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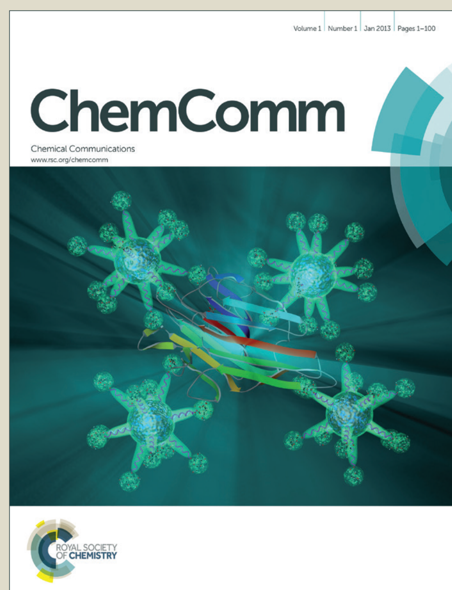

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Pseudomonas aeruginosa activates the quorum sensing LuxR response regulator through secretion of 2-aminoacetophenone

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In this study we identify a volatile compound produced by *Pseudomonas aeruginosa*, which can specifically activate the LuxR quorum-sensing response regulator of *Vibrio fischeri*. Comparative gas-chromatograph analysis between *P. aeruginosa* wild type and a Δ*lasR* mutant strain implied that the active volatile is 2-aminoacetophenone. Use of synthetic 2-aminoacetophenone and *in silico* docking analyses, verified the activity of the molecule and provided putative interacting residues within the binding site.

A fascinating manifestation of bacterial interactions is quorum sensing (QS), whereby various responses are modulated in accordance with population density, through the constant synthesis and perception of small signalling molecules. These signalling molecules bind to cognate receptors, and once their concentration reaches a specific threshold, they trigger a signal transduction cascade.[1] Recent studies indicated that several airborne compounds produced by plants can either activate or suppress the pathways of bacterial QS.[2-4] Similar studies were also conducted on bacterial volatiles, where it was demonstrated that Dimethyl disulfide produced by *Pseudomonas fluorescens* and *Serratia plymuthica* can inhibit QS related pathways of various bacterial species, such as *Agrobacterium tumefaciens*, *Chromobacterium violaceum* and *Pectobacterium carotovorum*.[5] However, a direct antagonistic effect on the relevant response regulators has not been demonstrated, and to the best of our knowledge, QS-activating bacterial volatiles have yet to be found.

Here we tested the hypothesis that specific bacterial volatiles may act as either agonists or antagonists of QS systems. For this purpose, we chose to examine the effect of total volatiles from *Pseudomonas aeruginosa*, a well-studied Gram negative bacterium. *P. aeruginosa* is a ubiquitous microorganism found in various environments such as soil, water, sewage, plants and hospitals. [6–9] It is a common opportunistic human pathogen, known for the highly antibiotic-resistant biofilms it forms together with other microbial species in the lungs of cystic fibrosis patients. [10,11]

In order to identify potential volatile substances that can act as either QS agonists or antagonists, we examined the effect of total volatiles produced by *P. aeruginosa* on several QS bioreporters. Initial screening was conducted using bi-partite Petri dishes that have separate compartments but a joint headspace allowing only the exchange of volatile substances. The bioreporters used in this study respond to various Acyl homeserine lactone (AHL) molecules ranging in carbon chain length from four to 12 carbons. These molecules are the most common QS signalling molecules used by Gram negative bacteria for communication. The reporter strains used in this study with their designated response regulators are summarized in table S1.

We found that volatiles of *P. aeruginosa* PAO1 strain significantly induced a positive luminescence response in the *Escherichia coli*/pSB401 reporter strain, regulated by *Vibrio fischeri* LuxR response regulator ($P<0.05$; ANOVA on Ranks and Student–Newman–Keuls post hoc test) (Fig. 1). Moreover, the effect of *P. aeruginosa's* volatiles was synergistic to the induction obtained by 1 pmol of N-3-oxo-hexanoyl-homoserine lactone (3-oxo-C6-HSL) (Fig. 1). Notably, *P. aeruginosa's* volatiles did not affect any of the other examined response regulators (data not shown), nor did they act as antagonists to LuxR (Fig. 1). These results imply that a certain compound/s from *P. aeruginosa's* total volatiles can specifically activate the LuxR response regulator.

P. aeruginosa possesses three different QS systems that are crucial for its full virulence and persistence within the host. Two systems, *las* and *rhl*, are activated by N-3-oxo-dodecanoyl-homoserine lactone and N-butanoyl-homoserine lactone, respectively.[12] The third system, *mvfR*, is activated by the quinolones signals 4-hydroxy-2-heptylequinolone and Pseudomonas quinolone signal (PQS).[13]

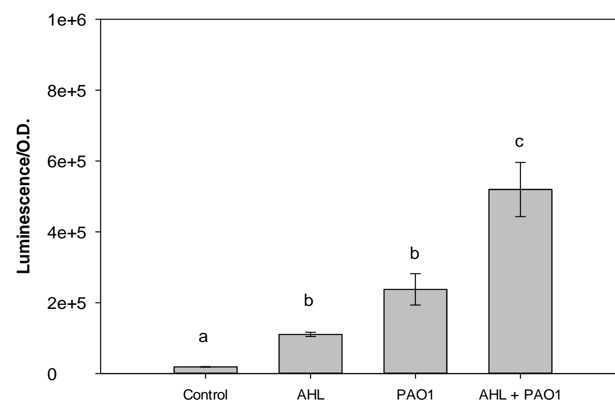

Figure 1. The effect of *Pseudomonas aeruginosa's* total volatiles on a LuxR-expressing biosensor. Luminescence levels of *E. coli*/pSB401 reporter strain, expressing LuxR response regulator, in response to total volatiles of *P. aeruginosa* (PAO1) or 1pmol of 3-oxo-C6-HSL (AHL). For antagonistic or synergistic assays the effect of volatiles was also examined with addition of 1 pmol of 3-oxo-C6-HSL to the reporter (PAO1 + AHL). n=8; Error bars are the standard error of the mean. Different letters indicate a statistical difference ($P<0.05$) according to ANOVA on Ranks and Student–Newman–Keuls post hoc test.

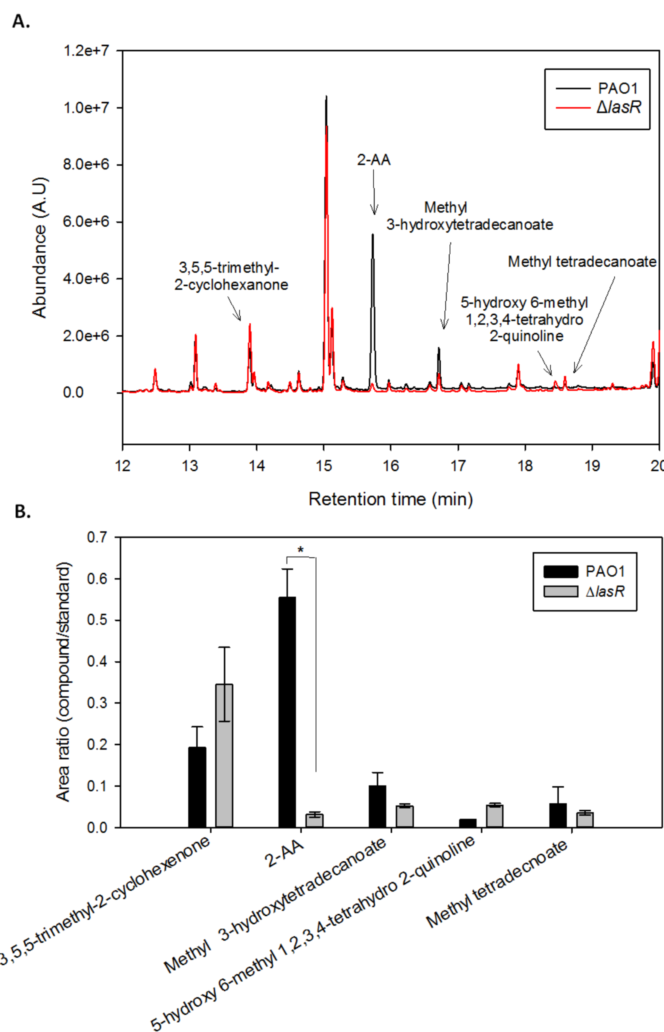

Figure 2. Volatiles profile of *P. aeruginosa* PAO1 wild type and a *lasR* mutant. A: Gas chromatogram of a wild type (PAO1- black line) and a *lasR* mutant (Δ*lasR* – red line) of *P. aeruginosa*. B: Abundance of five volatiles in the wild type and Δ*lasR* strains. n=3; Error bars represent standard error of the mean, asterisks represents significant difference between Δ*lasR* and PAO1 wild type according to student t-test (P<0.05). To aid visualization, results are presented only for retention times of 12-20 min. Beyond these retention times there were no substantial differences in volatile profiles of mutant compared to wild type strains.

Overall, more than 10% of *P. aeruginosa*'s genome is under the regulation of QS[14]. We therefore examined whether a QS mutant could maintain its ability to activate the LuxR reporter strain. Because the three QS systems *of P. aeruginosa* are hierarchically organized such that the *las* QS system is dominant over the *rhl* and PQS,[15,16] we used a Δ*lasR* mutant, deficient in the production of the LasR response regulator. We found that in contrast to the WT strain, total volatiles of the *ΔlasR* mutant did not activate the LuxR response regulator (Fig. S1). In order to identify the volatile substance/s responsible for the activation of the LuxR response regulator by *P. aeruginosa*, we conducted a comparative gas-chromatograph mass-spectrometer (GC/MS) analysis of the Δ*lasR* and WT strains. As seen in Fig. 2 the main volatile affected from mutations in *lasR* was 2-aminoacetophenone (2-AA). This is in agreement with a recent study, which demonstrated that 2-AA synthesis is controlled by the QS pathway through the *mvfR* system.[17] We therefore hypothesized that 2-AA is the volatile compound responsible for the activation of the LuxR response regulator. To test this hypothesis, we added different concentrations of synthetic 2-AA, either as volatiles or in a dissolved state, to cultures of *E. coli*/pSB401 and *E. coli* JLD271/pAL103 reporter strains. Consistent with our hypothesis, we found that 2-AA was able to significantly induce the LuxR-regulated luminescence of the reporter strains when applied both as a liquid (Fig. 3) and as a volatile (Fig.S2) (p<0.05; ANOVA and Dennett post hoc test, and student's t-test, respectively).

Similarly to the effect of *P. aeruginosa*'s total volatiles, addition of 2-AA to the biosensors inoculated with a fixed concentration of AHL (10 nM) further induced the LuxR-regulated luminescence as compared to the value measured with 10 nM of AHL without 2-AA (Fig. 3, green bars).

However, there was a difference in the range of concentrations having a synergistic effect on *E. coli*/pSB401 biosensor (25-500 µm) compared to *E. coli* JLD271/pAL103 biosensors (100 and 500 µm). In order to verify that the observed induction of luminescence by 2-AA occurred via LuxR activation, 2-AA was applied to an *E. coli* JLD271/pAL104 reporter strain, which harbours the same plasmid as pAL103 but lacks the gene encoding LuxR.

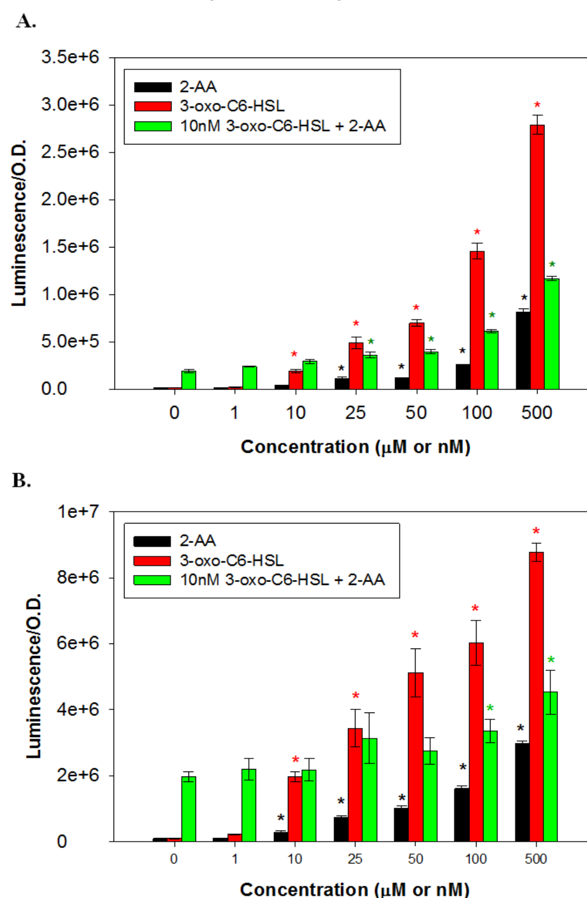

Figure 3. The effect of 2-AA and 3-oxo-C6-HSL on LuxR-expressing biosensors. 2-AA (0-500 µM) and 3-oxo-C6-HSL (0-500 nM) were added to *E. coli*/pSB401 (A) and *E. coli* JLD271/pAL103 (B) reporter strains in order to evaluate the effect of 2-AA on LuxR response regulator. For antagonistic/synergistic assays (3-oxo-C6-HSL + 2AA), 0-500 nM of 2-AA was added to the reporter strain in presence of 10 nM of 3-oxo-C6-HSL. Presented values are the luminescence measurements taken 12 h post exposure to signalling molecules. n=4; Error bars represent standard error of mean, asterisks indicate a statistical difference (P<0.01) compared to control, according to ANOVA and Dunnett post hoc test.

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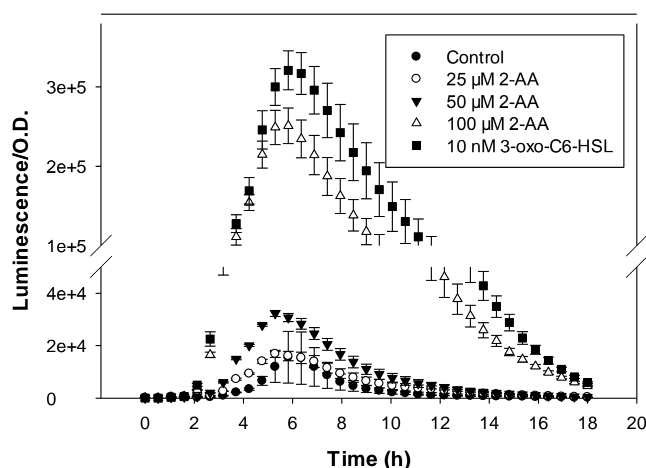

Figure 4. The effect of 2-AA on LuxR-regulated luminescence of *Vibrio fischeri*. Luminescence of *Vibrio fischeri* MJ-1 in absence (Control) and presence of 25, 50 and 100 µM of 2-AA and 10 nM of 3-oxo-C6-HSL, measured every half hour during 18 hours incubation.

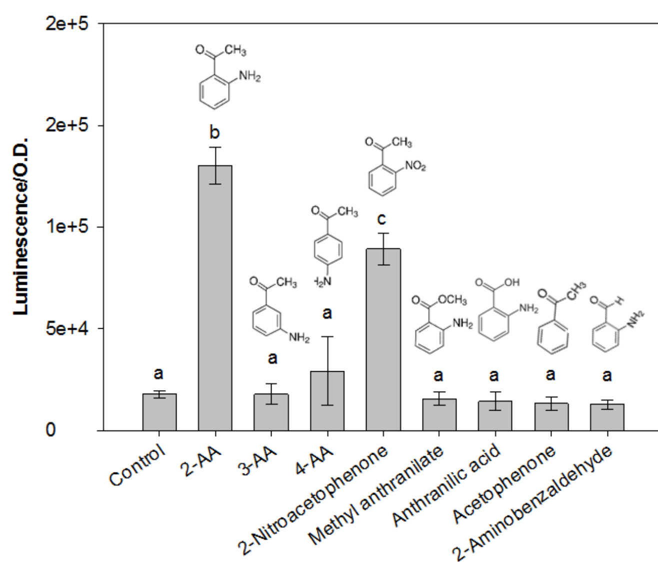

Figure 5. The effect of 2-AA and related modified molecules on LuxR-expressing biosensor. Relative luminescence of *E. coli* pSB401 reporter strain upon exposure to 50µM of the following substances: 2-aminoacetophenone (2-AA), 3-aminoacetophenone (3-AA), 4-aminoacetophenone (4-AA), 2-nitroacetophenone, methyl anthranilate, anthranilic acid, acetophenone and 2-aminobenzaldehyde. Measurements are after 20h of incubation. n=6; Error bar represent standard error of the mean. Different letters indicate a statistical difference ($P<0.05$) according to ANOVA and Student–Newman–Keuls post hoc test. Structure of each molecule is depicted above the corresponding bar.

No effect of 2-AA on the LuxR-negative reporter was observed suggesting that indeed 2-AA interacts directly with the LuxR receptor (Fig. S3). Although volatiles of *P. aeruginosa* only activated the LuxR response regulator, we still examined possible cross reaction of the synthetic 2-AA compound with the additional bioreporters strains described above. Similar to the results obtained with total volatiles of *P. aeruginosa*, 2-AA did not induce the activity of *P. aeruginosa* cognate QS receptors, RhlR and LasR (Fig. S4). 50 and 100 µM of 2-AA slightly inhibited (20 and 16%, respectively) RhlR-regulated luminescence in the presence of AHL. However, 2-AA also slightly inhibited luminescence in absence of AHL to the same level (20%), implying that this inhibition is not via ligand-response regulator interaction. 500 µM of 2-AA exhibited more significant inhibition (decrease of 40%) towards both LasR- and RhlR-regulated luminescence. According to the O.D. measurements, the observed decrease in luminescence was not due to growth inhibition. Thus, it is possible that at such high concentrations (500 µM) , 2-AA directly inhibits LasR and RhlR, but as far as we know such concentrations are much higher than those measured in cultures of *P. aeruginosa* and are probably biologically irrelevant. Notably, 2-AA did not exhibit any significant activity towards any of the other response regulators examined in this study (*i.e.* TraR, SdiA, CepR, AhyR and AhlR) (data not shown).

Our work shows that 2-AA can activate the luxR response regulator in *E. coli* based biosensor strains. To fully evaluate the biological significance of this finding we have examined the activity of 2AA on the LuxR-regulated natural luminescence of wild type *V. fischeri* MJ-1. Under the conditions set in the experiment, without edition of exogenous HSL, MJ-1 exhibited relatively low levels of luminesce. Nevertheless, addition of 10 nM of 3-oxo-C6-HSL resulted in a significant increase in the luminescence (Fig. 4). The addition of 2-AA to MJ-1 also significantly increased the luminescence, in a dose dependant manner, similarly to the activation of the LuxR-harboring reporter strains.

AHLs and 2-AA are quite different in structure, thus the nature of the interaction between AHL-binding LuxR and 2-AA is not clear. In order to better understand the apparent specificity and interaction of 2-AA with the AHL-binding receptor, we examined the effect of several 2-AA analogues (4-aminoacetophenone, 3-aminoacetophenone, acetophenone, 2-nitroacetophenone, methyl anthranilate, anthranilic acid and 2-aminobenzaldehyde) on luminescence of the *E. coli*/pSB401 reporter strain. These analogues either have their amine-group in alternate positions, or a substitution in the ketone group. Deletion or translocation of the amine group to third and fourth positions completely abolished the induction of luminescence exhibited by the reporter strain, as well as substitution of the ketone group with a carboxylic acid, an ester or an aldehyde ($P<0.05$; ANOVA and Student–Newman–Keuls post hoc test) (Fig. 5). Substitution of the amine group with a nitro group partially reduced LuxR activation compared to 2-AA, indicating that 2-nitroacetophenone could also activate LuxR, but to a lesser extent compared to 2-AA. Additionally, no dose dependency was achieved with 2-nitroacetophenone, as well as synergistic effects in the presence of AHL. All the analogues, except of 2-nitroacetophenone, exhibited neither significant inducing activity in absence of exogenous AHL, nor synergistic/antagonistic activities in presence of AHL (Fig. S5). These results indicate that the presence of the ketone group and the position of the amine group are crucial factors in LuxR-2-AA interactions and activation. Further, we conducted *in silico* docking analyses of 2-AA and LuxR's cognate ligand, 3-oxo-C6-HSL, into a LuxR model in order to study the LuxR interaction with 2-AA. Overlap of the docked 2-AA and 3-oxo-C6-HSL revealed a similar position of the 2-AA ring and the ring of 3-oxo-C6-HSL within the binding pocket of LuxR (Fig. S6). AHL docking suggested that Trp66, Asp79 and Tyr70 are the crucial residues in AHL-LuxR interactions by interacting with AHL via hydrogen bonds (Fig. 6A). In addition to these interactions, hydrophobic interactions with Pro48, Met51, Ile56, Ile76, and Val82 were suggested to stabilize the carbon chain. Docking of 2-AA into LuxR model revealed that some of the LuxR conserved residues that participate in 3-oxo-C6-HSL interactions (Trp66, Tyr70 and Asp 79)

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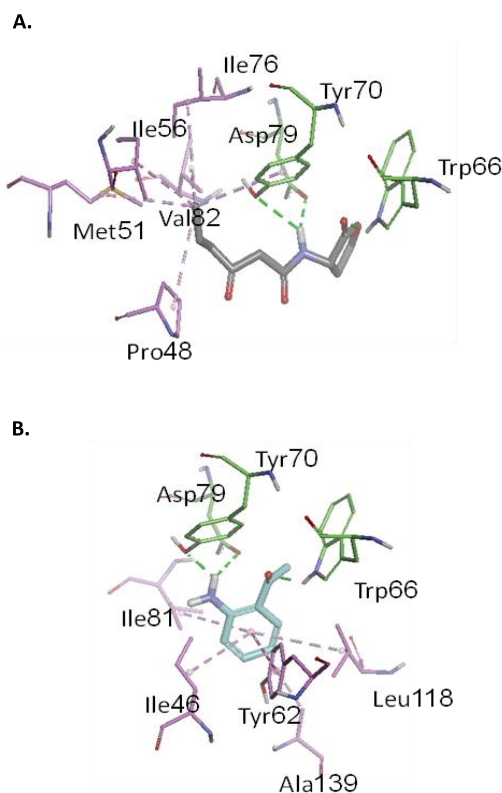

Figure 6. ***In silico*** **docking of 3-oxo-C6-HSL (panel A) and 2-AA (panel B) into LuxR model.** Interactions are shown as dotted lines and colored according to interaction type. Hbonds - green, hydrophobic interactions – pink.

also play a role in the interactions between 2-AA and the receptor (Fig. 6B). Tyr70 and Asp79 could form hydrogen bonds with the amine group, Trp66 with the carbonyl group. Additionally, Tyr62, Leu118, Ala139, Ile46, Ile81 were suggested to be involved in hydrophobic interaction. The combination of all five residues forming the hydrophobic interactions is not conserved and is unique to LuxR compared to SdiA, LasR and TraR (Fig. S7). This could explain the specificity of 2-AA towards LuxR.

Conclusions

2-AA is a low molecular weight volatile compound produced by *P. aeruginosa* in relatively high amounts (up to 80 μM).[18] Several studies described its role in the persistence of the pathogen and its interaction with the host[17,18-19]. The work described here suggests yet another possible role for 2-AA as a specific activator of the LuxR response regulator. As apparent from Fig. 3 and Fig. 4, the affinity of 3-oxo-C6-HSL towards LuxR is approximately three orders of magnitude higher than that of 2-AA. Nevertheless, such differences in affinity are still biologically relevant because the concentrations of 3-oxo-C6-HSL measured in bacterial cultures of *Vibrio* spp. varied between 1 to 10 nM,[20] whereas those of 2-AA in *P. aeruginosa* cultures reached 80 μM.[18]

Because 2-AA did not activate the QS receptors of *P. aeruginosa*, we hypothesize that it might serve as an inter-species signal, activating QS systems in other bacteria. 2-AA has been detected in total volatiles of several bacterial species inhabiting various environments ranging from the human body to marine sediments.[21,22] Moreover, BLAST analysis revealed that several bacterial species other than *V. fischeri*, such as *Aliivibrio logei*, *Vibrio mimicus*, *Photobacterium leiognathi* and *Vibrio parahaemolyticus*, possess highly similar LuxR homologs that include all the residues that were suggested to interact with 2-AA but lack in SdiA, TraR and LasR (Fig. S8). Thus, the potential involvement of 2-AA in LuxR-regulated QS cross talk by AHL-mimicking could be wide spread and ecologically significant. Future studies examining the interactions between bacterial species producing 2-AA and those possessing a LuxR response regulator, could provide important insights regarding the role of volatiles in microbial interspecies interactions in nature.

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Notes and references

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1 B. L. Bassler, *Curr. Opin. Microbiol.*, 1999, **2**, 582–587.
2 A. Mandabi, H. Ganin, P. Krief, J. Rayo, and M. M. Meijler, *Chem. Commun.*, 2014, **50**, 5322–5325.
3 S. A. Burt, V. T. Ojo-Fakunle, J. Woertman, and E. J. Veldhuizen, *PloS one*, 2014, **9**, e93414.
4 B. Jaramillo-Colorado, J. Olivero-Verbel, E. E. Stashenko, I. Wagner-Döbler, and B. Kunze, *Nat. Prod. Res.*, 2012, **26**, 1075–1086.
5 L. Chernin, N. Toklikishvili, M. Ovadis, S. Kim, J. Ben-Ari, I. Khmel, and A. Vainstein, *Environ. Microbiol. Rep.*, 2011, **3**, 698–704.
6 S. K. Green, M. N. Schroth, J. J. Cho, S. D. Kominos, and V. B. Vitanza-Jack, *Appl. Microbiol.*, 1974, **28**, 987–991.
7 S. D. Kominos, C. E. Copeland, B. Grosiak, and B. Postic, *Appl. Microbiol.*, 1972, **24**, 567–570.
8 J. Glazebrook, R. Campbell, G. Hutchinson, and N. Stallman, *Aust. J. Exp. Biol. Med. Sci.*, 1978, **56**, 147–156.
9 D. Wheater, D. Mara, L. Jawad, and J. Oragui, *Wat. Res.*, 1980, **14**, 713–721.
10 S. Moreau-Marquis, B. A. Stanton, and G. A. O'Toole, *Pulm. Pharm. Therap.*, 2008, **21**, 595–599.
11 L. Eberl and B. Tümmler, *Int. J. Med Microbiol*, 2004, **294**, 123–131.
12 R. S. Smith and B. H. Iglewski, *Curr. Opin. Microbiol.*, 2003, **6**, 56–60.
13 E. C. Pesci, J. B. Milbank, J. P. Pearson, S. McKnight, A. S. Kende, E. P. Greenberg, and B. H. Iglewski, *PNAS*, 1999, **96**, 11229–11234.
14 V. E. Wagner, D. Bushnell, L. Passador, A. I. Brooks, and B. H. Iglewski, *J. Bacteriol.*, 2003, **185**, 2080–2095.
15 A. Latifi, M. Foglino, K. Tanaka, P. Williams, and A. Lazdunski, *Mol. Microbiol.*, 1996, **21**, 1137–1146.
16 D. S. Wade, M. W. Calfee, E. R. Rocha, E. A. Ling, E. Engstrom, J. P. Coleman, and E. C. Pesci, *J. Bacteriol.*, 2005, **187**, 4372–4380.
17 M. Kesarwani, R. Hazan, J. He, Y. Que, Y. Apidianakis, B. Lesic, G. Xiao, V. Dekimpe, S. Milot, E. Deziel, and others, *PLoS path*og., 2011, **7**, e1002192.
18 C. D. Cox and J. Parker, *J. Clin. Microbiol*. 1979, **9**, 479–484.
19 Y.-A. Que, R. Hazan, B. Strobel, D. Maura, J. He, M. Kesarwani, P. Panopoulos, A. Tsurumi, M. Giddey, J. Wilhelmy, and others, *PloS one*, 2013, **8**, e80140.
20 D. L. Milton, V. J. Chalker, D. Kirke, A. Hardman, M. Cámara, and P. Williams, *J. Bacteriol.*, 2001, **183**, 3537–3547.
21 V. Thiel, T. Brinkhoff, J. S. Dickschat, S. Wickel, J. Grunenberg, I. Wagner-Döbler, M. Simon, and S. Schulz, *Org. Biomol. Chem.*, 2010, **8**, 234–246.
22 J. S. Dickschat, T. Martens, T. Brinkhoff, M. Simon, and S. Schulz, *Chem. Biodivers.*, 2005, **2**, 837–865.

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