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Nonantibiotic-Based *Pseudomonas aeruginosa* Biofilm Inhibition with Osmoprotectant Analogues

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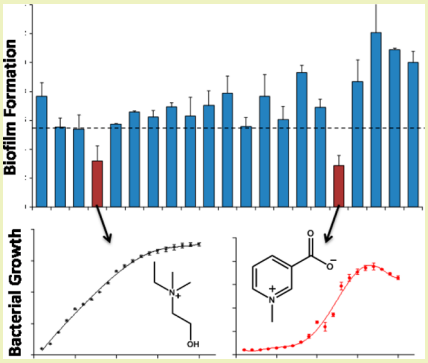
Supporting Information

ABSTRACT: The current unsustainable usage of antibiotics for clinical and agricultural purposes has resulted in a global spread of antibiotic-resistant bacterial strains. This rapid reduction in antimicrobial potency is directly caused by the strong evolutionary selection pressure of classical antibiotics. One promising strategy to avoid this problem is the development of antivirulence compounds, which seeks to alleviate the aforementioned selection pressure by specifically mitigating pathogenic phenotypes, for example, toxin secretion or biofilm formation, but without killing the bacteria. In this work, we propose a new target for this emerging antivirulence approach and identify through molecular design followed by chemical screening that bacterial osmoprotectant analogues can be used as antivirulence metabolites against biofilm formation. Among the 19 compounds tested, ethylcholine, a biosynthetic precursor of ethyl glycine betaine and previously reported to induce glycine betaine catabolism, was found to most effectively inhibit biofilm establishment without detectable biological toxicity. The potential complementary usage of this molecule with traditional antibiotic compounds and its other impacts on bacterial physiology were also herein explored and discussed.



KEYWORDS: *Antibiotic resistance, Antivirulence, Biofilm, Antimicrobial agents*

INTRODUCTION

Bacterial biofilm formation on human tissue or implantable devices has been known to lead to immune-evasion and chronic infection by many bacterial species.[1−3] While traditional antibiotics are no doubt effective in various clinical applications, several obstacles exist when applying them in preventing and eradicating biofilm formation. Some of these difficulties include the lack of efficacy in killing biofilm-embedded bacterial cells,[4,5] the induction of nascent biofilm formation at a sublethal antibiotic dosage[6] and, above all, the quick development as well as spread of the antibiotic-resistant mutants within the bacterial population.[7,8] In fact, the past several decades saw the rapid emergence of antibiotic-resistant strains far outpacing the discovery and development of antibiotic compounds, leading to a global concern over the diminishment of existing tools in fighting bacterial infection and the worrying possibility of returning to a preantibiotics age.

In light of the current unsustainable usage of traditional antibiotics, several alternatives have been proposed. One promising strategy to avoid the problem of bacteria quickly developing a defense mechanism against the chemical therapeutic agents is to shift the drug targets away from the pathogenic bacteria viability and instead aim at reducing specific virulent phenotypes. Currently, many such antivirulence compounds are under development with their biological targets ranging from bacterial toxin production to biofilm formation.[9,10] As the successful applications of these antivirulence compounds do not rely on the elimination or growth inhibition of drug-sensitive bacterial cells, the evolutionary pressure for bacteria under treatment is thus considerably weaker in comparison to the bacterial population exposed to traditional antibiotics, resulting in a much reduced rate in the emergence and spread of resistant strains.[11]

While past efforts to develop antivirulence drugs against biofilm establishment have largely revolved around the search for quorum sensing inhibitors,[9,10] more recent microbiological studies based on mutant library screening and physiological analysis have significantly expanded the list of potential antivirulence targets by identifying other genetic pathways essential for biofilm formation but not for bacterial survival. These genetic determinants for biofilm establishment include pH homeostasis,[12] arginine metabolism,[12] and iron uptake,[13] as well as osmotic adaptation.[12,14] More specifically, for osmotic adaptation, as bacteria transit from a free-living planktonic state to a matrix-dwelling biofilm phenotype, bacterial cells experience a major increase in extracellular osmotic pressure.[14,15] To prevent the quick loss of cellular water, bacteria in response accumulate osmoprotectant molecules, perhaps most famously glycine betaine, via active transport and partial or *de novo* synthesis to balance this cross-membrane difference in ionic strength.[16] In the particular case of *Pseudomonas aeruginosa*, a bacterial species notorious for causing biofilm-related clinical infections, the intracellular increase of glycine betaine concentration can be achieved via either direct

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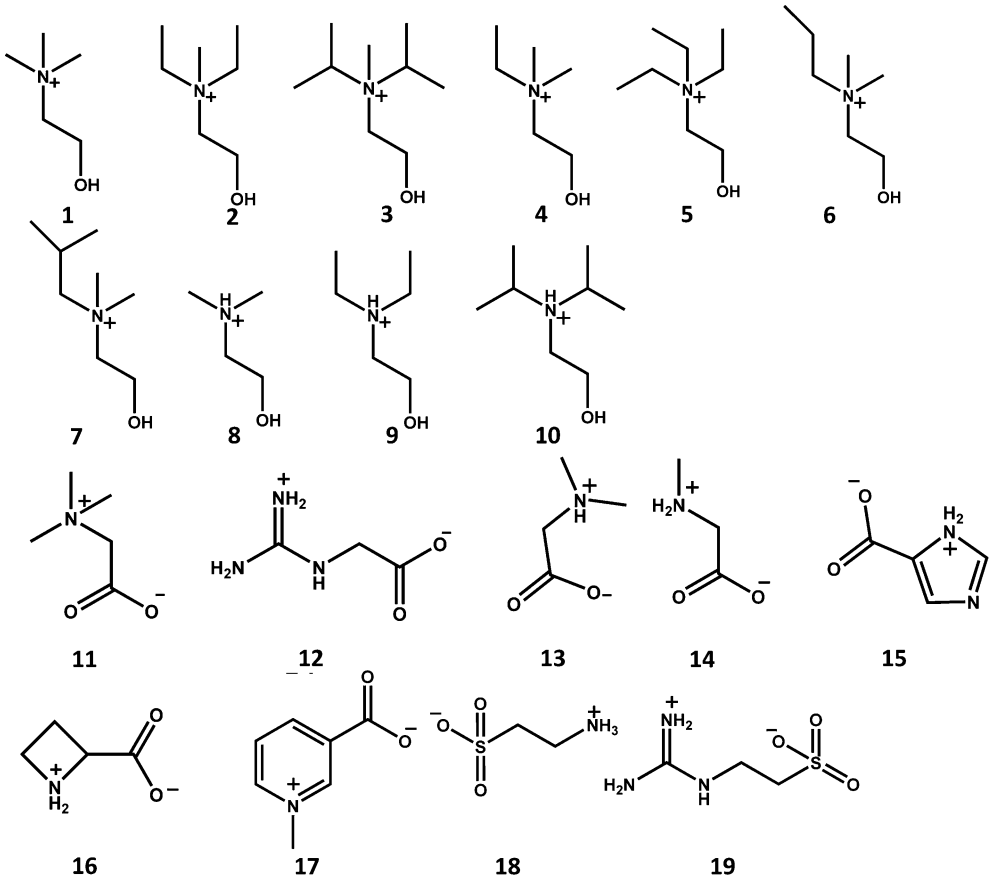

Figure 1. Chemical structures of osmoprotectant analogues together with glycine betaine and choline tested in this study.

molecular import or a partial synthesis from its choline precursor.[17]

Compared with other genetic determinants for biofilm formation, the interfering bacterial osmoprotection mechanism as a potential antibiofilm route offers the following advantages: First, various chemical analogues of natural osmoprectants are structurally simple, thus reducing the technical burden of creating a chemical screening library. Second, these structural mimics generally show low or no toxicity (see below), making it difficult for bacteria to develop resistance. Third, certain glycine betaines and their precursor choline transmembrane transporters in *P. aeruginosa* showed a medium to low substrate specificity, making it plausible to design small molecular analogues to actually enter into the cells and exert their biological activities.[18] The bioactivity of such molecular analogues may include competitive binding to osmoprotectant membrane importers, inhibition of various osmoprotectant synthetic pathways, or acceleration of catabolic degradation of indigenous osmoprotective molecules, all of which can result in compromised bacterial defense against high osmotic pressure. Thus, the central goal of this work is using the integration of molecular design and small-scale chemical screening to examine the feasibility of reducing biofilm formation with osmoprotectant analogues as potential antivirulence metabolites. To this end, the bioactivity of 17 osmoprotectant analogues together with indigenous choline and glycine betaine were tested against *P. aeruginosa* biofilm formation. The impacts of these compounds on other aspects of bacterial physiology including growth curves and susceptibility against conventional antibiotic compounds were also herein explored.

EXPERIMENTAL SECTION

Chemicals. Six new choline analogue structures were synthesized in this study. In a typical reaction, 1 molecular equivalent of tertiary ammine alcohol was mixed with 1.2 molecular equivalent of alkylation agent iodoalkane in acetonitrile. The reaction was then stirred vigorously under nitrogen protection at 60 °C for 24 h. After reaction, the solvent was removed in a rotary evaporator (BÜCHI, Switzerland). The product was subsequently precipitated and washed with anhydrous diethyl ether in excess. The resulting white powder was characterized using 1H nuclear magnetic resonance (NMR) in D_2O to give the correct structures for diethylcholine (Figure S1, Supporting Information), diisopropylcholine (Figure S2, Supporting Information), ethylcholine (Figure S3, Supporting Information), triethylcholine (Figure S4, Supporting Information), n-propylcholine (Figure S5, Supporting Information), and isopropylcholine (Figure S6, Supporting Information). All other chemicals tested were purchased from Sigma-Aldrich and used without further purification.

Bacterial Strains and Culture Conditions. The *P. aeruginosa* PAO1 wild type strain was purchased from ATCC. Various *P. aeruginosa* mutant strains as well as MPAO1 (the wild type strain used to construct the mutant library) were purchased from the University of Washington Manoil Lab.[19] All bacteria were cultured in Lysogeny broth (LB) media at 37 °C with or without the addition of specified compounds.

Growth Curve Measurement and Biofilm Quantification. The bacteria growth curve was measured by first diluting a *P. aeruginosa* overnight culture to an optical density at wavelength 600 nm (O.D.600) to 0.05. The second culture was subsequently incubated at 37 °C. At every predetermined time point, the bacterial culture was thoroughly mixed, and a new O.D.600 value was measured as an indication for bacterial growth.

A 24 h biofilm formation was quantified using standard crystal violet staining. In a typical measurement, a *P. aeruginosa* overnight culture

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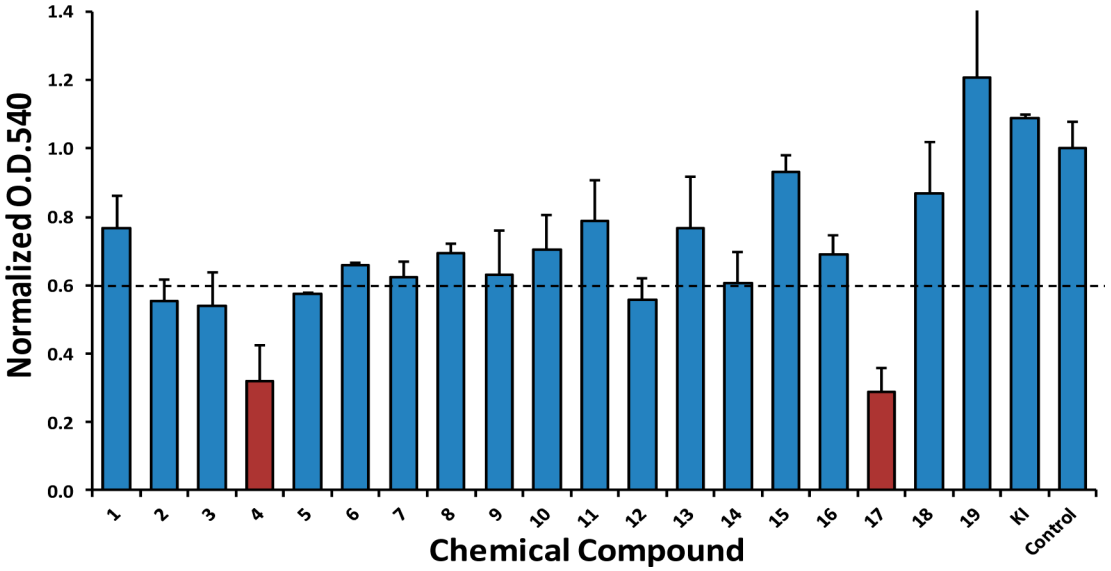

Figure 2. Twenty-four hour biofilm formation of *P. aeruginosa* PAO1 supplemented with various chemical compounds at 10 mM in LB media. All O.D.540 readings from standard crystal violet staining were normalized to the result of the negative control.

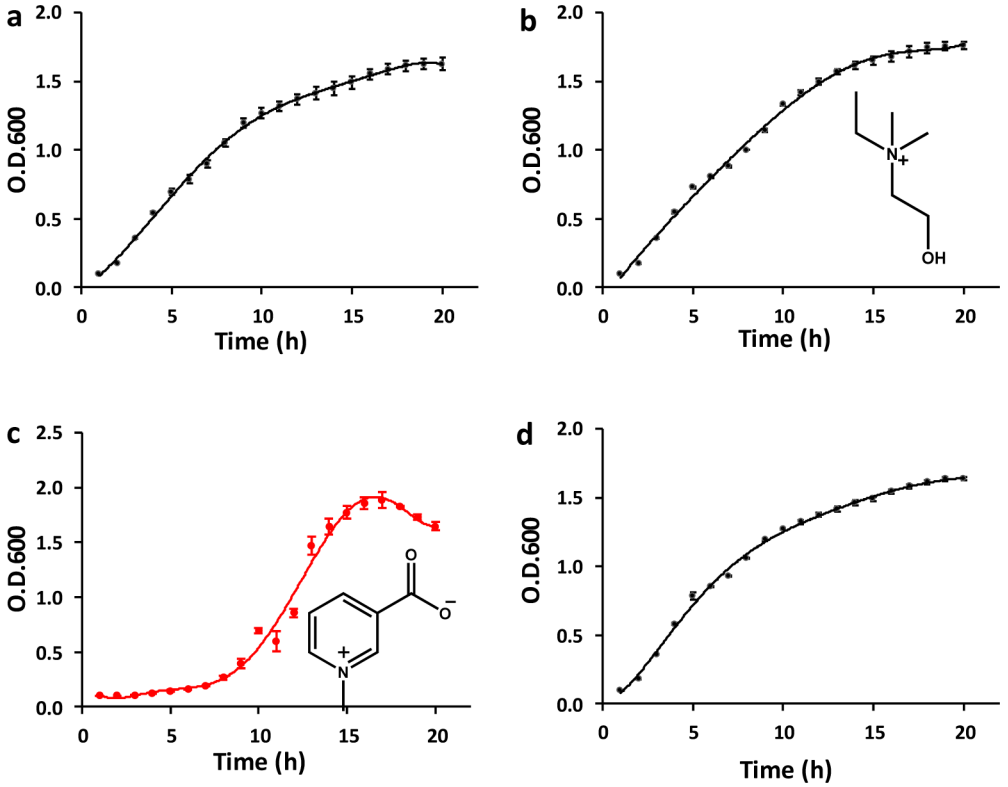

Figure 3. Growth curves of *P. aeruginosa* PAO1 in LB media (a), LB media supplemented with 10 mM compound 4 ethylcholine (b), LB media supplemented with 10 mM compound 17 trigonelline (c), and LB media supplemented with 10 mM potassium iodide (d).

was diluted in LB media with or without the addition of testing chemicals to a final O.D.600 = 0.05. A total of 500 μL of the second culture was subsequently added into a 48-well plate and incubated at 37 °C for 24 h to allow biofilm formation. After 24 h, the bacteria-containing media was removed, and each well was washed gently with sterilized PBS three times before adding 1 mL 0.1% w/v crystal violet solution into each well. The staining was carried out at room temperature for 20 min. After 20 min, the staining solution was removed, and each well was washed gently with sterilized PBS three times before finally adding 95% ethyl alcohol to dissolve the crystal violet stain absorbed into the biofilm matrix. The O.D.540 reading of the resulting alcohol solution was used as an indication of *P. aeruginosa* biofilm formation.

Antibiotic Susceptibility Test. The *P. aeruginosa* overnight culture was diluted in LB media with or without the addition of testing chemicals to a final O.D.600 = 0.05. After 24 h incubation at 37 °C, various antibiotics were added into the bacterial culture to its predetermined concentration (20 μg/mL tobramycin, 10 μg/mL gentamicin, or 60 μg/mL nalidixic acid) for an additional 6 h. After exposure, the bacterial cells were washed via three cycles of centrifugation and resuspension in sterilized PBS. The final bacterial suspension was diluted and plated on LB agar plates. The subsequent

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reduction in the colony number formed is used an indication for bacteria antibiotic susceptibility.

RESULTS AND DISCUSSION

Antibiofilm Effects of Osmoprotectant Analogues. A total of 19 chemicals (Figure 1), including indigenous choline (compound 1) and glycine betaine (compound 11), were tested for their effects on the *P. aeruginosa* PAO1 24 h biofilm formation in LB media using a standard crystal violet staining. Among these 19 chemicals, compounds 2−10 were structural mimics of choline, whereas compounds 12−19 were zwitterions and shared similarities with glycine betaine to various degrees. In particular, compounds 17 (trigonelline) and 18 (taurine) are natural products Trigonelline is commonly found in coffee and has previously been suggested to affect bacterial surface adhesion.[20] Meanwhile, taurine exists widely in animal tissues and is a major component of bile.[21]

At the relatively high 10 mM concentration used in the initial screening, 6 of the 19 testing chemicals lead to a more than 40% reduction in 24 h biofilm formation (Figure 2). The most significant differences were observed for compounds 4 (ethylcholine) and 17 (trigonelline), with 32% and 28% of the biofilm biomass, respectively, in comparison to the negative control as determined from standard crystal violet staining. However, unlike ethylcholine, which did not alter the bacterial growth curve, trigonelline slowed *P. aeruginosa* PAO1 proliferation significantly as manifested by a prolonged initial lag phase (Figure 3). As the goal of this study is to identify potential osmoprotectant analogues that specifically reduce bacterial biofilm phenotypes without inhibiting cell growth, we hence limit our focus on ethylcholine for the rest of the study. Iodoalkanes were used in the syntheses of various choline analogues, resulting an iodide anion associated with every molecule of cationic chonline analogue synthesized. To rule out possible anion interference on experimental results, LB media supplemented with 10 mM potassium iodide were also tested and confirmed to have no appreciable effects on bacterial growth and biofilm formation.

This capability to inhibit the *P. aeruginosa* PAO1 biofilm phenotype was largely retained at 5 mM and 1 mM for ethylcholine (33% at 5 mM and 45% at 1 mM in comparison to the wild type biofilm formation), indicating the biological potency of this molecule (Figure 4). An essentially indistinguishable ethylcholine dosage response was also observed for another *P. aeruginosa* PAO1 strain (labeled MPAO1), which was used in a previous construction of a near- saturation *P. aeruginosa* transposon insertion mutant library and utilized in a later part of this study (see below).[19] This extra caution in testing wild type strains from different sources is necessary as significant genome differences due to decades of separate culture between PAO1 (deposited in ATCC) and MPAO1 has been previously reported.[22]

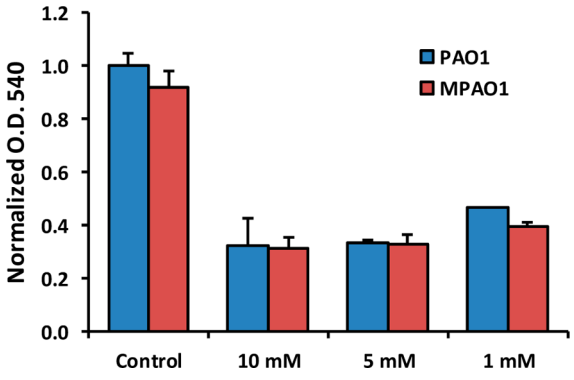

Figure 4. Twenty-four hour biofilm formation of *P. aeruginosa* PAO1 and *P. aeruginosa* MPAO1 at 10, 5, and 1 mM ethylcholine concentration. All O.D.540 readings from standard crystal violet staining were normalized to the result of the PAO1 negative control.

Ethylcholine and Bacterial Antibiotic Susceptibility. A chief motivation behind our efforts to search for antibiofilm osmoprotectant analogues was to identify and demonstrate a potential new route of mitigating this key bacterial virulent phenotype without necessarily incurring a strong selection pressure for its resistance. Ethylcholine was so far proven a promising candidate for this purpose due to its antibiofilm activity and minimal cytotoxicity, that is, not affecting the bacterial growth curve. To get a more complete understanding of the evolutionary footprint of ethylchoine on bacteria physiology and, more particularly, its effect on bacterial survivability under stress, we tested the *P. aeruginosa* PAO1 antibiotic susceptibility to three commonly used antibiotics with or without the presence of 10 mM ethylcholine (Figure 5).

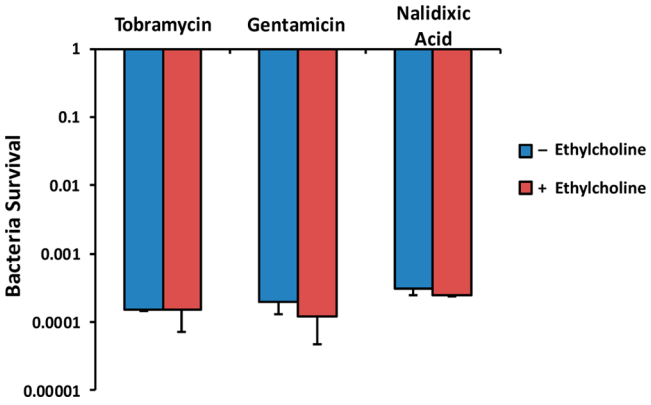

Figure 5. *P. aeruginosa* PAO1 survival after exposure to tobramycin (20 ug/mL), gentamicin (10 μg/mL), or nalidixic acid (60 μg/mL) with or without additional ethylcholine at 10 mM.

Our results showed that in all three cases—tobramycin (20 μg/mL), gentamicin (10 μg/mL), and nalidixic acid (60 μg/mL)—the coexistence of ethylcholine in the culturing environment does not change the survival percentage of *P. aeruginosa* PAO1 under antibiotic treatment. This observation suggests the relative evolutionary neutrality of ethylcholine for bacteria under different growth conditions and, more attractively, the potential of using osmoprotectant analogues to combat bacterial infection in combination with traditional antibiotic compounds while not significantly accelerating the development for resistance.

Metabolic Fate of Ethylcholine and Its Effects on Isogenic Mutants. In a recent study aiming to characterize the *P. aeruginosa* GbdR regulon, Hampel and co-workers reported that ethylcholine can be used as a noncatabolizable chemical probe to strongly induce the glycine betaine catabolic pathway transcription in a GbdR-controlled fashion.[23] Evidence was also presented in the same work that ethylcholine is transported into *P. aeruginosa* cells as a choline analogue and oxidized by BetAB to its zwitteroinic form—ethyl glycine betaine—before exerting its biological function. (Figure 6a).[23] Interestingly, supplementing the *gbdR* knockout mutant, which

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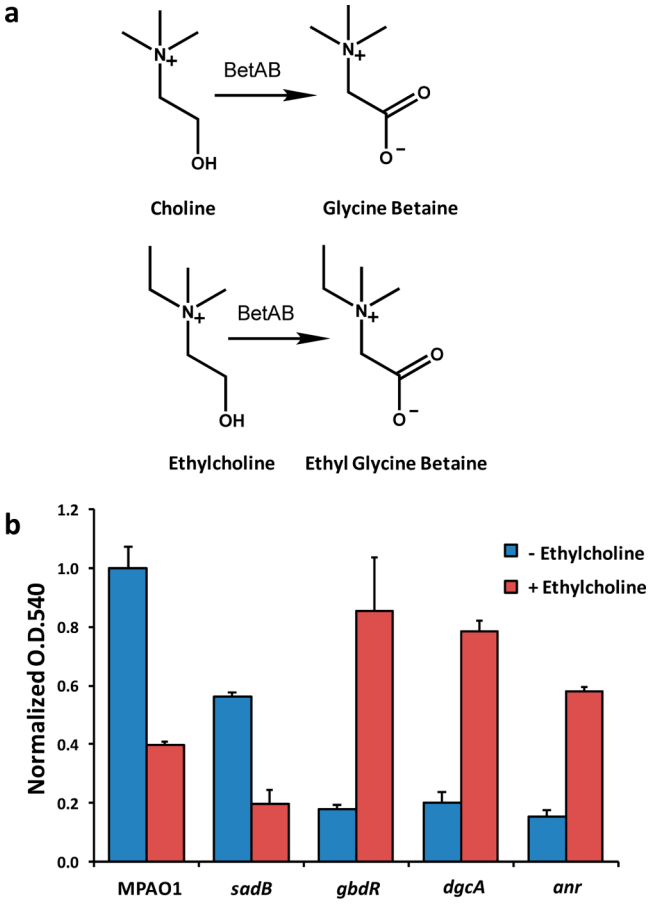

Figure 6. (a) Conversion of choline and ethylcholine into their respective zwitterionic forms in *P. aeruginosa* by BetAB; (b) 24 h biofilm formation of *P. aeruginosa* MPAO1 and various isogenic mutants with or without additional 1 mM ethylcholine in the growth media. All O.D.540 readings from standard crystal violet staining were normalized to the result of the MPAO1 negative control.

has previously been reported to have a defective biofilm phenotype,[12] with 1 mM ethylcholine in the growth medium saw a near 5-fold increase in subsequent biofilm formation (Figure 6b). As ethylcholine suppressed the biofilm phenotype in the wild type MPAO1 strain, this unexpected reverse effect on *gbdR* mutant biofilm formation makes it tempting to speculate that the reduction of biofilm formation by ethylcholine in a wild type strain at 1 mM may in part involve a GbdR-independent hyperactivation of glycine betain catabolism due to the primary or secondary metabolic effects of ethylcholine. This physiological shift following the exposure to ethylcholine might then be partially offset by a genetic knockout (*gbdR*, in this case) controlling this catabolic pathway. A similar restoration of biofilm formation capability was also observed in *dgcA* and *anr* mutant strains; both mutants are closely related to glycine betaine catabolism and display defects in biofilm formation.[12,23,24] In contrast, adding 1 mM ethylcholine into the growth media of a *P. aeruginosa* surface attachment deficient (*sad*) mutant, which has a defective biofilm phenotype due to unregulated cellular motility,[25] resulted in a further decrease in its biofilm formation.

CONCLUSIONS

Several antivirulence compounds are currently under development in response to the rapid emergence of antibiotic-resistant bacterial strains including the now infamous vancomycin-resistant *Enterococcus* species and methicillin-resistant *Staphylococcus aureus* (MRSA).[9] This relatively more defensive stance against pathogenic bacteria aims to alleviate the disease symptoms without inadvertently selecting for genetic mutants that are immune to the original chemical attacks. In this work, we propose and demonstrate the application of osmoprotectant analogues as one group of such antivirulence metabolites to specifically reduce biofilm formation. The chemical simplicity and low cytotoxity (only 2 out of 19 chemicals tested in this study resulted in observable retardation in bacterial growth at 10 mM) make osmoprotectant analogues promising new candidates for the development of future evolutionarily neutral and sustainable antimicrobial strategies.

ASSOCIATED CONTENT

Supporting Information

NMR spectra of synthesized compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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