miguel, 2010). Other EO constituents are represented by terpenoids, and allyl- and propenylphenols (phenylpropanoids) (Swamy et al., 2016; Miguel, 2010).

In nature, EOs serve several important ecological roles. They attract desirable insects that disperse pollen or seeds and repel the undesirable ones. Essential oils also act as defensive substances against microorganisms and herbivores, and mediate allelopathic communication between plants (Bakkali et al., 2008; Miguel, 2010). Human use of EOs includes their application as antibacterials, antivirals, antifungals, insecticides and herbicides (Burt, 2004; Miguel, 2010). In medicine, EOs are used as components of anticancer, antinociceptive, antiphlogistic, antiviral, antibacterial and antioxidant products (Buchbauer, 2010). Some EOs have antioxidant properties and can be used to prevent diseases caused by

free radicals, such as brain dysfunction, cancer, heart disease, immune system decline, and inflammatory responses (Aruoma, 1998; Kamatou and Viljoen, 2010; Miguel, 2010). The antioxidant properties of EOs also reflect their use as food preservatives (Miguel, 2010).

Due to their complex composition, the EOs do not have a particular cellular target (Carson et al., 2002; Bakkali et al., 2008). Most EOs are hydrophobic and can exhibit some level of cytotoxicity associated with the leakage of macromolecules and cell lysis (Bakkali et al., 2008). As antimicrobials, EOs were reported to function by disrupting membrane integrity, ATP synthesis, protein and quorum sensing pathways (Nazzaro et al., 2013; Yap et al., 2014; Amato et al., 2017b). In bacteria, EOs also can coagulate cytoplasm (Gustafson et al., 1998) and affect the interactions of lipids and proteins in the membrane (Ultee et al., 2002; Burt, 2004). Due to multiple targets of EOs, no particular resistance or adaptation to EOs has been described so far (Bakkali et al., 2008).

2.3. 4-methoxybenzaldehyde (p-anisaldehyde)

4-methoxybenzaldehyde is a metabolite from seeds of star anise (*Pimpinella anisum)*, which is a standard part of the diet in Northern India and is widely cultivated in the Mediterranean basin and South Asia (Shi et al., 2017). This phytoaldehyde exhibits antifungal activity against several species of yeast and molds in laboratory media, fruit purees, and fruit juices (Lu et al., 2010, Shreaz et al., 2011). This compound also exhibits fungicidal, as opposed to fungistatic activity against the wild-type and antibiotic-resistant strains of *Candida* spp. (Shreaz et al., 2011). The combined treatment by 4-methoxybenzaldehyde and bacteriocin nisin was shown to have a long-term inhibitory effect on the growth of the Gram-positive bacterium *Staphylococcus aureus* (Shi et al., 2017). The compound has also been shown to act synergistically with nisin against *Listeria*

monocytogenes, which is a common and economically important food-borne pathogen (Zhao et al., 2016).

2.4. Pro-Antimicrobial Networks via Degradable Acetals (PANDAs)

Many components of EOs, including phytoaldehydes, are hydrophobic and highly volatile (Turek & Stintzing, 2013). In anise oil, 4-methoxybenzaldehyde is one of the major volatile compounds that constitute approximately 0.42% of this EO (Topuz et al., 2016). Amato et al. (2017b) recently addressed this problem of volatility and instability of phytoaldehydes by incorporating them into Pro-Antimicrobial Networks via Degradable Acetals (PANDAs) via thiol-ene photopolymerization. Thiol-ene allows step-wise addition of 4-methoxybenzaldehyde acetal and therefore, the occupation of every cross-link junction by the acetal (Amato et al., 2017b). The degradation of PANDAs is triggered by low pH and humidity and results in the release of the active antimicrobial 4-methoxybenzaldehyde and inactive low molecular weight degradation byproducts (Figure 1) (Amato et al., 2017a).

PANDAs were shown to have antimicrobial effect against both bacterial and fungal pathogens, including *P. aeruginosa* PAO1, *Salmonella typhimurium* ATCC 6539, *Escherichia coli* ATCC 43895 (serotype O157:H7), and *Histoplasma capsulatum* G217B, and exhibited a minimum cytotoxic effect on KB (HeLa) cells (Amato et al., 2017b). The concept was further improved by synthesizing co-PANDAs, which combine synergistically interacting 4-methoxybenzaldehyde and 4-bromobenzaldehyde (Amato et al., 2018). The co-PANDAs have higher antimicrobial activity and negligible cytotoxicity to mammalian cells (Amato et al., 2018). Despite the increased antimicrobial activity of co-PANDA, there is potential to further improve PANDAs by incorporating natural compounds that target important structures or pathways in microbes.

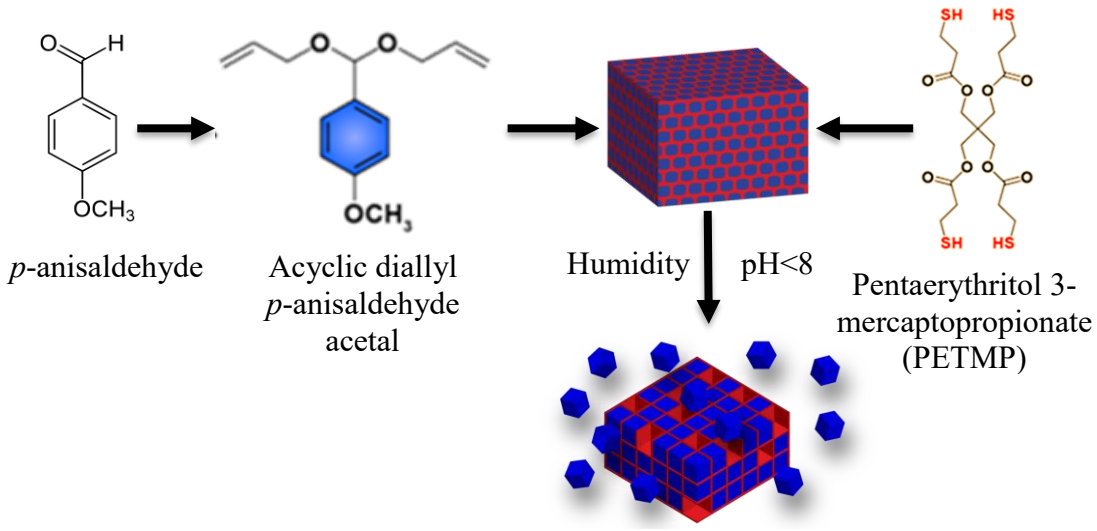

Figure 1. Synthesis of PANDAs and their degradation. The scheme is adapted from Amato et al. (2018).

2.5. Multidrug efflux pumps

Efflux pumps are membrane transporter proteins that represent a significant component of the intrinsic and acquired antibiotic resistance mechanisms in *P. aeruginosa* (Poole, 2011). When antibiotics reach the intracellular milieu of the bacterial cell, they are rapidly expelled by efflux pumps, which allows the organism to survive (Lomovskaya et al., 2001). While drug-specific efflux pump genes are harbored in mobile genetic elements, multidrug-resistance pumps are encoded on bacterial chromosomes, signifying their function in natural bacterial environments (Lomovskaya et al., 2001, Fernández & Hancock, 2012). There are two main classes of efflux pumps: ATP-binding cassette (ABC) transporters and secondary multidrug transporters (Fernández & Hancock, 2012). The ABC transporters use the energy released from the hydrolysis of ATP to move solutes across the membrane, facilitating the uptake of nutrients against a concentration gradient (Schneider and Hunke, 2002). Their other functions include signal transduction, protein secretion, antibiotic

resistance, antigen presentation, bacterial pathogenesis, and sporulation (Schneider and Hunke, 2002). The secondary multidrug transporters fall into four superfamilies, including the major facilitator superfamily , the small multidrug-resistance family, the multidrug and toxic compound extrusion family, and the resistance-nodulation-cell division (RND) superfamily (Fernández & Hancock, 2012).

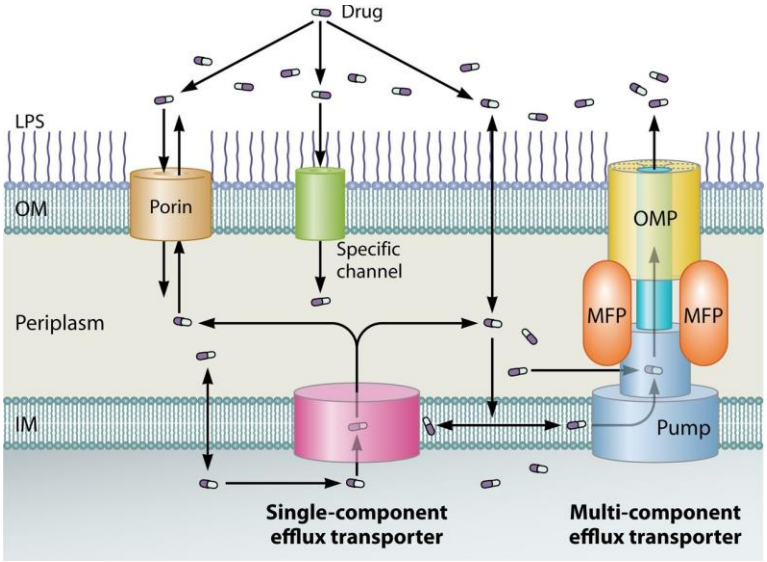

Figure 2. Function of efflux pumps in the influx and efflux of antimicrobials in *P. aeruginosa*. The figure is acquired through Li et al. (2015). Outer membrane [OM]; inner membrane [IM]; OM channel protein [OMP]; membrane fusion protein [MFP].

Members of the RND family play a major role in antimicrobial resistance in *P. aeruginosa* (Poole, 2011). An RND-type efflux system is a tripartite complex that consists of a pump embedded in the inner membrane, the periplasmic adapter protein (or membrane fusion protein), and the outer membrane channel (Figure 2) (Li et al., 2015). The RND pumps are thought to take up the antibiotics from the periplasm and cooperate with single-component or singlet pumps to extrude them out of the cell (Li et al., 2015).

Twelve RND-type efflux systems have been identified in *P. aeruginosa* (Poole, 2011), and four of these, MexAB-OprM, MexCD-OprJ, MexEF-OprN, and MexXY-OprM

(OprA), make the most significant contribution to the multidrug-resistance in clinical isolates (Li et al., 2015). The expression of *P. aeruginosa* RND-type efflux pump genes is induced in response to various external stress factors including reactive oxygen species (MexAB-OprM, MexXY-OprM), reactive nitrogen species (MexEF-OprN), membrane-damaging agents (MexCD-OprJ), and ribosome-blocking substances (MexXY-OprM), (Lister et al., 2009; Morita et al., 2014; Poole, 2014). MexAB-OprM is constitutively expressed in wild-type strains and contributes to the intrinsic resistance to β-lactams and other antimicrobials (Poole et al., 1993; Li et al., 1995; Yoneda et al., 2005). The MexXY-OprM pump also contributes to the intrinsic resistance of *P. aeruginosa* to several agents such as fourth-generation cephems, tetracyclines, erythromycin, and gentamicin (Yoneda et al., 2005). On the other hand, the expression of MexCD-OprJ and MexEF-OprN is tightly regulated in wild-type *P. aeruginosa* and their role is limited to acquired resistance (Kohler et al. 1997; Yoneda et al., 2005).

2.6. Efflux pump inhibitors (EPIs)

Efflux activity mediated by RND-type pumps contributes significantly to the intrinsic drug resistance in many pathogenic microorganisms (Li et al., 2015). Thus the employment of efflux pump inhibitors (EPIs) represents an attractive approach in counteracting the multidrug resistance in *P. aeruginosa* and other Gram-negative pathogens (Askoura et al., 2011). The synergistic application of antibiotics and EPIs reduces the invasiveness of *P. aeruginosa* and the MICs of many antibiotics (Hirakata et al., 2009). The inhibition of efflux pumps increases the intracellular drug concentration (Lomovskaya et al., 2001), restores the drug activities against the resistant strains, and reduces the chance of the further development of antibiotic resistance (Askoura et al., 2011).

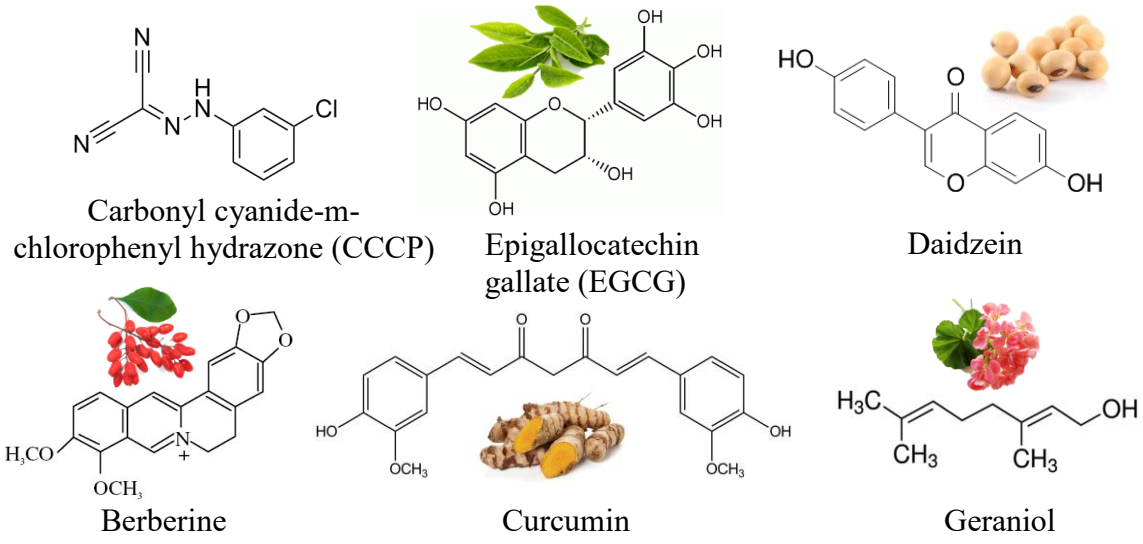

Figure 3. Chemical structures of natural efflux pump inhibitors.

Several synthetic compounds have been reported to inhibit RND-type efflux pumps, including phenyl-arginine-β-naphthylamide (PAβN), D13-9001, and MBX2319 (Li et al., 2015). Although these EPIs increase the potency of various antibiotics against multidrug-resistant pathogens, their clinical application is hindered by the high degree of cytotoxicity (Li et al., 2015). Due to the constant exposure to bacteria in the soil, plants have developed diverse defense systems and are therefore considered to be a promising source of new EPIs with low toxicity and high tolerability (Prasch & Bucar, 2015). Over the last decade, various plant-derived compounds with antimicrobial and efflux pump inhibitory activity have been discovered (Prasch & Bucar, 2015). In this study, we tested the natural EPIs epigallocatechin gallate, daidzein, berberine, curcumin and geraniol for synergistic activity with 4-methoxybenzaldehyde against *P. aeruginosa* PAO1 (Figure 3).

Epigallocatechin gallate (EGCG) is the main polyphenol component of green tea, which exhibits antibacterial activity and also inhibits the activity of bacterial gelatinase

(Blanco et al., 2003), P-glycoprotein, and efflux pumps (Roccaro et al., 2004). Daidzein is an isoflavone isolated from soybeans, which was shown to have an inhibitory effect on efflux pumps of *Mycobacterium smegmatis*, *P. aeruginosa* and *E. coli*, as well as on P-glycoprotein of cancer cells (Prasch & Bucar, 2015). Berberine is a natural benzylisoquinoline alkaloid produced by plants of the *Berberidaceae* family. This compound has antimicrobial activity and kills microorganisms by interacting with the cell division protein FtsZ (Boberek et al., 2010) and inhibiting efflux pumps in Gram-positive bacteria (Morita et al., 2016). Geraniol is isolated from *Geranium* species and was shown to be a potent EPI in Gram-negative bacteria (Lorenzi et al., 2009). Lastly, curcumin is extracted from rhizomes of the plant *Curcuma longa* (*Zingiberaceae*) and has multiple biological activities such as anti-inflammatory, antiviral, antioxidant, anticancer, and inhibition of the expression of efflux pumps (Negi et al., 2014).

3. Materials and Methods

3.1. Bacterial strain, growth conditions and compounds

Pseudomonas aeruginosa PAO1 was used in all experiments listed in this study. The strain was routinely cultured in Luria-Bertani (LB) medium (Difco), while Mueller-Hilton II (MH) broth and agar (Becton, Dickinson and Co.) were used for all antimicrobial assays. Cultures were grown at 37 °C with shaking at 220 rpm where necessary. The selection of transposon mutants was performed by amending the growth medium with tetracycline (Tc) (Affymetrix) at the concentration of 100 μg/mL. The stock solution of 4-methoxybenzaldehyde (Fischer Scientific) was prepared by ultrasonicating the compound for 5 min in 0.2% (w/v) agar. The stock concentrations of EGCG (10 mg/ml), geraniol (5

mg/ml), daidzein (5 mg/ml), berberine (2 mg/ml), and curcumin (5 mg/ml) were prepared by dissolving the chemicals in dimethylsulfoxide (DMSO; Alfa Aesar).

3.2. Transposon mutagenesis and screening of the mutants

Electrocompetent cells of *P. aeruginosa* PAO1 were prepared using a modified procedure of Choi et al. (2006). Six milliliters of an overnight culture in LB medium were aliquoted into four microcentrifuge tubes and centrifuged at room temperature for 2 min at 16,000 x g. The cells were washed twice with 1 mL of 300 mM sucrose, and each cell pellet was resuspended in 25 μL of 300 mM sucrose and combined in one tube. The transposon mutagenesis was performed with the EZ-Tn5™ Transposomes (Epicentre) following the manufacturer's instruction. Briefly, 0.7 μL of the transposome was added to 100 μL of electrocompetent cells, and the mixture was transferred to a 2 mm gap width electroporation cuvette. After applying a pulse (2.5 kV, 10 μF, 600 Ω) with an Electroporator 2510 (Eppendorf) the cells were immediately mixed with one milliliter of room temperature LB medium and transferred to a glass tube (16 x 150 mm). The transformants were incubated for 1.5 hours at 37 °C with shaking at 250 rpm. The cells were then diluted 1:10 in LB medium and 100 μL of the suspension were spread-plated onto an LB-Tc_{100} plate. The plates were incubated at 37 °C for 24 hours or until colonies appeared. Negative controls included cells electroporated without the EZ-Tn5 transposome mixture.

The resultant transposon-bearing clones (confirmed via PCR) were transferred into wells of 96-well microplates pre-filled with 100 μL of MH medium. The transposon mutant library was incubated at 37 °C for 24 hours and replicated with a 96-prong replicator (VP Scientific) into new microplates containing MH medium with 0.2% agar amended with 4-methoxybenzaldehyde at 1.2, 1.5, and 1.7 mg/mL concentrations, respectively. The

inoculated microplates were incubated at 37 °C for 24 hours, and bacterial growth was recorded by measuring optical density at 600 nm (OD_{600}) using a Synergy 2 reader (Biotek). Cultures grown in the unamended MH medium were mixed with dimethyl sulfoxide (7% final), and frozen at −80 °C for long-term storage. Assays were repeated twice with three replicates for each treatment condition. The cultures of transposon mutants that had grown to an $OD_{600} < 0.05$ were selected as sensitive mutants. From the second screening, the factor of inhibition (F_1) of 4-methoxybenzaldehyde was calculated by dividing OD_{600} of the untreated culture by that of the treated culture for each mutant (Campen et al., 2015).

3.3. Mapping of Tn5 transposon insertions by inverse PCR and sequencing

Genomic DNA was extracted from overnight LB cultures of the sensitive mutants using a DNeasy UltraClean Microbial Kit (Qiagen), and 250-ng aliquots of the DNA samples obtained were digested with the restriction endonuclease *Sac*II (New England Biolabs). The reactions were incubated for 3 hours at 37 °C, and the enzyme was inactivated at 65 °C for 20 min. The digested DNA was self-ligated by incubating it overnight at 16 °C in the presence of T4 DNA ligase (New England Biolabs).

The ligation products served as templates for inverse PCR with the Q5 High-Fidelity DNA Polymerase (New England Biolabs) and transposon-specific primers (Table 1; Eurofins) (Amato et al., 2018). Cycling conditions included 98 °C for 30 s, followed by 34 cycles of 98 °C for 10 s, 72 °C for 2 min and 72 °C for 2 min, and a final extension at 72 °C for 5 min. The PCR products were analyzed by gel electrophoresis (1% w/v agarose gel, 120 V, 20 min) and amplicons were purified using a GeneJET PCR Purification Kit (Thermo Scientific). The purified DNA was sequenced by Eurofins USA, and areas flanking the EZ-

Tn5 <TET-1> integration sites were mapped to the *P. aeruginosa* PAO1 genome using the BLASTn web tool of the Pseudomonas database (Winsor et al., 2016).

Table 1. Oligonucleotide primers used in the inverse PCR.

Primer	Primer or probe	Reference
TET-1FP-3	5′-GCATCTCGGGCACGTTGGGTCCT-3′	EZ-Tn5™ <TET-1> Insertion Kit, *Epicentre*
TET-1RP-4	5'-CGAGGATGACGATGAGCGCATTGTTAG-3'	

3.4. Testing interactions of efflux pump inhibitors (EPIs) with 4-methoxybenzaldehyde

The interactions of 4-methoxybenzaldehyde with EPIs were tested using a modified broth microdilution technique with bacteria cultured in MH broth supplemented with non-inhibitory concentrations of EGCG (150 µg/mL), daidzein (1 mg/mL), curcumin (400 µg/mL), berberine (400 µg/mL), geraniol (600 µg/mL), and the uncoupler of proton motive force carbonyl cyanide m-chlorophenylhydrazone (CCCP) (25 µg/mL), respectively. The MIC of 4-methoxybenzaldehyde against *P. aeruginosa* PAO1 was determined in the presence and absence of each of these compounds. Bacteria suspended in unamended MH medium served as a positive control, while MH medium mixed with 4-methoxybenzaldehyde in 0.2% agar was used as a negative control. Microtiter plates were then incubated at 37 °C for 24 hours and OD_{600} of the cultures were measured to determine the nature of interactions ($OD_{600} < 0.05$ were considered negative for bacterial growth). Each experiment was repeated three times with three replicates for each treatment.

3.5. Using RT-qPCR to measure the effect of 4-methoxybenzaldehyde and EGCG on the expression of RND-type efflux pump genes

The expression of *mexA*, *mexC*, *mexE*, *mexX*, and *rpoD* was determined with quantitative reverse transcription PCR (RT-qPCR) using antimicrobial exposure and RNA

extraction protocols adapted from Liu et al. (2017). An overnight culture was diluted to an OD_{600} of 0.01 in MH medium and incubated at 37 °C in a 96-well microtiter plate. At an OD_{600} of 0.6, the culture was treated with 4-methoxybenzaldehyde (1 mg/mL), EGCG (150 μg/mL), and the combination of 4-methoxybenzaldehyde (0.5 mg/mL) and EGCG (150 μg/mL) prepared in MH medium. Each treatment had three independent biological replicates. The control culture was amended MH medium ultrasonicated for 5 min to standardize the conditions.

After 1 hour of exposure at 37 °C, approximately 2.5 x 10^8 cells were fixed by mixing with two volumes of RNAprotect Bacteria reagent (Qiagen), and total RNA was extracted using an RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. Total RNA was treated at 37 °C for 30 min with DNase I (Ambion) and purified using RNA Clean and Concentrator columns (Zymo Research). The concentration and purity of RNA samples were determined using the QuantiFlour RNA System (Promega). cDNA was synthesized from 1 μg of total RNA using the iScript Reverse Transcription Supermix (Bio-Rad), and RT-qPCR was performed with the Luminaris Probe qPCR Master Mix (Thermo Scientific) according to the manufacturer's instructions. The analysis was performed using a CFX96 Real-Time PCR Detection System and CFX Maestro software (Bio-Rad). RNA samples not treated with reverse transcriptase served as a negative control to ensure the absence of contaminating genomic DNA. The primers and probes used for RT-qPCR studies are listed in Table 2 (Quale et al., 2006) The expression of mRNA for the genes of interest was normalized to that of the housekeeping gene *rpoD*. This gene was shown to be constitutively expressed in *P. aeruginosa* (Salvi et al., 2003; Quale et al., 2006).

Table 2. Oligonucleotide primers and probes used in the RT-qPCR experiment.

Gene	NCBI Accession	Primer or probe		Reference
rpoD	NP_249267.1	Fwd	5'-GGGCTGTCTCGAATACGTTGA-3'	Quale et al., 2006
		Rev	5'-ACCTGCCGGAGGATATTTCC-3'	
		Probe	[DFAM]TGCGGATGATGTCTTCCACCTGTTCC[DTAM]	
mexA	NP_249116.1	Fwd	5'-AACCCGAACAACGAGCTG-3'	Quale et al., 2006
		Rev	5'-ATGGCCTTCTGCTTGACG-3'	
		Probe	[DFAM]CATGTTCGTTCACGCGCAGTTG[DTAM]	
mexC	NP_253289.1	Fwd	5'-GGAAGAGCGACAGGAGGC-3'	Quale et al., 2006
		Rev	5'-CTGCACCGTCAGGCCCTC-3'	
		Probe	[DFAM]CCGAAATGGTGTTGCCGGTG[DTAM]	
mexE	NP_251183.1	Fwd	5'-TACTGGTCCTGAGCGCCT-3'	Quale et al., 2006
		Rev	5'-TCAGCGGTTGTTCGATGA-3'	
		Probe	[DFAM]CGGAAACCACCCAAGGCATG[DTAM]	
mexX	Part of a genome record NC_002516	Fwd	5'-GGCTTGGTGGAAGACGTG-3'	Quale et al., 2006
		Rev	5'-GGCTGATGATCCAGTCGC-3'	
		Probe	[DFAM]CCGACACCCTGCAGGGCC[DTAM]	

Fwd, forward; Rev, reverse.

4. Results

4.1. Sensitive transposon mutants and the corresponding defective genes

Previously, a smaller transposon mutant library of *P. aeruginosa* PAO1 was generated and screened for the sensitivity to 4-methoxybenzaldehyde. That screen revealed that disruption of the RND pump gene *mexA* increased the sensitivity of *P. aeruginosa* to 4-methoxybenzaldehyde (Amato et al., 2018). We hypothesized that generation of additional transposon mutants would help to identify other cellular pathways that mediate resistance to phytoaldehydes. To this end, we generated and screened 7,000 mutants of *P. aeruginosa* PAO1, bringing the size of the transposon mutant library to 10,000 clones. Using the genome coverage formula of $m = 1 - e^{-L/G}$ (Campen et al., 2015) for a given size of library (L), the

number of unique mutants (m), and the number of genes (G), we estimate that our transposon library covered approximately 83% of *P. aeruginosa* genes.

Pseudomonas aeruginosa PAO1 was transformed with EZ-Tn5 transposomes, and the efficiency of transposon insertion was calculated to be between 1 x 10^5 and 1 x 10^8 colony forming unit/µg. Mutants were screened against sub-inhibitory concentrations of 4-methoxybenzaldehyde (1.2, 1.5 and 1.7 mg/mL). We then selected the mutants that grew (OD_{600} > 0.05) in the control medium but not in the medium amended with 4-methoxybenzaldehyde as sensitive. An example of a sensitive mutant is shown in Figure 4. In total, we identified the disrupted genes of 25 mutants with increased sensitivity to 4-methobenzaldehyde. The transposon insertion sites in the sensitive mutants were mapped, and the sequences were annotated.

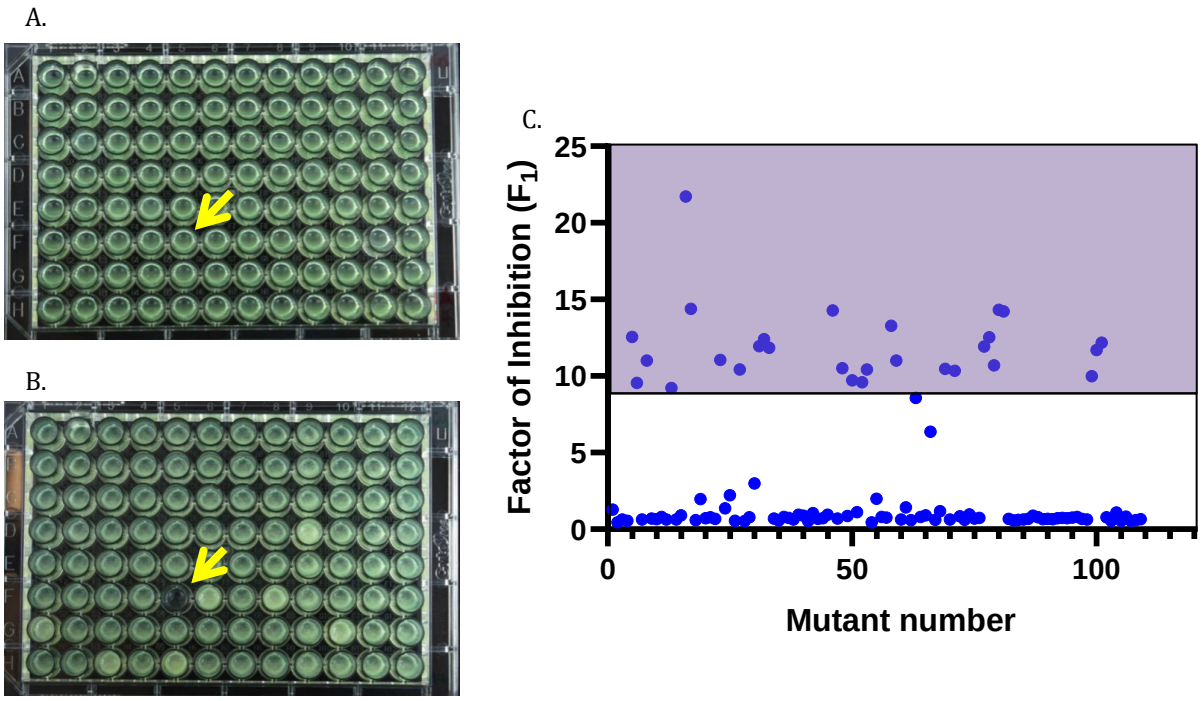

Figure 4. Selection of hypersensitive mutants. Mutants that grew in (A) control medium but did not grow in (B) medium supplemented with 1.5 mg/mL of 4-methoxybenzaldehyde were selected as hypersensitive. (C) The mutants with F_1 of 9 and above are defined as hypersensitive.

The sensitive mutants, genes disrupted by transposon insertions, and their putative functions are summarized in Table 3. The major cellular pathways involved in the interaction of *P. aeruginosa* with 4-methoxybenzaldehyde included multiple components of the cell envelope, as well as proteins involved in the transport of molybdenum and biosynthesis of molybdenum cofactor, energy metabolism, nucleotide biosynthesis, and two-component regulatory systems. A significant proportion (24%) of the recovered mutants had a defect in genes encoding conserved hypothetical proteins. Mutations in energy metabolism and nucleotide biosynthesis genes resulted in the highest sensitivity to 4-methobenzaldehyde (1.2 mg/mL). The *nqrE*, *moeB*, *nqrF*, and *ccoN1* mutants failed to grow in the presence of 1.5 mg/mL, while the rest of the mutants stopped growing at 1.7 mg/mL 4-methoxybenzaldehyde.

Table 3. Genes disrupted by EZ-Tn5 in mutants with sensitivity to 4-methoxybenzaldehyde.

Mutant	ORF (Disrupted gene)	Function	Functional category
218	PA0590 (*apaH*)	Bis(5'-nucleosyl)-tetraphosphatase	Nucleotide biosynthesis and metabolism, purine metabolism
144	PA0928 (*gacS*)	Sensor/response regulator hybrid	Two component regulatory system, biofilm formation
184*	PA1554 (*ccoN1*)	Cytochrome C oxidase, cbb3-type, CcoN subunit	Central intermediary metabolism, Energy metabolism
219, 239, 169	PA1861 (*modC*)	Molybdenum transport protein ModC	Transport of small molecules, ABC transporters
171	PA1862 (*modB*)	Molybdenum transport protein ModB	Transport of small molecules
186, 201	PA1880	Probable oxidoreductase	Putative enzymes
161**	PA2991 (*sth*)	Soluble pyridine nucleotide transhydrogenase	Nucleotide biosynthesis and metabolism
191	PA2993	Conserved hypothetical protein	Unknown, thiamine diphosphate biosynthesis
209, 217*, 237	PA2994 (*nqrF*)	Na^+-translocating NADH:quinone oxidoreductase, subunit Nqr6	Energy metabolism, Polycyclic aromatic hydrocarbon degradation
146*	PA2995 (*nqrE*)	Na^+-translocating NADH:quinone oxidoreductase subunit Nqr5	Energy metabolism

215, 216**	PA2999 (*nqrA*)	Na^+-translocating NADH:ubiquinone oxidoreductase subunit Nrq1	Energy metabolism
188	PA3000 (*aroP1*)	Aromatic amino acid transport protein AroP1	Transport of small molecules
151	PA3029 (*moaB2*)	Molybdopterin biosynthetic protein B2	Biosynthesis of cofactors, prosthetic groups and carriers, molybdopterin biosynthesis
197	PA3271	Probable two-component sensor	Two component regulatory system
190	PA4617	Hypothetical protein	Unknown
170	PA4618	Hypothetical protein	Unknown
155*	PA4663 (*moeB*)	Molybdopterin biosynthesis MoeB protein	Biosynthesis of cofactors, prosthetic groups and carriers, sulfur relay system
196	PA4856 (*retS*)	RetS (Regulator of exopolysaccharide and Type III secretion)	Two-component regulatory systems, biofilm formation
143	PA5339	Conserved hypothetical protein	Unknown

**Sensitive to 1.2 mg/mL of 4-methoxybenzaldehyde;*sensitive to 1.5 mg/mL of 4-methoxybenzaldehyde; the rest of the mutants were sensitive to 1.7 mg/mL of 4-methoxybenzaldehyde.

4.2. Interaction of efflux pump inhibitors (EPIs) with 4-methoxybenzaldehyde

Although the transposon mutagenesis did not yield any additional efflux pump mutants, we proceeded to test the effect of EPIs on the susceptibility of *P. aeruginosa* PAO1 to 4-methoxybenzaldehyde based on the previous result by Amato et al. (2018). We exposed bacterial suspensions to non-inhibitory concentrations of plant-derived EPIs and determined their effect on MICs of 4-methoxybenzaldehyde.

Results of these assays are summarized in Table 4 and show that daidzein did not affect the MIC of 4-methoxybenzaldehyde, while berberine, curcumin, and geraniol exhibited partial synergism by moderately decreasing the MIC values. In contrast, the treatment of *P. aeruginosa* with the synthetic efflux pump inhibitor CCCP (uncouples proton motive force) significantly reduced the MIC of 4-methoxybenzaldehyde. A similar effect was observed with EGCG, which was the most effective natural EPI and improved the efficacy of 4-methoxybenzaldehyde by 60% (Table 4). As CCCP and EGCG showed the most robust

synergistic interaction with 4-methoxybenzaldehyde, we tested the MIC of 4-methoxybenzaldehyde in the presence of both EPIs with *mexA* mutant from the transposon mutant library generated by Amato et al. (2018).

Table 4. The effect of EPIs on MIC of 4-methoxybenzaldehyde and their interactions in wild-type *P.aeruginosa* PAO1.

EPI	Concentration (µg/mL)	MIC of 4-methoxybenzaldehyde (mg/mL)	Interaction
None	N/A	2.0	N/A
CCCP	25	0.6	Synergism
EGCG	150	0.8	Synergism
Daidzein	400	2	Indifference
Berberine	400	1.5	Partial synergism
Curcumin	400	1.5	Partial synergism
Geraniol	400	1.5	Partial synergism

Our findings confirmed that CCCP and EGCG potentiate the antimicrobial activity of 4-methoxybenzaldehyde both in the wild-type strain and the *mexA* mutant derivative. Importantly, we showed that only 30% and 40% of the total amount of 4-methoxybenzaldehyde usually required to inhibit *P. aeruginosa* was needed to effectively suppress the bacterial growth in the presence of CCCP and EGCG, respectively (Figure 5).

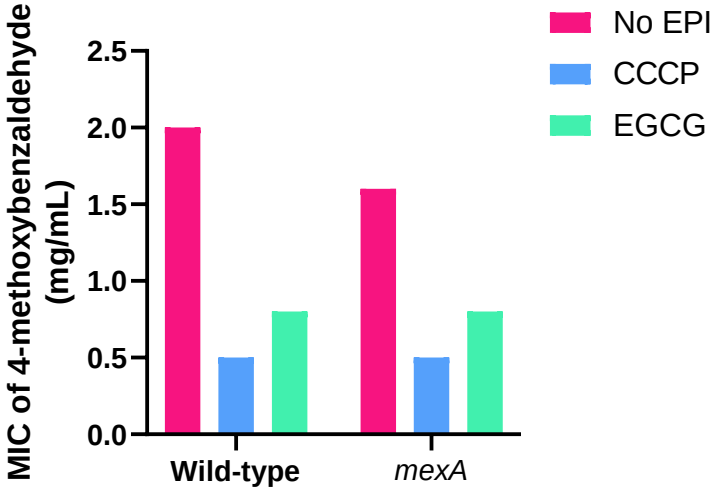

Figure 5. MICs of 4-methoxybenzaldehyde in the presence of cyanide m-chlorophenyl hydrazine (CCCP) (25 µg/mL) and EGCG (150 µg/mL) for *P.aeruginosa* PAO1 *mexA* mutant.

4.3. The response of RND-type efflux pump gene expression to 4-methoxybenzaldehyde and EGCG

As EPIs sensitized *P. aeruginosa* PAO1 and its mutants to 4-methoxybenzaldehyde, we proceeded to investigate if 4-methoxybenzaldehyde and EGCG perturb the expression of the genes contributing to the intrinsic and acquired types of resistance to antimicrobial compounds. We exposed the wild-type *P. aeruginosa* to 4-methoxybenzaldehyde, EGCG, and the combination of both compounds, and used RT-qPCR with specific primers and probes targeting genes encoding MexAB-OprM and components of other clinically-relevant RND-type efflux pumps (i.e., MexCD-OprJ, MexEF-OprN, and MexXY-OprM).

Results of this experiment revealed that 4-methoxybenzaldehyde induced the *mexC* and *mexE* genes by 54- and 21-fold, respectively, compared to the untreated control. In contrast, the addition of EGCG significantly decreased the expression of *mexC* and *mexE*, which were upregulated only by 18- and 12-fold, respectively, relative to the control (Figure 6). EGCG alone caused a slight (3.1-fold) upregulation of the *mexA* gene but did not affect levels of expression of other efflux genes.

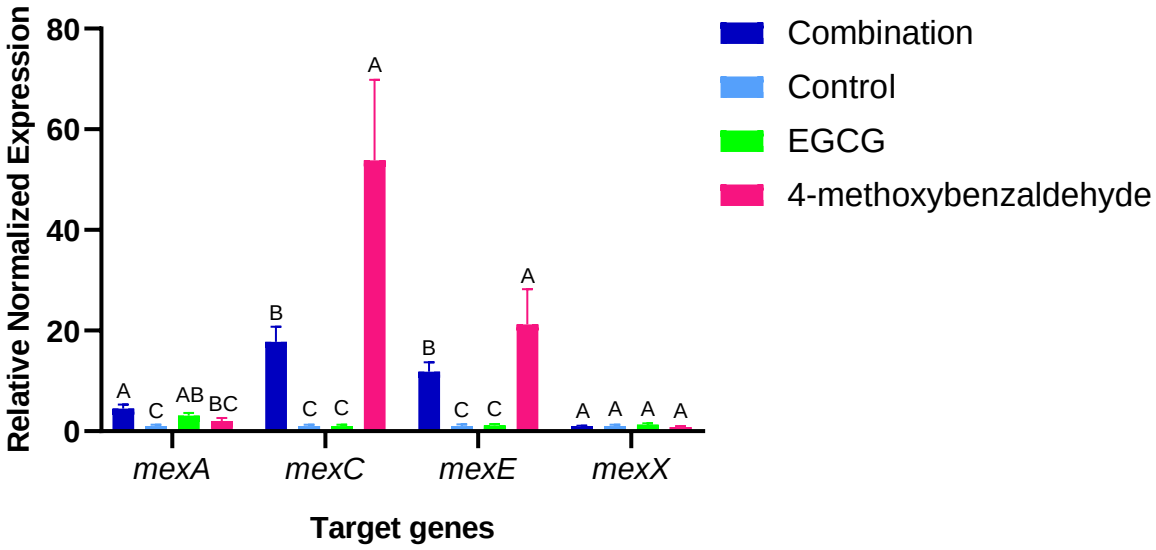

Figure 6. Relative expression of RND-type efflux pump genes in response to 4-methoxybenzaldehyde, EGCG and a combination of both compounds. Tukey-Kramer HSD test was used for statistical analysis. The significant difference represents the comparison between treatments for each gene.

5. Discussion

The increasing interest in medical applications of antimicrobial EO constituents has led to the exploration of their molecular interactions with many important pathogens. Previously, Amato et al. (2018) showed that *mexA* mutants from the transposon mutant library of *P. aeruginosa* PAO1 were hypersensitive to 4-methoxybenzaldehyde. This finding prompted us to expand the transposon screen in the hope of identifying more genes responsible for susceptibility to 4-methoxybenzaldehyde. The results of this screen revealed that the exposure of *P. aeruginosa* PAO1 to 4-methoxybenzaldehyde affects a diverse range of cytoplasmic and membrane pathways, including those involved in various transport functions, the central intermediary metabolism, energy metabolism, and biosynthesis of cofactors. We discovered that six out of 25 susceptible mutants contained transposon

insertions in genes encoding subunits of a Na^+-translocating NADH:quinone oxidoreductase. These results were consistent with the findings of Yu et al. (2010), who showed that 4-methoxybenzaldehyde upregulates the gene encoding an aryl-alcohol dehydrogenase involved in the oxidoreductase activity in *Saccharomyces cerevisiae*. The recovery of mutations in genes for the transport of molybdenum and synthesis of the molybdenum cofactor supported the study from Amato et al. (2018), which also observed multiple hypersensitive mutants that were deficient in these pathways. Currently, no evidence in the literature can explain the connection between the molybdenum cofactor pathways and 4-methoxybenzaldehyde, which warrants further investigation of these mutants. Interestingly, we also found that some hypersensitive phenotypes were associated with mutations in regulatory genes, such as the global two-component regulatory system GacAS. In *P. aeruginosa*, the GacAS system contributes to the formation of biofilms, secretion of secondary metabolites, and resistance to some antibiotics (Brinkman et al., 2001). However, the antibiotic resistance mechanism mediated by GacAS is unclear (Gooderham & Hancock, 2009). Finally, we found several hypersensitive mutants that were deficient in the production of conserved hypothetical proteins which play an unknown role in the biology of *P. aeruginosa*.

We also tested several plant-derived efflux pump inhibitors (EPIs) for the capacity to improve the antimicrobial efficacy of 4-methoxybenzaldehyde. Results of this testing revealed that epigallocatechin gallate (EGCG) from green tea acted synergistically and significantly reduced the minimal inhibitory concentration of 4-methoxybenzaldehyde. Our findings agreed with the data of Roccaro et al., (2004), who reported that EGCG interfered with the function of efflux pumps in *S. aureus*. The mechanism behind this interference

remains unclear, and it has been suggested that EPIs may inhibit efflux by interfering with the expression of efflux pump genes, disrupting the assembly of the efflux pump components, or by interfering with the energy source of efflux pumps (Askoura et al., 2011). Finally, we measured the expression of genes encoding transfusion membrane proteins of RND-type efflux pumps MexAB-OprM, MexCD-OprJ, MexEF-OprN, and MexXY-OprM in response to 4-methoxybenzaldehyde and EGCG. We found that 4-methoxybenzaldehyde significantly upregulated components of the MexCD-OprJ and MexEF-OprN efflux pumps, which are known to act on aromatic hydrocarbons and may thus recognize 4-methoxybenzaldehyde as a substrate (Askoura et al., 2011). The induction of *mexC* and *mexE* in the presence of 4-methoxybenzaldehyde and EGCG was significantly lower, thus suggesting that EGCG has a suppressive effect on the expression of these genes. The *mexX* gene did not respond to any of the treatments, indicating that 4-methoxybenzaldehyde may not be an essential substrate for this pump under the tested experimental conditions.

6. Conclusion

In summary, this study has demonstrated that antimicrobial phytoaldehydes kill bacteria by perturbing different classes of cellular pathways, including components of RND-type efflux pumps, which also play an important role in the resistance to different types of clinical antibiotics. The susceptibility to phytoaldehydes can be improved by treating *P. aeruginosa* with synthetic and natural efflux pump inhibitors. In particular, we demonstrated that the natural EPI epigallocatechin gallate (EGCG) interacts synergistically with 4-methoxybenzaldehyde. Collectively, our results highlighted the importance of active efflux in

the resistance of *P. aeruginosa* to phytoaldehydes and the potential of EPIs in improving the efficacy of these natural antimicrobials and phytoaldehyde-based PANDAs.

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