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The antimicrobial activity and cellular pathways targeted by *p*-anisaldehyde and epigallocatechin gallate in the opportunistic human pathogen *Pseudomonas aeruginosa*.

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ABSTRACT

Plant-derived aldehydes are constituents of essential oils that possess broad-spectrum antimicrobial activity and kill microorganisms without promoting resistance. In our previous study, we incorporated *p*-anisaldehyde from star anise into a polymer network called PANDAs (Pro-Antimicrobial Networks via Degradable Acetals) and used it as a novel drug delivery platform. PANDAs released *p*-anisaldehyde upon a change in pH and humidity, and controlled growth of the multi-drug resistant pathogen *Pseudomonas aeruginosa* PAO1. In this study, we identified cellular pathways targeted by *p*-anisaldehyde, by generating 10,000 transposon mutants of PAO1 and screened them for hypersensitivity to *p*-anisaldehyde. To improve the antimicrobial efficacy of *p*-anisaldehyde, we combined it with epigallocatechin gallate (EGCG), a polyphenol from green tea, and demonstrated that it acts synergistically with *p*-anisaldehyde in killing *P. aeruginosa*. We then used RNA-seq to profile transcriptomic responses of *P. aeruginosa* to *p*-anisaldehyde, EGCG, and their combination. The exposure to *p*-anisaldehyde altered the expression of genes involved in the modification of cell envelope, membrane transport, drug efflux, energy metabolism, molybdenum cofactor biosynthesis, and stress response. We also demonstrated that the addition of EGCG reversed many *p*-anisaldehyde-coping effects and induced oxidative stress. Our results provide an insight into the antimicrobial activity of *p*-anisaldehyde and its interactions with EGCG and may aid in the rational identification of new synergistically-acting combinations of plant metabolites. Our study also confirms the utility of the thiol-ene polymer platform for the sustained and effective delivery of hydrophobic and volatile antimicrobial compounds.

IMPORTANCE

Essential oils (EOs) are plant-derived products that have been long exploited for their antimicrobial activities in medicine, agriculture, and food preservation. EOs represent a promising alternative to conventional antibiotics due to the broad-range antimicrobial activity,

low toxicity to human commensal bacteria, and the capacity to kill microorganisms without promoting resistance. Despite the progress in the understanding of the biological activity of EOs, many aspects of their mode of action remain inconclusive. The overarching aim of this work was to address these gaps by studying molecular interactions between an antimicrobial plant aldehyde and the opportunistic human pathogen *Pseudomonas aeruginosa*. Results of this study identified microbial genes and associated pathways involved in response to antimicrobial phytoaldehydes and provided insights into molecular mechanisms governing the synergistic effects of individual constituents within essential oils.

INTRODUCTION

Pseudomonas aeruginosa is a ubiquitous Gram-negative bacterium that serves as an important model for opportunistic infections and biofilm research (1, 2). This organism is commonly found in soil, water, and plants, but can readily infect immunocompromised individuals causing septicemia and wound or urinary tract infections (3). It also responsible for pneumonia and causes increased morbidity and mortality in cystic fibrosis patients. *Pseudomonas aeruginosa* is resistant to multiple classes of antibiotics and belongs to a class of pathogens with increased virulence, persistence and transmissibility known as the ESKAPE group (also encompasses *Enterococcus faecium*, *Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii*, and *Enterobacter* species) (4). The Centers for Disease Control and Prevention (CDC) reports that, in the U.S. alone, *P. aeruginosa* is associated with 8% of all healthcare-acquired infections and over 400 deaths per year, which are often caused by multi-drug resistant (MDR) strains (5). These MDR variants are insensitive to nearly all the available β-lactams and aminoglycosides and are classified by the CDC as a serious threat that requires close monitoring and prevention activities (6). Therefore, there is a need for the development of antimicrobial agents, which do not promote antibiotic resistance and may help to mitigate the spread of MDR phenotypes in *P. aeruginosa* and other bacterial pathogens.

Plant essential oils (EOs) represent a rich source of alcohols, aldehydes, terpenes, ethers, ketones, and phenolic compounds with antimicrobial, antifungal, and antiparasitic activity (7). The antiseptic properties and low toxicity of EOs prompted their use in traditional food preservation and homeopathic medicine (8). However, despite the demonstrated biological activity, the wider acceptance of EOs and their constituents as antimicrobial agents is limited by the hydrophobicity, chemical instability, high concentrations needed to achieve sufficient antimicrobial effect, and undefined mode of action. In our previous work, we addressed some of these issues by incorporating the antimicrobial EO constituent *p*-anisaldehyde into a polymer network called Pro-Antimicrobial Networks via Degradable Acetals (PANDAs) (9, 10). The

resultant polymer material was designed to act as a pro-drug that releases *p*-anisaldehyde upon a change in pH and humidity. The incorporation into PANDAs increased the bioavailability and antimicrobial efficacy of *p*-anisaldehyde and related phytoaldehydes against *P. aeruginosa*, *Escherichia coli, Burkholderia cenocepacia, S. aureus,* and *Candida albicans.* We also demonstrated that the inactivation of the MexAB-OprM multidrug efflux pump sensitizes *P. aeruginosa* to the action of *p*-anisaldehyde (10).

MexAB-OprM is a member of the resistance-nodulation-cell-division (RND) superfamily of multidrug efflux pumps, which are recognized as essential contributors to the emergence of MDR phenotypes in pathogenic bacteria. These membrane transporters extrude a broad spectrum of antimicrobial compounds (11), intercellular signals, and virulence factors (12, 13), which makes them an attractive target for the development of anti-resistance drugs. Such drugs, known as efflux pump inhibitors (EPIs), interfere with the function, expression, or assembly of efflux pumps, and can significantly reduce or completely reverse resistance against otherwise ineffective antibiotics (14). Despite the development of a number of effective synthetic and semi-synthetic EPIs, none of these compounds are currently used in the treatment of bacterial infections because of the instability, low selectivity, and cytotoxic side effects (15). Plants, like animals, are attacked by bacterial pathogens and have evolved defense mechanisms to counteract such infections. This fact has prompted numerous plant-based studies aimed at the isolation of natural EPIs with lower toxicity and better tolerability. These efforts produced a growing list of promising candidates, some of which were patented and are being evaluated against different pathogens (16, 17).

In this study, we used *P. aeruginosa* PAO1 as a model organism to identify cellular pathways targeted in bacterial pathogens by *p*-anisaldehyde and structurally related compounds. We first subjected PAO1 to transposon mutagenesis and screened the resultant library of mutants for susceptibility to sub-inhibitory concentrations of *p*-anisaldehyde and the new *p*-anisaldehyde-containing antimicrobial polymer. This new polymer network relies solely

on diffusion to control the release of the active *p*-anisaldehyde from a non-degradable thiol-ene thermoset matrix (Fig. 1). The synthesis of the polymer was performed using only commercially available polyfunctional alkenes and polyfunctional thiols, which have eliminated the need to prepare and purify hydrolytically unstable monomers required for release of *p*-anisaldehyde. The new approach minimized the susceptibility of the monomers to atmospheric conditions during the network cure process and improved the batch-to-batch variability. The resultant polymer material had antimicrobial efficacy nearly identical to that of the previously reported degradable systems (9, 10).

We also screened a panel of plant-derived EPIs for the ability to potentiate the antimicrobial activity of phytoaldehydes and demonstrated that epigallocatechin gallate (EGCG), a polyphenol from green tea, acts synergistically with *p*-anisaldehyde and significantly reduces its minimal inhibitory concentration against *P. aeruginosa.* Finally, we profiled the transcriptomes of *P. aeruginosa* grown in the presence of *p*-anisaldehyde, EGCG, and a combination of thereof to gain insight into the possible mode of action of these plant-derived antimicrobial compounds. Our results revealed that *p*-anisaldehyde alters the expression of genes involved in membrane transport, lipids biosynthesis, stress response, energy metabolism, and biosynthesis of the molybdenum cofactor. The addition of EGCG reversed many of the *p*-anisaldehyde-coping responses and induced oxidative stress, which may contribute to the synergistic antimicrobial effect against *P. aeruginosa*.

RESULTS

Selection and characterization of mutants with hypersensitivity to *p*-anisaldehyde. The screening of the transposon mutant library yielded 39 clones that failed to grow in the presence of *p*-anisaldehyde. All hypersensitive mutants had F_1 values ≥ 9 and were at least 1.2 times more sensitive to *p*-anisaldehyde than the wild type PAO1 strain (Fig. 2A). Thirty-one mutants were sensitive to 1.7 mg mL^{-1} of *p*-anisaldehyde (0.85× MIC of the wild type strain), six

mutants were sensitive to 1.5 mg mL^{-1} (0.75× MIC), while two strains failed to grow at 1.2 mg mL^{-1} (0.6× MIC). The mapping of transposon insertion sites by inverse PCR and DNA sequencing revealed that the sensitivity to *p*-anisaldehyde was caused by mutations in 27 genes, which represented 24 unique cellular pathways (Table 1). We further chose 24 mutants and tested them against the *p*-anisaldehyde-containing polymer. Our results revealed that 21 of the tested strains were significantly more sensitive to the antimicrobial polymeric discs, which manifested as a significant ($P < 0.05$) increase in the zone of inhibition compared to the wild type PAO1 (Fig. 2B). Approximately 40% of the affected genes function in the energy metabolism and generation of ATP or participate in the uptake of molybdenum and synthesis of the molybdenum cofactor (Table 1). Other identified genes are involved in signal transduction, nucleotide metabolism, or membrane transport of small molecules. Interestingly, among mutants with increased sensitivity to *p*-anisaldehyde-containing polymeric discs were two isolates that carried mutations in *mexA*, which encodes the periplasmic linker component of the efflux pump MexAB-OprM (Table 1).

Epigallocatechin gallate potentiates the activity of *p*-anisaldehyde in *P. aeruginosa*. Since the transposon screen suggested the possible importance of efflux pumps for the resistance to phytoaldehydes, we tested a panel of known plant-derived EPIs for the ability to sensitize *P. aeruginosa* to *p*-anisaldehyde. The testing involved measuring the MIC of *p*-anisaldehyde in the presence of non-inhibitory concentrations of selected EPIs. Results of that screen revealed that the addition of daidzein had no effect, while berberine, curcumin, and geraniol exhibited partial synergism and moderately decreased the MIC of *p*-anisaldehyde in the wild-type PAO1 (Table 2). In contrast, the green tea polyphenol epigallocatechin gallate (EGCG) significantly reduced the MIC of *p*-anisaldehyde and exhibited a strong synergistic effect similar to that of the proton motive force uncoupler carbonyl cyanide m-chlorophenylhydrazone

(CCCP). Broth microdilution checkerboard assay between EGCG and *p*-anisaldehyde confirmed that both compounds interact synergistically with the ∑FIC value of 0.5 (Fig. S1).

The effect of *p*-anisaldehyde on the transcriptome of *P. aeruginosa* PAO1. In order to gain further insight into the antimicrobial activity of *p*-anisaldehyde, we profiled and compared transcriptomes of *P. aeruginosa* treated with *p*-anisaldehyde, EGCG, and a combination of both compounds. The RNA-seq generated a total of 554 million filtered reads, which were mapped to the reference PAO1 genome. Statistical analysis using the cut off criteria of log2 fold change $|\log_2 FC| \geq 1.5$ and false discovery rate (FDR) adjusted *p*-value ≤ 0.05 revealed that the highest number of differentially expressed genes (DEGs) was associated with exposure to *p*-anisaldehyde, which affected the expression of 265 genes, or 5% of the entire genome (Fig. 3A). The treatment with EGCG and a combination of both compounds altered, respectively, the expression of 29 and 86 genes. We also performed the pairwise comparison between the *p*-anisaldehyde and combination treatments to understand molecular mechanisms responsible for the synergistic effect of epigallocatechin gallate. The information on the differentially expressed gene ID tags, predicted functions, log2 fold change (log2FC) values, and *p*-values adjusted for the false discovery rate (FDR) are summarized in Table S1.

P. aeruginosa responded to *p*-anisaldehyde by upregulating the expression of 128 genes, many of which function in energy metabolism, membrane transport, signal transduction, and stress response (Fig. 4). The overall highest levels of induction were observed in genes encoding components of multidrug efflux pumps (5.9-fold), the two-component response regulator PhoP (PA1179) (4-fold), and several conserved hypothetical proteins (4-fold) (Table S1). The 137 downregulated genes included those encoding various transporters, and components of energy metabolism pathways, type III secretion apparatus, and respiratory nitrate reductase. Interestingly, a significant proportion (32%) of DEGs that responded to *p*-anisaldehyde genes encoded conserved hypothetical proteins of unknown function. We also matched the genes that were differentially expressed in response to *p*-anisaldehyde to genetic

loci identified during the transposon screen. Results of this comparison revealed that the two datasets shared three genes, which encoded a cytochrome oxidase (PA1554), a predicted oxidoreductase (PA1880), and a two-component sensor (PA3271) (Fig. 3B).

The Blast2GO analysis of *p*-anisaldehyde DEGs identified the intrinsic component of the membrane, plasma membrane, and cell periphery as dominant gene ontology (GO) terms in the cellular component category. The most common GO terms in molecular function and biological processes categories were, respectively, the binding of an organic cyclic compound, and cellular metabolic processes (Fig. 4A). A similar pattern of GO terms was observed in the Blast2GO profiling of genes interrupted by insertions of the EZ-Tn*5* <TET-1> transposon in *p*-anisaldehyde-sensitive mutants. Finally, the pathway enrichment analysis of upregulated DEGs revealed the overrepresentation (Fisher's exact test; FDR ≤ 0.05) of genes involved in the biosynthesis of lipids and response to chemicals and antibiotics. In contrast, the downregulated differentially expressed pathways were enriched, among others, in genes associated with ion transmembrane transport and translation (Fig. 4B).

EGCG modulates transcriptional changes caused by *p*-anisaldehyde in *P. aeruginosa*. In contrast to *p*-anisaldehyde, the exposure of PAO1 to subinhibitory levels of epigallocatechin gallate altered the expression of only 28 genes, one-third of which were classified as conserved hypothetical (Table S1). Among the upregulated DEGs with predicted functions were those encoding the efflux pump MuxABC-OpmB, transcriptional regulators (PA2525-PA2528 and PA2825) (3-fold), and components of the cell envelope and oxidative stress defense systems (PA0848, PA0849, PA4612, and PA4613) (2.5-fold). The six downregulated DEGs encoded a DNA mismatch repair protein (PA4946), an MFS transporter (PA2314) (2.4-fold), 1-phosphofructokinase (PA3561) (2.8-fold), and a FAD-binding subunit of glycolate oxidase (PA5354) (4.1-fold). The common GO terms in the cellular component category were linked with cell envelope, while the molecular function was associated with binding of heterocyclic compounds, oxidoreductase activity, transporter and peroxidase activity

(Fig. 5A). The dominant biological processes involved the response to chemical, regulation of cellular processes, and cellular detoxification. GO term enrichment analysis showed that most EGCG DEGs were involved in response to toxic substances and oxidative stress, while downregulated genes were associated with lipid biosynthesis, carbohydrate metabolism, and several other functions (Fig. 5B).

Interestingly, the treatment of *P. aeruginosa* with a combination of *p*-anisaldehyde and EGCG altered the expression of 86 genes. The majority of these genes (75 in total) were also present in *p*-anisaldehyde or EGCG datasets, and only 11 were uniquely associated with the mixed treatment (Fig. 3A). Three of these unique DEGs were downregulated and encoded a hypothetical protein (PA5406), a ribosomal protein (PA4433), and the cell division protein FtsE (PA0374) (Table S1). The unique upregulated genes encoded a hydrocarbon reductase (PA5236), an MFS transporter (PA5030), and a fosfomycin resistance protein (PA1129). The GO term enrichment analysis of the combination treatment revealed an overrepresentation of DEGs involved in the transmembrane transport, response to antibiotics and efflux, iron binding, and peptide biosynthesis (Fig. 6B).

The interaction between *p*-anisaldehyde and EGCG affects multiple categories of cellular pathways in *P. aeruginosa*. Our transcriptomic analysis revealed that *p*-anisaldehyde strongly induced genes encoding the efflux pumps MexCD-OprJ, MexEF-OprN, and MexKJ, as well as the putative transporter of the small multidrug resistance (SMR) family PA1541. Additionally, genes that encode regulators of the *mexAB-oprM* operon (i.e., the antirepressor gene *armR* (PA3719) and *nalC* (PA3721)) were differentially expressed in *p*-anisaldehyde-treated cells. In contrast, the treatment with EGCG induced only one efflux pump, MuxABC, suggesting that this transporter plays a specific role in the efflux of epigallocatechin gallate. We further validated the RNA-seq data by RT-qPCR with oligonucleotide primers and probes targeting components of seven clinically relevant *P. aeruginosa* efflux pumps. The results of this experiment revealed a strong induction of *mexC*, *mexE,* and *mexK* in response to *p*-

anisaldehyde, which was in agreement with the results of RNA-seq (Fig. 7). Interestingly, although, *mexC* and *mexE* were also upregulated in the combination treatment, the addition of EGCG resulted in significantly lower levels of expression compared to the *p*-anisaldehyde-only treatment. Contrary to results of the transposon mutagenesis, we did not detect any measurable induction of genes encoding components of the MexAB-OprM efflux pump. We attribute this discrepancy to differences in the length of the exposure to *p*-anisaldehyde between the transposon screen and gene expression experiments.

In addition to efflux pumps, we observed that the presence of *p*-anisaldehyde modulated the expression of almost 30 other membrane transporter genes (Table S1). The GO term enrichment analysis of these DEGs revealed an overrepresentation of pathways associated with the transmembrane transport of ions and inorganic molecules (Fig. 4). Like in the case of efflux pumps, some of these genes were also differentially expressed between the *p*-anisaldehyde and *p*-anisaldehyde/EGCG treatments. The comparison of gene expression profiles also revealed five ABC transporter genes (*agtA*, *agtB*, *ihpM*, *gltG,* and *yrbE*) that were downregulated by *p*-anisaldehyde, but that effect was significantly reversed in cultures treated with a combination of *p*-anisaldehyde and EGCG (Fig. 8). The effect was especially pronounced in the case of *yrbE* (PA4455), which encodes an ABC transporter involved in resistance to acidified nitrite, EDTA, and several antibiotics (18). A similar response to *p*-anisaldehyde and EGCG was observed in genes encoding components of the potassium translocating ATPase KdpFABC. Conversely, the addition of EGCG significantly induced the cation diffusion facilitator (CDF) transporter gene *yiiP*, whose expression was unaffected by *p*-anisaldehyde.

Our analysis also revealed that *p*-anisaldehyde and EGCG differentially modulate the activity of several genes involved in the modification of the *P. aeruginosa* cell envelope. The exposure to *p*-anisaldehyde induced genes of the *arnBCADTEF* cluster, which functions to modify the lipid A component of the lipopolysaccharide thereby leading to increased resistance to cationic antimicrobial peptides (19). This induction was not observed in the EGCG-treated

cells, and the treatment with both compounds significantly reduced the expression of *arnBCF* (Fig. 8). Similar alterations were observed in the expression level of the *oprH-phoPQ* operon, which encodes an outer membrane protein H and a two-component signal transduction system that regulates the activity of the *arnBCADTEF* operon (20). Interestingly, *oprH*, which was induced by *p*-anisaldehyde but had a lower level of expression in the presence of EGCG, represents part of the Mg^{2+} stimulon and contributes to the resistance to polymyxin B and aminoglycosides (21).

p-Anisaldehyde and EGCG differentially affected expression of multiple cellular pathways associated with the stress response. The comparative analysis revealed that genes encoding several oxidative stress response enzymes, molecular chaperones, and a component of the DNA mismatch repair system were differentially expressed between the *p*-anisaldehyde, EGCG, and combination treatments. The EGCG and combination treatments upregulated genes encoding the KatB catalase (PA4613) (22) and its accessory ankyrin-like protein AnkB (PA4612) (23), the alkyl hydroperoxide-reducing protein AhpB (PA0848), the thioredoxin reductase TrxB2 (PA0849) (24), and two proteins, PA3237 and PA3287, that are upregulated in response to H_2O_2 (25, 26) (Fig. 8). In contrast, *p*-anisaldehyde upregulated the expression of molecular chaperones GroES and GroEL, but this effect was reversed by the presence of EGCG. In *mutL*, which functions to stabilize components of the mismatch repair machinery, the combination treatment completely negated the effect of individual *p*-anisaldehyde and EGCG treatments, which both strongly downregulated this gene.

Finally, our results revealed that the treatment with *p*-anisaldehyde and EGCG modulated the expression of the *narK1K2GHJI* operon (PA3872-PA3877), which encodes the respiratory nitrate reductase and nitrate transporter (Fig. 8). *p*-Anisaldehyde downregulated genes of the nitrate reductase pathway, but this repression was significantly weaker in the combination treatment. The exposure of *P. aeruginosa* to EGCG alone had little effect on the expression of this pathway.

DISCUSSION

In this study, we used a combination of transposon mutagenesis and RNA-seq to characterize cellular pathways targeted by *p*-anisaldehyde and epigallocatechin gallate in the multidrug-resistant human pathogen *P. aeruginosa*. *p*-anisaldehyde is a constituent of essential oils and a member of the phenylpropanoid family of metabolites, which are synthesized by plants as derivatives of the amino acid phenylalanine (27). Therefore, our findings provide insight into the biological activity of a larger group of structurally related compounds with antimicrobial, antifungal, and antibiofilm properties (28, 29). Although the antimicrobial activity of EOs is traditionally attributed to their ability to affect the integrity of cellular membranes (30), our results revealed that the response to *p*-anisaldehyde involves a broad range of cellular pathways. We observed that the exposure to *p*-anisaldehyde resulted in the downregulation of genes encoding cytochrome oxidases (*cyoA*, *ccoN1*), a phosphofructokinase (*fruK*), a pyruvate carboxylase (*pycA*), and the NADH:ubiquinone oxidoreductase NqrAEF. In the course of our transposon screen, we recovered several mutants with defects in genes involved in the transport of molybdenum and biosynthesis of the molybdenum cofactor. Molybdenum cofactor is an essential component of several important molybdoenzymes, including respiratory nitrate reductases (31). Our transcriptomic data revealed that exposure to *p*-anisaldehyde significantly downregulated the *narK1K2GHJI* operon that in *P. aeruginosa* encodes components of the inner membrane-bound nitrate reductase complex. This dissimilatory nitrate reductase catalyzes the respiratory reduction of nitrate to nitrite and helps generate ATP in the absence of oxygen (32). Interestingly, nitrate reduction decreases the *trans* fatty acid content and inhibits the formation of biofilms in *P. aeruginosa* (33). The biofilm lifestyle and alterations in the fatty acid profile are associated with the response to environmental stress and toxic substances (34). Hence, it is plausible that the repression of nitrate reductase represents a defense response to

317 *p*-anisaldehyde by favoring the formation of resistant biofilms and densely packed membranes
318 with higher *trans* fatty acid content.
319 Other notable categories of defense response to *p*-anisaldehyde in *P. aeruginosa*
320 included the upregulation of molecular chaperones and efflux transport. The treatment with *p*-
321 anisaldehyde upregulated genes encoding heat-shock proteins (*grpE, htpX, ibpA, hslU*) and
322 chaperons (*dnaK, dnaJ, groES*), which function to refold and destroy proteins damaged by
323 extreme temperature, oxidative stress, disinfectants, heavy metals, and antibiotics (35-37). We
324 further observed an upregulation of *betAB* genes (PA5372, PA5373) that encode enzymes
325 involved in the conversion of choline to glycine betaine, a key microbial compatible solute. The
326 intracellular accumulation of glycine betaine and related compounds confers tolerance to
327 osmotic, thermal, oxidative, and denaturant forms of stress (38-40). Our transposon screen and
328 RNA-seq also revealed that *p*-anisaldehyde upregulates multiple transporters, including efflux
329 pumps of the RND superfamily, which expel antibiotics, metabolic inhibitors, detergents,
330 biocides, quorum sensing signals, and some virulence factors (41, 42). We observed the
331 induction of genes encoding the MexCD-OprJ, MexEF-OprN, and MexKJ efflux pumps, and
332 recovered a transposon mutant with a defect in MexAB-OprM. A similar upregulation of *mexB*,
333 *mexC*, *mexE*, and *mexY* genes was recently reported by Tetard et al. (43) in *P. aeruginosa*
334 PA14 treated with cinnamaldehyde. Interestingly, the induction of efflux pump transporters and
335 chaperones was also observed in *P. putida* exposed to toluene (44) thus suggesting that the
336 response to *p*-anisaldehyde represents part of a broader strategy used by *P. aeruginosa* to
337 cope with solvent stress.
338 Individual EO constituents are often less potent than antibiotics, which presents practical
339 problems for their use as antimicrobials or food preservatives. A possible way to overcome this
340 obstacle involves the exploitation of synergistic effects between different EO constituents.
341 Although a number of synergistic combinations of compounds were identified by trial and error
342 (7), the molecular mechanisms behind such interactions remain poorly understood. The results

of this study may help to address this gap in knowledge by probing synergistic interactions between *p*-anisaldehyde and EGCG. The RNA-seq profiling revealed that the two compounds target very different sets of cellular pathways in *P. aeruginosa*. Interestingly, we included EGCG in our experiments as a potential efflux pump inhibitor (45, 46), and then observed the induction of the efflux pump MuxABC-OpmB. Although the expression of other efflux pump genes was not affected (or even somewhat repressed in cultures treated with a combination of *p*-anisaldehyde and EGCG), we suggest that the EPI properties of epigallocatechin gallate should be investigated further. The analysis of genes differentially expressed in the presence of EGCG also revealed components of several pathways (*katB, ahpB, trxB2,* PA2826, PA3237, PA3287) that are associated in *P. aeruginosa* with the response to oxidative stress and exposure to H_2O_2 (25). These findings provide an insight into the antimicrobial mode of action of epigallocatechin gallate and agree with reports of the intercellular release of H_2O_2 in *E. coli* O157:H7 treated with subinhibitory levels of EGCG (47), and results of Liu et al. (48), who profiled the transcriptomic response of *P. fluorescens* to EGCG. Surprisingly, apart from the oxidative stress genes, our RNA-seq data did not significantly overlap with the *P. fluorescens* dataset, which had over 400 genes whose expression was altered in response to EGCG. We attribute these discrepancies to differences in the biology of the two *Pseudomonas* species, higher concentrations of EGCG used by Liu et al. (48), and more stringent fold change cutoff ($|\log_2 FC| \geq 1.5$ vs. $|\log_2 FC| \geq 1$) to identify the differentially expressed *P. aeruginosa* genes.

Collectively, our results suggest that *p*-anisaldehyde affects *P. aeruginosa* by first interfering with the integrity of its cell envelope, which then allows it to accumulate intracellularly and adversely affect proteins, by causing their misfolding and aggregation. In contrast, epigallocatechin gallate poisons bacteria by inducing oxidative stress, which may explain its ability to complement and potentiate the antimicrobial action of *p*-anisaldehyde. The synergistic antimicrobial effect is further enhanced by the capacity of EGCG to partially or completely reverse the upregulation of many genes by *p*-anisaldehyde, including those encoding various

transporters and key multidrug efflux pumps. Interestingly, the treatment with a combination of *p*-anisaldehyde and EGCG also significantly repressed a gene (PA0374) encoding FtsE, which is membrane protein located in the septal ring. This may represent yet another facet of the synergism because studies in *E. coli* and several other species demonstrated that *ftsE* mutants grow poorly and exhibit division defects (49). In gram-negative bacteria, the failure to complete cell division is associated with increased sensitivity to antibiotics, detergents, and defensins (50).

In conclusion, this study provides an insight into the antimicrobial activity of *p*-anisaldehyde and its synergistic interactions with epigallocatechin gallate. Our results may aid in the rational identification of new synergistically-acting combinations of plant metabolites and their exploitation for the control of pathogenic microorganisms. Our study also confirms the utility of the thiol-ene polymer platform for the sustained and effective delivery of hydrophobic and volatile antimicrobial compounds.

MATERIALS AND METHODS

Bacterial strain, growth conditions and compounds. All experiments conducted in this study were performed with the reference strain *Pseudomonas aeruginosa* PAO1. The organism was routinely cultured at 37°C in Difco Luria-Bertani (LB) medium (Becton Dickinson, Franklin Lakes, NJ), while Mueller-Hilton II (MH) broth and agar (Becton Dickinson) were used for all antimicrobial assays. The selection of transposon mutants was performed by amending the growth medium with tetracycline (Tc) (Thermo Scientific, Waltham, MA) at the concentration of 100 µg mL^{-1}. *p*-Anisaldehyde (pA), epigallocatechin gallate (EGCG), geraniol, daidzein, berberine, curcumin, 2-hydroxy-2-methylpropionate (Darocur 1173), carbonyl cyanide m-chlorophenylhydrazone (CCCP), 1,3,5-triallyl-1,3,5-triazine-2,4,6(1*H*,3*H*,5*H*)-trione (TTT), and pentaerythritol tetrakis(3-mercaptopropionate) (PETMP) were obtained from Thermo Scientific in the highest purity available and used without further purification. The stock solution of *p*-

anisaldehyde (12 mg mL^{-1}) was prepared by ultrasonicating the compound for 5 min in MH broth amended with 0.75% DMSO. The stocks of CCCP (5 mg mL^{-1}), EGCG (20 mg mL^{-1}), geraniol (25 mg mL^{-1}), daidzein (30 mg mL^{-1}), berberine (35 mg mL^{-1}), and curcumin (25 mg mL^{-1}) were prepared by dissolving the chemicals in dimethyl sulfoxide (DMSO) (Alfa Aesar, Haverhill, MA).

Construction of the transposon mutant library. The transposon mutagenesis was conducted by electroporating *P. aeruginosa* with transposomes, which are stable complexes formed between the EZ-Tn*5* <TET-1> transposon and the EZ-Tn*5* Transposase (both from Lucigen, Middleton, WI). Briefly, cells from an overnight culture of PAO1 were collected and washed twice in 0.3 M sucrose. One hundred microliters of electrocompetent bacteria, equivalent to 10^{10} viable cells, were mixed with 0.7 µL of the EZ-Tn5™ <TET-1> transposomes in a 1-mm gap width electroporation cuvette. The mixture was electroporated with an Electroporator 2510 (Eppendorf, Hauppauge, NY) at 2.5 kV, 10 µF, and 600 Ω. The transformed cells were immediately suspended in LB broth, incubated with shaking at 37°C for 1.5 h, spread plated onto LB-Tc_{100}, and incubated at 37°C until the appearance of individual colonies. The tetracycline-resistant colonies were transferred individually into 96-well microtiter plates prefilled with LB broth amended with 7% DMSO and stored at -80°C. A total of 10,000 transposon-bearing mutants were collected, which is estimated to cover approximately 83% of the PAO1 genome using the formula: $m = 1 - e^{-L/G}$, where *G* is equal to the number of genes in the PAO1 genome (5,572 protein-coding genes) and for a given size library (*L*) (51).

Screening of the transposon library for the sensitivity to *p*-anisaldehyde. The transposon mutants of PAO1 were screened for hypersensitivity to *p*-anisaldehyde by replicating the library with a 96-prong replicator (VP Scientific, San Diego, CA) into microplates pre-filled with MH broth supplemented with 0.6× MIC (1.2 mg mL^{-1}), 0.75× MIC (1.5 mg mL^{-1}), and 0.85× MIC (1.7 mg mL^{-1}) of *p*-anisaldehyde. The inoculated plates were incubated at 37°C for 24 h, and bacterial growth was recorded by measuring optical density at 600 nm (OD_{600})

using a Synergy 2 reader (BioTek Instruments, Winooski, VT). The sensitivity of individual mutants was determined by defining their factor of inhibition (F_1) values, which are calculated as the reciprocal of the OD_{600} ratio between the treated and untreated conditions (51). All mutants with F_1 values of ≥9 were considered as hyper susceptible to *p*-anisaldehyde. The screening was performed twice in duplicates for each tested concentration of *p*-anisaldehyde.

Mapping transposon insertion sites in hypersensitive mutants. Genomic DNA was extracted from overnight cultures of the sensitive mutants grown in LB broth using a DNeasy UltraClean Microbial Kit (Qiagen, Germantown, MD), and 250 ng of the DNA was digested with the restriction endonuclease *Sac*II (New England Biolabs, MA, USA). The endonuclease reaction was incubated for 3 hours at 37°C, and the enzyme was inactivated at 65°C for 20 min. The digested DNA was self-ligated with T4 DNA ligase (New England Biolabs) by incubating it overnight at 16°C. The ligation products served as a template for inverse PCR with the Q5 High-Fidelity DNA Polymerase (New England Biolabs), and transposon-specific primers (Table 3). 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 amplicons were purified using a GeneJET PCR Purification Kit (Thermo Scientific) and sequenced at Eurofins MWG Operon (Huntsville, AL). Areas flanking the EZ-Tn*5* <TET-1> integration sites were mapped to the *P. aeruginosa* PAO1 genome using the BLASTn web tool of the *Pseudomonas* database (52).

Synthesis of *p*-anisaldehyde-releasing polymeric discs. A stock resin solution was prepared by adding 2 g of TTT (8.04 mmol, 24.12 mmol ene), 2.95 g of PETMP (6.03 mmol, 24.12 mmol SH), and 118 mg Darocur 1173 (0.72 mmol, 3 mol percent relative to SH) to a scintillation vial and mixing thoroughly. Two-gram portions of the resin were then mixed with 800 mg *p*-anisaldehyde (28.6 wt %) to form the active resin. Eighty-microliter aliquots of resin were dispensed onto glass slides spaced with Teflon spacers (0.75 mm in thickness) and cured using an OmniCure S1000-1B light source (Lumen Dynamics, Mississauga, Ontario, Canada) with a

100 W mercury lamp (λmax = 365 nm, 320–500 nm filter) for 20 s at an intensity of 200 mW cm^{-2}. Control disks were prepared in the same fashion from the stock resin. Network conversion was confirmed via kinetic data collected through real-time FTIR (RT-FTIR) spectroscopy to monitor the disappearance of thiol and alkene functional groups. RT-FTIR spectra were recorded using a Nicolet 8700 FTIR spectrometer (Thermo Scientific) equipped with a KBr beam splitter and MCT/A detector using an OmniCure S1000 320–500 nm filtered ultraviolet light source. Each sample was exposed to a UV light with an intensity of 200 mW cm^{-2}. Series scans were collected with a data spacing of 2 scans per second with a resolution of 4 cm^{-1}. Thiol conversion was monitored via integration of the SH peak between 2500 and 2620 cm^{-1} and alkene conversion was monitored via the peak between 3050 and 3125 cm^{-1}.

Evaluating the sensitivity of transposon mutants to the of *p*-anisaldehyde-releasing polymeric discs. The antimicrobial activity of polymeric discs against the wild type PAO1 strain and select hypersensitive mutants was determined via a zone of inhibition (ZOI) assay. Briefly, overnight bacterial cultures were adjusted to OD_{600} of 0.1, and then further diluted 1:5 with fresh MH broth. Aliquots (200 µL) of diluted bacterial suspensions were mixed with 4 mL of lukewarm molten soft agar and overlaid on MH agar, after which an 80-mm^3 *p*-anisaldehyde polymeric disc was placed at the center of each inoculated plate. Plates were incubated for 24 h at 37°C, and the ZOI was measured in mm. In order to compare the sensitivity of mutants, the average ZOI of each mutant was normalized to that of the WT strain, which represented the level of sensitivity of 100%. All treatments were replicated three times, and each mutant was tested twice.

Screening plant-derived EPIs for synergistic interactions with *p*-anisaldehyde. Several plant-derived EPIs were evaluated for the synergistic antimicrobial activity with *p*-anisaldehyde using a modified broth microdilution technique (53). This was done by determining the MIC of *p*-anisaldehyde against *P. aeruginosa* PAO1 in the presence of non-inhibitory concentrations of EGCG (150 µg mL^{-1}), daidzein (1 mg mL^{-1}), curcumin (400 µg mL^{-1}), berberine

(400 µg mL^{-1}), or geraniol (600 µg mL^{-1}). The positive control was treated with the uncoupler of proton motive force carbonyl cyanide m-chlorophenylhydrazone (25 µg mL^{-1}), whereas the negative control was treated with *p*-anisaldehyde, and bacteria cultured in the unamended MH medium served as growth control. The assay was conducted in 96-well microtiter plates, which were incubated at 37 °C for 24 hours before measuring OD_{600} to determine the nature of interactions (OD_{600} < 0.05 was considered negative for bacterial growth). Each experiment was repeated three times, with three replicates per treatment.

Confirmation of synergistic interactions between EGCG and *p*-anisaldehyde. The synergistic interaction between EGCG and *p*-anisaldehyde was verified using a broth microdilution checkerboard technique. Stock concentrations of both compounds were diluted to MIC, 0.5× MIC, 0.25× MIC, and 0.125× MIC, which corresponds to 300, 150, 75 and 37.5 µg mL^{-1} for EGCG, and 2, 1, 0.5 and 0.25 mg mL^{-1} of *p*-anisaldehyde. Next, 25-µL aliquots of both compounds were combined in a checkerboard manner with 50 µL of bacterial suspension adjusted to 10^5 CFU mL^{-1}. Bacteria grown in the MH broth without EGCG and *p*-anisaldehyde served as a control. Microtiter plates were incubated at 37°C for 24 h, and OD_{600} was measured to determine the fractional inhibitory concentration (FIC) (10), with FIC ≤ 0.5 and FIC ≥ 4 indicating, respectively, synergism and antagonism. Each treatment included three replicates, and the entire experiment was repeated three times. In all assays, the final concentration of DMSO was maintained below 1%, the level at which this organic solvent does not interfere with the growth of *P. aeruginosa* PAO1 (54).

Extraction and processing of RNA. Overnight broth culture of WT *P. aeruginosa* PAO1 was diluted to an OD_{600} 0.01, after which 100-µL aliquots of the bacterial suspension were dispensed into wells of a microtiter plate and incubated statically at 37°C. At an OD_{600} of 0.6, each microtiter plate well received 100 µL of MH broth amended with 1.5% DMSO and ½ MIC concentrations of *p*-anisaldehyde (1 mg mL^{-1}), EGCG (150 µg mL^{-1}), or a combination of both compounds. The experiment included three biological replicates of each treatment plus a

control, which was cultured in MH broth amended with the same amount of DMSO. After 1 h of exposure to antimicrobials at 37°C, 0.4 mL of culture containing approximately 2.5×10^8 cells were fixed by mixing with two volumes of RNAprotect Bacteria Reagent (Qiagen), and total RNA was extracted using RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. Total RNA was treated with RNase-free DNase I (Ambion, Austin, TX) and purified with RNA Clean and Concentrator-25 columns (Zymo Research, Irvine, CA). The concentration of RNA was measured using a NanoDrop OneC spectrophotometer (Thermo Scientific) and a QuantiFlour RNA System (Promega, Madison, WI), while its integrity was determined using an Agilent 2100 Bioanalyzer and an RNA 6000 Nano Kit (both from Agilent Technologies, Santa Clara, CA). Samples of total RNA (RIN > 9; $A_{260}/_{280}$ ratio ~2.0) were shipped to the Center for Genome and Research and Biocomputing (Oregon State University, Corvallis, OR), where they were treated with a Ribo-Zero rRNA Removal Kit (Bacteria) (Illumina, San Diego, CA), and the efficacy of ribodepletion was confirmed using a Bioanalyzer RNA 6000 Pico Kit (Agilent Technologies). The stranded RNA-Seq libraries were prepared, quantified by qPCR, and sequenced on a HiSeq 3000 instrument (Illumina) in 150 bp single-end mode.

Bioinformatic analysis of transcriptomic data. The analysis of RNA-seq data was performed using the KBase suite of expression analysis tools (55). Briefly, the raw reads in fastq format were filtered and processed with Trimmomatic (56), and the quality of filtered data was assessed with FastQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). The processed reads were then aligned to the reference PAO1 genome (GenBank accession number NC_002516.2, downloaded from http://www.pseudomonas.com) with HISAT2-v2.10 (57), and full-length transcripts were assembled with StringTie v1.3.3b (58). The differential expression analysis of the assembled transcripts was carried out with DESeq2 v1.20.0 (59), and genes demonstrating greater than a 1.5-fold (log2) difference in expression and an adjusted p value ≤ 0.05 between control and experimental treatments were used in downstream analysis.

The functional annotation and pathway enrichment analyses were performed with the Blast2GO suite (60).

RT-qPCR analysis of genes encoding components of RND efflux pumps. The response of *P. aeruginosa* efflux pump genes to *p*-anisaldehyde, EGCG, and a combination of both compounds was validated by the quantitative reverse transcription PCR (RT-qPCR). Briefly, 1 µg of total RNA was converted to cDNA using the iScript Reverse Transcription Supermix (Bio-Rad, Hercules, CA, USA), and used in the RT-qPCR assay performed with the Luminaris Probe qPCR Master Mix (Thermo Scientific) and oligonucleotide primers and probes targeting *mexA*, *mexC*, *mexE*, *mexX*, *mexK*, *muxB*, and PA1541 (61) (Table 3). Samples of RNA untreated with reverse transcriptase served as a negative control to confirm the absence of contaminating genomic DNA. The analysis was performed with a CFX96 Real-Time PCR Detection System and CFX Maestro software (Bio-Rad). The expression of selected efflux pump genes was normalized to that of the housekeeping gene *rpoD*.

Data availability. The RNA-seq data used in this study have been deposited in the NCBI Sequence Read Archive (SRA) database under the accession number PRJNA579575.

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750 **Figure legends**

751 **Figure 1.** Synthesis of the *p*-anisaldehyde-releasing antimicrobial polymer.

752

753 **Figure 2.** The sensitivity of transposon mutants to *p*-anisaldehyde. (A) The distribution of F_1
754 values of EZ-Tn*5*<TET-1> mutants. Red dots indicate strains with $F_1 \geq 9$ that were selected for
755 further analysis. (B) The inhibition of growth caused by *p*-anisaldehyde polymeric discs in
756 selected transposon mutants ($F_1 \geq 9$) and the wild type PAO1 strain. Asterisks indicate mutants
757 that were more sensitive to *p*-anisaldehyde than the parental strain (one-tailed *t*-test at $P<0.05$).

758

759 **Figure 3.** Venn diagram comparing the number of differentially expressed genes between *P.*
760 *aeruginosa* exposed to *p*-anisaldehyde, EGCG, and the combination of both compounds (A).
761 The comparison of genes implicated in the response to *p*-anisaldehyde in transposon mutants
762 and cultures profiled by RNA-seq (B).

763

764 **Figure 4.** Gene ontology (GO) classification of differentially expressed genes (DEGs) in
765 response to *p*-anisaldehyde. Functional annotation of DEGs and transposon mutagenesis
766 genes (A), and the GO term enrichment analysis using Fisher's exact test (False Discovery
767 Rate adjusted *p*-value ≤ 0.05) (B).

768

769 **Figure 5.** Gene ontology (GO) classification of *P. aeruginosa* genes that were differentially
770 expressed in response to epigallocatechin gallate (A), and the GO term enrichment analysis of
771 these differentially expressed genes using Fisher's exact test (False Discovery Rate adjusted *p*-
772 value ≤ 0.05) (B).

773

774 **Figure 6.** Gene ontology (GO) classification of *P. aeruginosa* genes that were differentially
775 expressed in response to a combination of *p*-anisaldehyde with epigallocatechin gallate (A), and

776 the GO term enrichment analysis of differentially-expressed genes using Fisher's exact test
777 (False Discovery Rate adjusted *p*-value ≤ 0.05) (B).
778
779 **Figure 7**. Relative expression of RND-type efflux pump genes in response to *p*-anisaldehyde,
780 EGCG, and a combination of both compounds. Bars with different letters indicate significant
781 differences in gene expression as determined by Tukey-Kramer HSD test ($P \leq 0.05$).
782
783 **Figure 8**. Changes in the *P. aeruginosa* transcriptome in response to *p*-anisaldehyde, EGCG,
784 and the combination of both compounds.
785
786
787
788

1 **TABLE 1** Genes disrupted by EZ-Tn*5*<TET-1> in transposon mutants with increased sensitivity to *p*-anisaldehyde.

Functional category	PA number	Gene	Predicted function	Number of mutants
Membrane transport	PA1777	*oprF*	Outer membrane porin F	1
	PA3000	*aroP1*	Aromatic amino acid permease	1
	PA3336		Major facilitator superfamily (MFS) transporter	1
	PA5068	*tatA*	Sec-independent protein translocase	2
Signal transduction	PA0928	*gacS*	Two-component sensor kinase	1
	PA3271		Two-component sensor kinase	1
	PA4856	*retS*	Hybrid sensor histidine kinase/response regulator	1
Energy metabolism	PA1554	*ccoN1*	Cbb3-type cytochrome c oxidase subunit I	1
	PA4465		Nucleotide-binding protein	1
	PA0337	*ptsP*	Phosphoenolpyruvate-protein phosphotransferase	2
	PA1880		Probable oxidoreductase	2
	PA2993		FAD:protein FMN transferase	1
	PA2994	*nqrF*	Na(+)-translocating NADH-quinone reductase subunit F	3
	PA2995	*nqrE*	Na(+)-translocating NADH-quinone reductase subunit E	1
	PA2999	*nqrA*	Na(+)-translocating NADH-quinone reductase subunit A	3
Transport of molybdenum	PA1861	*modC*	Molybdenum import ATP-binding protein	3
and synthesis of Mo co-	PA1862	*modB*	Molybdenum transport system permease	1
factor	PA3028	*moeA2*	Molybdopterin molybdenum transferase	2
	PA3029	*moaB2*	Molybdenum cofactor biosynthesis protein B	1
	PA4663	*moeB*	Molybdopterin biosynthesis MoeB protein	1
Response to antibiotics	PA0425	*mexA*	MexAB-OprM efflux system, periplasmic linker component	2
	PA5485	*ampDh2*	N-acetylmuramoyl-L-alanine amidase	1
Nucleotide metabolism	PA0590	*apaH*	Bis(5'-nucleosyl)-tetraphosphatase	1

and modification	PA2991	*sth*	Soluble pyridine nucleotide transhydrogenase	1
	PA5339	*ridA*	2-aminoacrylate deaminase	1
	PA4617		rRNA large subunit methyltransferase G	1
Unknown	PA4618		Hypothetical protein	2

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1 **TABLE 2** The effect of plant-derived EPIs on the MIC of *p*-anisaldehyde in *P. aeruginosa* PAO1.

EPI	Concentration, μg mL^{-1}	MIC of *p*-anisaldehyde, mg mL^{-1}	Type of interaction
None	N/A	2.0	N/A
CCCP	25	0.6	Synergism
EGCG	150	0.8	Synergism
Daidzein	400	2.0	Indifference
Berberine	400	1.5	Partial synergism
Curcumin	400	1.5	Partial synergism
Geraniol	400	1.5	Partial synergism

2

3

1 **TABLE 3** Oligonucleotide primers and qPCR probes used in this study.

Primer or probe	Sequence	Reference
TET-1FP-3	5'-GCATCTCGGGCACGTTGGGTCCT-3'	Lucigen
TET-1RP-4	5'-CGAGGATGACGATGAGCGCATTGTTAG-3'	Lucigen
rpoD-F	5'-GGGCTGTCTCGAATACGTTGA-3'	61
rpoD-R	5'-ACCTGCCGGAGGATATTTCC-3'	61
rpoD-P	5'-[FAM]-TGCGGATGATGTCTTCCACCTGTTCC-[BHQ1]-3'	61
mexA-F	5'-AACCCGAACAACGAGCTG-3'	61
mexA-R	5'-ATGGCCTTCTGCTTGACG-3'	61
mexA-P	5'-[FAM]-CATGTTCGTTCACGCGCAGTTG-[BHQ1]-3'	61
mexC-F	5'-GGAAGAGCGACAGGAGGC-3'	61
mexC-R	5'-CTGCACCGTCAGGCCCTC-3'	61
mexC-P	5'-[FAM]-CCGAAATGGTGTTGCCGGTG-[BHQ1]-3'	61
mexE-F	5'-TACTGGTCCTGAGCGCCT-3'	61
mexE-R	5'-TCAGCGGTTGTTCGATGA-3'	61
mexE-P	5'-[FAM]-CGGAAACCACCCAAGGCATG-[BHQ1]-3'	61
mexX-F	5'-GGCTTGGTGGAAGACGTG-3'	61
mexX-R	5'-GGCTGATGATCCAGTCGC-3'	61
mexX-P	5'-[FAM]-CCGACACCCTGCAGGGCC-[BHQ1]-3'	61
mexK-F	5'-GAGTTCGGCACCACCTA-3'	This study
mexK-R	5'-CAGGCGGTCGGCATAGTC-3'	This study
mexK-P	5'-[FAM]-CAAGGGCTTCGACTACGCGGTG-[BHQ1]-3'	This study
muxB-F	5'-ATGGTGGCGATCCTGCTC-3'	This study

muxB-R	5'-GATGGTCGGGTAGTCCACTTC-3'	This study
muxB-P	5'-[FAM]-GATCGCCTACCGCTTCCTGCCG-[BHQ1]-3'	This study
PA1541-F	5'-TGATCGGCCTGTCCTATTTCTTC-3'	This study
PA1541-R	5'-GTGATCAGGACGATGCCAATG-3'	This study
PA1541-P	5'-[FAM]-CCCTGGCGGTCAAGCGTGTC-[BHQ1]-3'	This study

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