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Phenazine antibiotic inspired discovery of potent bromophenazine antibacterial agents against *Staphylococcus aureus* and *Staphylococcus epidermidis*†

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Nearly all clinically used antibiotics have been (1) discovered from microorganisms (2) using phenotype screens to identify inhibitors of bacterial growth. The effectiveness of these antibiotics is attributed to their endogenous roles as bacterial warfare agents against competing microorganisms. Unfortunately, every class of clinically used antibiotic has been met with drug resistant bacteria. In fact, the emergence of resistant bacterial infections coupled to the dismal pipeline of new antibacterial agents has resulted in a global health care crisis. There is an urgent need for innovative antibacterial strategies and treatment options to effectively combat drug resistant bacterial pathogens. Here, we describe the implementation of a *Pseudomonas* competition strategy, using redox-active phenazines, to identify novel antibacterial leads against *Staphylococcus aureus* and *Staphylococcus epidermidis*. In this report, we describe the chemical synthesis and evaluation of a diverse 27-membered phenazine library. Using this microbial warfare inspired approach, we have identified several bromophenazines with potent antibacterial activities against *S. aureus* and *S. epidermidis*. The most potent bromophenazine analogue from this focused library demonstrated a minimum inhibitory concentration (MIC) of 0.78–1.56 μM, or 0.31–0.62 μg mL^{-1}, against *S. aureus* and *S. epidermidis* and proved to be 32- to 64-fold more potent than the phenazine antibiotic pyocyanin in head-to-head MIC experiments. In addition to the discovery of potent antibacterial agents against *S. aureus* and *S. epidermidis*, we also report a detailed structure–activity relationship for this class of bromophenazine small molecules.

Introduction

The emergence of multidrug resistant bacterial infections has lead to a serious global crisis. Every class of antibiotic that has been introduced into the clinic has been met with the development of drug resistant bacteria.[1,2] Despite the growing need for new antibacterial agents, many pharmaceutical companies have abandoned their antibacterial discovery programs as the anticipated success with target-based, high-throughput screening (HTS) campaigns has yet to be realized.[2–5] The health care emergency that has resulted from drug resistant bacterial infections has been gaining momentum over the past four decades as only two new classes of antibiotics have been introduced into the clinic.[2,4] Innovative antibacterial strategies are desperately needed to meet the biomedical challenge of resistant bacterial infections.

It is without question that microorganisms produce potent antibiotics as agents of microbial warfare and competition. As a result, the large majority of our antibiotic arsenal is based on such natural products discovered in the antibiotic golden era between the 1940s and 1960s (*i.e.*, penicillin, streptomycin, erythromycin, tetracycline, vancomycin) or their synthetic derivatives.[2] In fact, very few clinically useful treatment options for bacterial infections have been developed from purely synthetic origins (*i.e.*, sulfonamides, quinolones, oxazolidinones).

Considering that many past successes in antibiotic discovery have been grounded on bacterial warfare agents/strategies from microorganisms, it stands to reason that future antibacterial treatments will also depend on the discovery and implementation of innovative microbial-inspired antibacterial strategies. One such strategy that we have become interested in is the use of redox-active phenazine antibiotics by *Pseudomonas* during competition with other bacteria and fungi through the formation of reactive oxygen species (ROS).[6,7] One example of this competition is in young cystic fibrosis (CF) patients.[7] Many times, individuals with CF first develop *Staphylococcus aureus* lung infections when they are young. As the CF patient ages, *Pseudomonas aeruginosa* co-infects the lung and successfully competes against *S. aureus* for this niche using redox-active phenazine antibiotics.

We have initiated a research program focused on the discovery of novel antibacterial agents against *S. aureus* and *S. epidermidis* inspired by phenazine antibiotics (Fig. 1,

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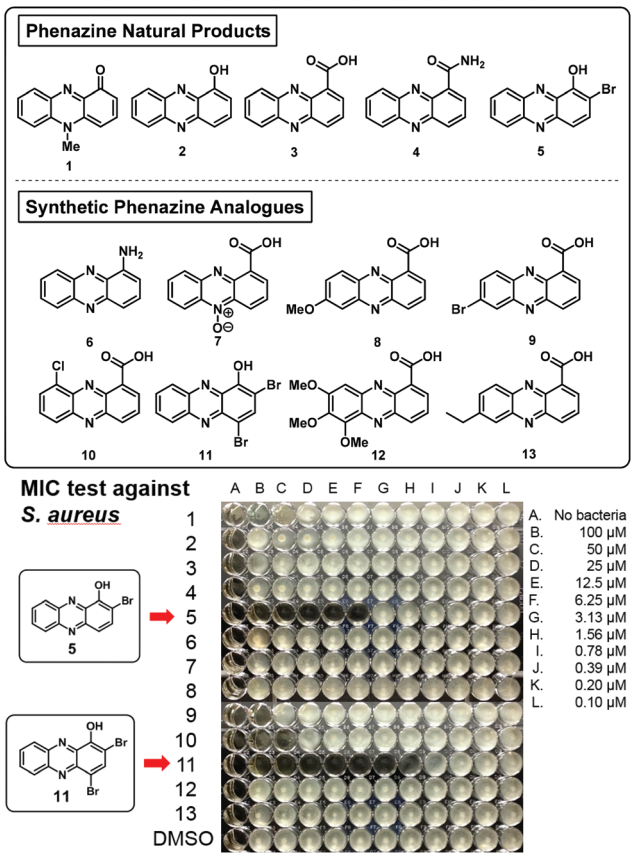
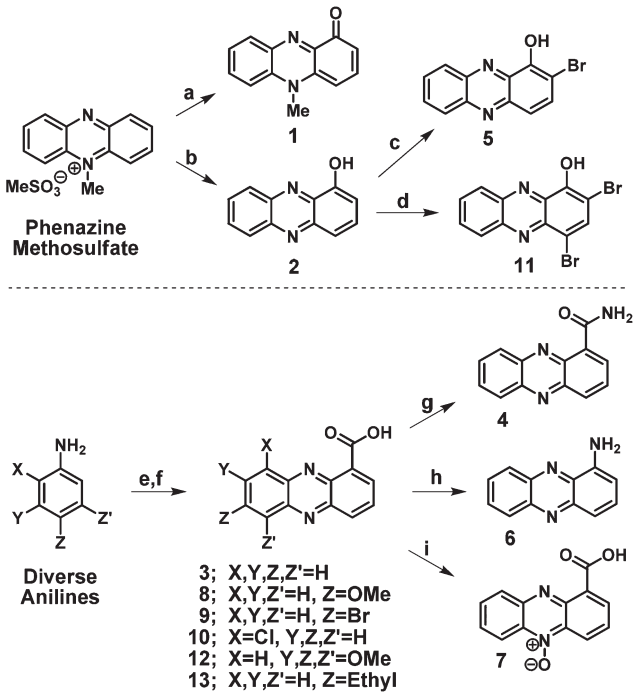


Fig. 1 Initial library of 5 phenazine natural products and 8 synthetic phenazines in head-to-head MIC experiments against *Staphylococcus aureus*.



Scheme 1 Synthesis of 13 diverse phenazines for screening against *S. aureus* and *S. epidermidis*. (a) H_2O, sunlight, 50%; (b) H_2O, sunlight; NaOH, 37%; (c) 1.2 eq. NBS, PhMe, 21%; (d) 2.2 eq. NBS, PhMe, 99%; (e) 2-bromo-3-nitrobenzoic acid, CuCl, Cu°, *N*-ethylmorpholine, 2,3-butanediol, 36–73% (6 analogues); (f) $NaBH_4$, NaOEt, 7–54% (6 analogues); (g) $SOCl_2$, PhMe; NH_3 (aq.), 53%; (h) DPPA, THF–TEA; H_2O, 61%; (i) H_2O_2, AcOH, 58%.

phenazines **1–5**). *S. aureus* is a human pathogen that is notorious for life-threatening drug resistant infections in hospitals and the community.[8] In fact, in the United States alone there are more annual deaths from methicillin-resistant *Staphylococcus aureus* (MRSA) related infections than AIDS.[9] *Staphylococcus epidermidis* is also a pathogen of great importance as it is particularly prevalent in persistent catheter related infections.[10]

Here we describe the synthesis and evaluation of electronically diverse phenazine natural products and synthetic analogues against *S. aureus* and *S. epidermidis*. Reduction potential and redox-cycling capabilities of the phenazine are electronically influenced by functional group substitutions on the phenazine heterocycle.[6,11] We hypothesized that an electronically diverse library of phenazine small molecules would serve as a fruitful starting point for the discovery of promising lead antibacterial agents against *S. aureus* and *S. epidermidis* based on a ROS-based competition strategy model employed by *Pseudomonas aeruginosa*.

Results and discussion

We first set out to synthesize a focused library of electronically diverse phenazines. We were able to rapidly synthesize five naturally occurring phenazines: pyocyanin **1**, 1-hydroxyphenazine **2**, phenazine-1-carboxylic acid (PCA) **3**, phenazine-1-carboxamide (PCN) **4**, and 2-bromo-1-hydroxyphenazine **5**. In addition to naturally occurring phenazine antibiotics, we also synthesized eight non-natural phenazine small molecules (compounds **6–13**) for our initial screen against *S. aureus* and *S. epidermidis* (Scheme 1). Phenazines **1**, **2**, **5** and **11** were synthesized from phenazine methosulfate using previously reported synthetic protocols.[12–14] In addition, several electronically diverse PCA analogues (compounds **3**, **8–10**, **12**, **13**) were synthesized using a previously described route.[15] PCA **3** was diversified *via* amidation reaction to the naturally occurring phenazine PCN **4** using thionyl chloride followed directly by treatment with aqueous ammonia. Curtius rearrangement of PCA **3** readily afforded 1-aminophenazine **6** while oxidation of **3** with hydrogen peroxide yielded PCA *N*-oxide **7**.

This initial phenazine library was screened against *S. aureus* ATCC 25923 and *S. epidermidis* ATCC 12228 according to the Clinical and Laboratory Standards Institute (CLSI) recommendations for microdilution MIC experiments.[16] For these MIC experiments, a concentration range of 0.1–100 μM was used for phenazine/antibiotic compound made from eleven 2-fold dilutions in 96-well microtiter plates. From the initial screen of 13 phenazines, we identified two phenazine small molecules that demonstrated potent growth inhibition

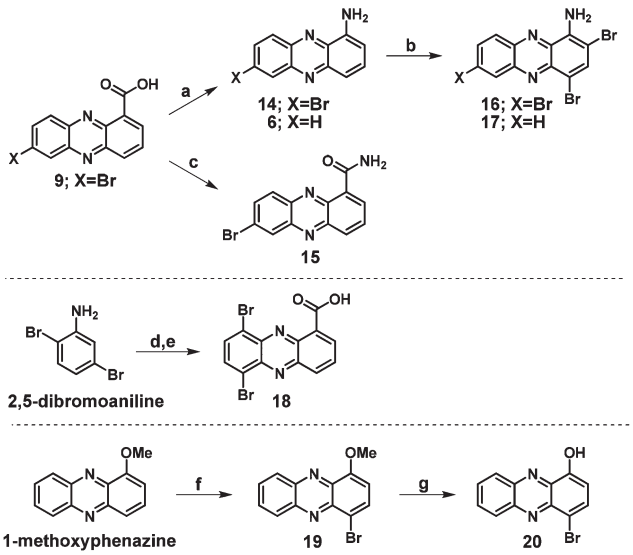
Table 1 Minimum inhibitory concentration (MIC) results for phenazines **1–27** and kanamycin using a microdilution protocol in 96-well plates

Phenazine	*S. aureus* (ATCC 25923) MIC (μM)	*S. aureus* (ATCC 25923) MIC (μg mL^{-1})	*S. epidermidis* (ATCC 12228) MIC (μM)	*S. epidermidis* (ATCC 12228) MIC (μg mL^{-1})	*P. aeruginosa* (PAO1) MIC (μM)
Pyocyanin 1	50	10.6	50	10.6	>100
2	>100	>19.7	>100	>19.7	>100
3	>100	>22.5	>100	>22.5	>100
4	>100	>22.4	>100	>22.4	>100
5	6.25	1.72	6.25	1.72	>100
6	>100	>19.6	>100	>19.6	>100
7	>100	>24.1	>100	>24.1	>100
8	>100	>25.5	>100	>25.5	>100
9	100	30.3	100	30.3	>100
10	50	13.0	50	13.0	>100
11	1.56	0.55	0.78–1.56	0.28–0.55	>100
12	>100	>31.5	>100	>31.5	>100
13	>100	>25.3	>100	>25.3	>100
14	>100	>27.4	—	—	>100
15	>100	>30.2	—	—	>100
16	>100	>43.2	—	—	>100
17	>100	>35.4	—	—	>100
18	>100	>38.3	—	—	>100
19	>100	>28.9	—	—	>100
20	>100	>27.5	—	—	>100
21	0.78–1.56	0.31–0.62	0.78	0.31	—
22	1.56	0.66	1.56	0.66	—
23	1.56	0.72	1.56	0.72	—
24	1.56	0.74	—	—	—
25	3.13	1.68	3.13	1.68	—
26	>100	>46.5	—	—	—
27	>100	>36.9	—	—	—
Kanamycin	1.56–6.25	0.76–3.03	0.78–1.56	0.38–0.76	—

MIC experiments were carried out in duplicate, several active compounds were assayed up to 5 times during these investigations; — is designated for compounds that were not tested against a particular bacterium.

activity against *S. aureus* and *S. epidermidis*. 2-Bromo-1-hydroxyphenazine **5** demonstrated impressive antibacterial activity with MIC values of 6.25 μM (1.72 μg mL^{-1}) against both *S. aureus* and *S. epidermidis* while 2,4-dibromo-1-hydroxyphenazine **11** had improved potency against both *Staphylococcus* strains (MICs of 1.56 μM/0.55 μg mL^{-1} against *S. aureus*; 0.78–1.56 μM/0.28–0.55 μg mL^{-1} against *S. epidermidis*; see Table 1). To our surprise, bromophenazine **11** was found to be 32-fold more potent than the phenazine antibiotic pyocyanin **1** in head-to-head MIC experiments against *S. aureus* and *S. epidermidis* (Fig. 1). Kanamycin (aminoglycoside antibiotic) was used as a positive control against *S. aureus* and *S. epidermidis*.

The potent antibacterial activity of bromophenazines **5** and **11** against *S. aureus* and *S. epidermidis* prompted us to synthesize a second small, yet diverse collection of bromophenazine small molecules (compounds **14–20**; Scheme 2). This set of bromophenazine compounds was a combination of designed compounds to probe various structural elements of compound **11** to establish an SAR (compounds **16**, **17**, **19**, **20**) in addition to evaluating unrelated bromophenazines (compounds **14**, **15**, **18**) against *S. aureus*. With this series of bromophenazines, we were primarily interested to know the impact



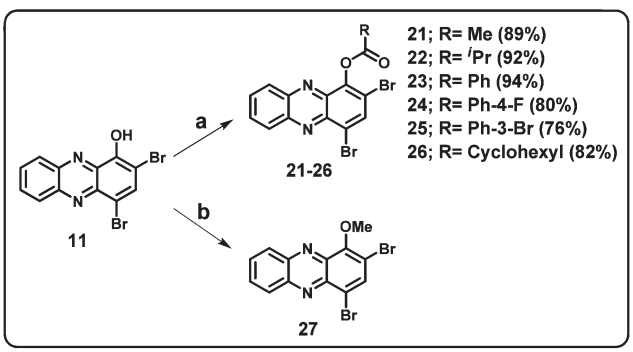
Scheme 2 Synthesis of 7 diverse bromophenazines for screening against *S. aureus*. (a) DPPA, THF–TEA; H_2O, 69% for **14**; (b) 2.1 eq. NBS, PhMe, 8% for **20**, 62% for **17**; (c) $SOCl_2$, PhMe; NH_3 (aq.) 98%; (d) 2-bromo-3-nitrobenzoic acid, CuCl, Cu°, *N*-ethylmorpholine, 2,3-butanediol, 29%; (e) $NaBH_4$, NaOH, 32%; (f) 1 eq. NBS, PhMe–MeCN, 89%; (g) BBr_3, CH_2Cl_2, 27%.

of substituting the hydroxyl group in **11** for an amine group (compounds **16** and **17**) since the synthetic route we used to make 1-amino-2,4-dibromophenazines would be ideal for the synthesis of a more structurally diverse bromophenazine small molecule library.

Phenazine **9** was converted into amine **14** through Curtius rearrangement. The carboxylic acid of **9** was also transformed to the corresponding primary amide in **15** using thionyl chloride followed directly by treatment with aqueous ammonia. The 1-aminophenazines **6** and **14** were dibrominated using *N*-bromosuccinimide to yield bromophenazines **16** and **17**. 2,5-Dibromoaniline was converted to 6,9-dibromophenazine-1-carboxylic acid **18** using the 2-step protocol to make PCA analogues (Jourdan–Ullmann coupling, followed by reductive ring closure with sodium borohydride). Finally, 1-methoxyphenazine was brominated in the 4-position to make **19**, which was demethylated to make **20** using a known route.[14] All bromophenazines **14–20** were evaluated against *S. aureus* in MIC experiments and found to be inactive as growth inhibitors at the highest concentrations tested (100 μM). Although we were surprised at this result, this small set of compounds was useful in establishing a detailed structure–activity relationship (SAR) for this class of bromophenazines (Scheme 3).

Phenazines **1–20** were then screened for growth inhibition activity against *P. aeruginosa* strain PAO1. In MIC experiments, none of these phenazines demonstrated growth inhibition against PAO1 at the highest concentration tested (100 μM). It is well established that *Pseudomonas* is resistant to pyocyanin-induced death at very high concentrations.[17] The lack of growth inhibition against PAO1 is supportive of phenazines **5** and **11** demonstrating potent antibacterial activity against *S. aureus* and *S. epidermidis* through a ROS-generating mechanism.

We then functionalized the phenolic hydroxyl group of bromophenazine **11**. We synthesized a small collection of diverse ester analogues **21–26** by condensing bromophenazine **11** with various acid chlorides. Additionally, we synthesized the corresponding methyl ether **27** by refluxing bromophenazine **11** with methyl iodide in acetone.[14]



Scheme 3 Synthesis of 7 diverse ester/ether bromophenazine analogues for screening against *S. aureus*. (a) acid chloride, TEA, CH_2Cl_2, 76–94% (6 analogues); (b) K_2CO_3, acetone; iodomethane, 29%.

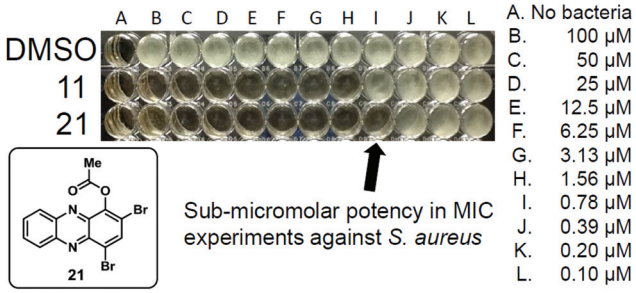


Fig. 2 Lead bromophenazines **11** and **21** in head-to-head evaluation in MIC experiments against *S. aureus*.

Bromophenazines **21–27** were evaluated against *S. aureus* and bromophenazine **21** demonstrated the most potent activity (Fig. 2) with an MIC of 0.78–1.56 μM (0.31–0.62 μg mL^{-1}) against *S. aureus* and an MIC of 0.78 μM (0.31 μg mL^{-1}) against *S. epidermidis*. The potent growth inhibition demonstrated by **21** corresponds to a 32- to 64-fold increase in potency against *S. aureus* and *S. epidermidis* when compared head-to-head against pyocyanin **1**. Bromophenazines **22**, **23** and **24** also demonstrated impressive growth inhibition activities against *S. aureus* and *S. epidermidis* (**24** not tested against *S. epidermidis*) reporting MIC values of 1.56 μM while bromophenazine **25** had an MIC of 3.13 μM against *S. aureus* and *S. epidermidis*. Interestingly, bromophenazines **26** and **27** were completely inactive against *S. aureus* at 100 μM in MIC experiments. Bromophenazines **11** and **21** were found to be more potent or equipotent to kanamycin in head-to-head MIC experiments while the naturally occurring bromophenazine **5** demonstrated slightly lower potency than kanamycin against *S. aureus* and *S. epidermidis* (Table 1).

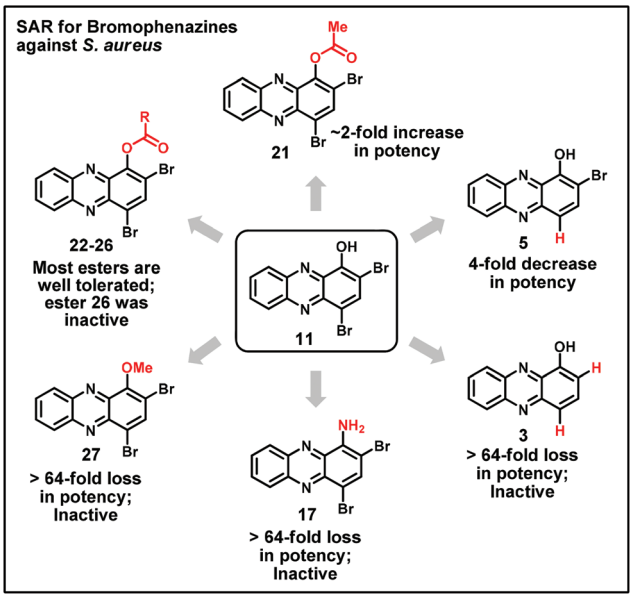


Fig. 3 Detailed structure–activity relationship (SAR) for this novel class of antibacterial agents against *S. aureus*.

If we use bromophenazine **11** (MIC 1.56 μM) as a starting point to compare other analogs against, a detailed SAR emerges from our investigations against *S. aureus* (Fig. 3). The loss of the bromine atom at the 4-position of **11** results in a 4-fold reduction in potency (phenazine **5**; MIC 6.25 μM). Substituting an amine group (compound **17**; MIC >100 μM) for the hydroxyl of **11** leads to a loss in activity against *S. aureus*. Ester analogues of **11** can either enhance potency (compound **21** MIC 0.78–1.56 μM) or abolish growth inhibition (compound **26**; MIC >100 μM), but most ester moieties evaluated during our investigation are well tolerated and potent against *S. aureus* (compounds **22–25** had MICs of 1.56–3.13 μM). The methyl ether **27** demonstrated no growth inhibition against *S. aureus* in MIC experiments at the highest concentration tested (100 μM). We can also note that the loss of a bromine atom at the 2-position of the phenazine ring (compounds **16** and **19**; not shown in Fig. 3) results in the complete loss of activity against *S. aureus* (MIC >100 μM). This SAR will be beneficial in guiding future analogue development pertaining to this class of bromophenazine small molecules.

Conclusions

We have discovered a class of bromophenazine antibacterial agents that demonstrate potent growth inhibition against *S. aureus* and *S. epidermidis* inspired by a microbial warfare strategy. These bromophenazines originated from a focused library of 13 diverse phenazine compounds, including five naturally occurring "phenazine antibiotics" that was evaluated against *S. aureus* and *S. epidermidis* in MIC assays. These findings are indeed timely as novel compounds that are potent antibacterial against *S. aureus* and *S. epidermidis* are of great importance as these pathogens are notorious for their drug-resistant infections in humans.

Experimental section

Synthetic procedures, ^{1}H and ^{13}C NMR spectra and HRMS are reported in the ESI.†

Antibiotic susceptibility tests (MIC assay protocol)

The minimum inhibitory concentration (MIC) for each phenazine was determined by the broth microdilution method as recommended by the Clinical and Laboratory Standards Institute (CLSI).[16] In a 96-well plate, eleven two-fold serial dilutions of each compound were made in a final volume of 100 μL Luria Broth (one column served as a blank). Each well was inoculated with 10^5 bacterial cells at the initial time of incubation, prepared from a fresh log phase culture (OD_{600} of 0.5). The MIC was defined as the lowest concentration of compound that prevented bacterial growth after incubating 16 to 20 hours at 37 °C. The concentration range tested for each phenazine/antibiotic during this study was 0.10 to 100 μM. All phenazine compounds were prepared for biological evaluation as 10 mM DMSO stock solutions and were stored at room temperature in the absence of light. DMSO served as our vehicle and negative control in each microdilution MIC assay. DMSO was serially diluted at the same concentration as the phenazine compounds with a top concentration of 1% v/v. Bacterial strains used during these investigations were *S. aureus* (ATCC 25923), *S. epidermidis* (ATCC 12228) and *P. aeruginosa* (PAO1).

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