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COMMUNICATION

A bacterial biosensor encoding a genetically modified LuxR receptor exhibits improved detection of *Pseudomonas aeruginosa*'s biomarker molecule 2-aminoacetophenone

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2-Aminoacetophneone (2-AA) is a volatile molecule produced in high amounts by the opportunistic pathogen *Pseudomonas aeruginosa.* We have previously shown that 2-AA activates the quorum sensing (QS) LuxR receptor of *Aliivibrio fischeri*. In the present study we were able to improve LuxR's affinity and detection limit for 2-AA by genetic modification of three amino acids within the binding pocket of the receptor. Expression of the modified LuxR receptor in a luminescent bacterial biosensor provided an efficient detection assay of 2-AA in clinical *P. aeruginosa* strains isolated from blood and lung infections, as well as in phlegm samples obtained from subjects suffering from lung infections.

Pseudomonas aeruginosa is a ubiquitous opportunistic human pathogen that is common in lung, ear, diabetic wounds, blood and corneal infections.[1–6] It is also the most common respiratory pathogen in cystic fibrosis patients, leading to high rates of morbidity and mortality.[7] As part of its growth cycle, *P. aeruginosa* produces high amounts of the volatile 2-aminoacetophenone (2-AA), an aromatic amine with a grape-like odour.[8] Several studies reported that 2-AA has a role in the persistence phenotype of *P. aeruginosa* and in its interaction with the host.[8,9] Because it is produced in high amounts by *P. aeruginosa* it was suggested to serve as an efficient biomarker for the presence of this bacterium in infected tissues.[10] We have previously reported that, although not its cognate signal, 2-AA specifically activated the LuxR quorum sensing (QS) receptor of the marine bacterium *Aliivibrio fischeri*.[11] We also demonstrated that activation of the LuxR receptor by 2-AA could be exploited to diagnose *P. aeruginosa* infections through the use of a LuxR encoding bacterial biosensor.[12] However, for efficient diagnosis in clinical samples, improvement of the detection limit and specificity of the LuxR receptor towards 2-AA is required.

For this purpose, we randomly changed the DNA sequence of *luxR* using error-prone PCR (details in Section 1.2 of the Supplementary material). Variants of the luminescent biosensor *Escherichia coli* MJ109/pSB401 were produced by replacing the native *luxR* sequence with the randomly mutated sequences. The variants were then screened for receptor activation with and without 50 µM of 2-AA. One variant, termed LuxR-M22, exhibited higher luminescence levels in the presence of 2-AA, relative to the biosensor encoding the native LuxR receptor (Fig. 1A). In addition to the increased sensitivity towards 2-AA, modification of the amino acids in the LuxR-M22 receptor resulted in a decreased response towards LuxR cognate ligand 3-oxo-C6-acyl homoserine lactone (3-oxo-C6-HSL) (Fig. 1B). Detection limit of the LuxR-M22 biosensor was compared to that of the native LuxR biosensor using various concentrations of 2-AA (i.e. 0.1 – 10 µM). Our results show that the detection limit of the LuxR-M22 variant was improved by one order of magnitude compared to the detection limit of the native LuxR biosensor. The latter could detect 2-AA of 10 µM and higher, whereas the modified LuxR-M22 biosensor could detect 2-AA concentration of as low as 1 µM (Fig. 2).

Sequencing of the DNA encoding the modified receptor revealed that the increased sensitivity and specificity of the modified receptor to 2-AA occurred due to three point-mutations in the *luxR* gene. Those resulted in the following changes in the amino acid sequences: Asp72 to Gly72, Val102 to Ile102 and Met135 to Thr135 (Fig. S1). The mutations were termed M22A, M22B and M22C, respectively. In order to examine the role of each mutation we produced three single mutants in each of these amino acid encoding positions, as well as different combinations of double mutations.

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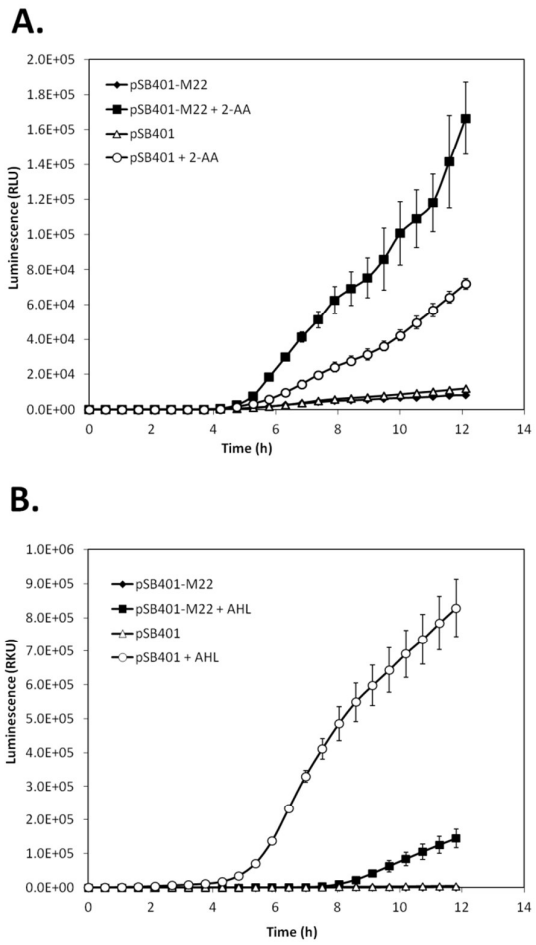

Figure 1. Response of the native and modified LuxR based biosensors towards AHL and 2-AA. A) Detection of 2-AA by the native LuxR receptor (pSB401) and by the modified LuxR receptor (pSB401-M22). Measurements were carried out with (+2-AA) or without addition of 50µM of 2-AA during 12h of incubation. Error bars represent standard deviation of 4 replicates. B) Detection of AHL, the cognate signal of LuxR, by the native LuxR receptor (pSB401) and its modified variant (PSB401-M22). Measurements were carried out with and without addition of 10nM of 3-oxo-C6-HSL (AHL) during 12h of incubation. Error bars represent standard deviation of 4 replicates.

Analysis of the response of the three LuxR variants (i.e. M22A, M22B, M22C) towards 2-AA, indicated that the single mutations of M22A and M22C resulted in improved responses towards 2-AA relative to the native receptor, but variant M22B exhibited a slightly decreased response (Fig. S2). Nevertheless, the response of the double mutant M22AC towards 2-AA was lower than that exhibited by the triple mutant (M22), indicating a synergistic effect of all three mutations (Fig. S2).

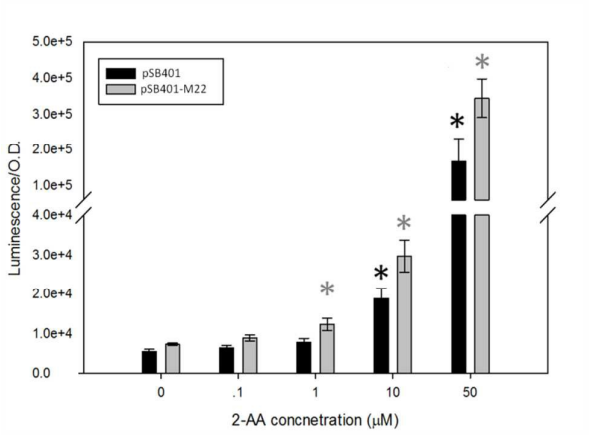

Figure 2. Detection limit of the native and modified LuxR biosensors. Bars show luminescence produced by the native (pSB401) and modified (pSB401-M22) LuxR biosensors in the presence of 0, 0.1, 1, 10 and 50 µM of 2-AA. The luminescence was measured after 20 h of incubation with 2-AA. Error bars represent standard deviation of 4 replicates. Asteriks represent statistical difference ($p<0.01$) from control (0 µM of 2-AA) for each of the biosensors, according to one-way ANOVA and Holm-Sidak post hoc.

We performed *in silico* docking analysis to compare the interactions of the native, as well as modified (M22) LuxR with 2-AA and 3-oxo-C6-HSL. Results of *in silico* docking of 3-oxo-C6-HSL within the native receptor model suggested that Trp66, Asp79 and Tyr62 are crucial residues in ligand-LuxR binding, interacting with 3-oxo-C6-HSL via hydrogen bonds (Fig 3A). The participation of these residues in the interaction between LuxR and AHL was also shown in a study published by Koch et al.[13]. In addition to these interactions, hydrophobic interactions with Pro48, Met51, Ile56, Ile58, Tyr70 and Ile76 were suggested to stabilize the carbon chain. Docking of 3-oxo-C6-HSL to the modified LuxR-M22 receptor suggested that, while the hydrogen bond interactions were maintained, there was a significant reduction in the hydrophobic interactions (three instead of six) (Fig 3B). This may explain the impaired activation of the LuxR-M22 receptor by 3-oxo-C6-HSL, relative to the native receptor.

Docking of 2-AA within the native LuxR receptor model suggested that two of the conserved residues of LuxR that participate in interaction with AHL (Tyr70 and Tyr62) also have a role in the interaction with 2-AA. In addition, anion-pi interaction was formed between Asp79 and the benzene ring of 2-AA and hydrophobic interactions between Ala113 and Leu118 and the ring of 2-AA. Docking of 2-AA within the model of the modified LuxR-M22 revealed that the ligand adapted a flipped conformation, relative to its position in the native LuxR receptor. In this position, in addition to the pi-donor, hydrogen bond and hydrophobic interactions that have been present both in the native and the modified receptors, a new pi-pi

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interaction was formed between the modified receptor and 2-AA. This interaction, with an energy value of -3.8 kcal/mol, possibly contributed to the observed improved sensitivity towards 2-AA compared to the native receptor.

2-AA is produced by *P. aeruginosa* PA14 strain in relatively high amounts (up to ~50 μM).[8] However, it is known that clinical strains of *P. aeruginosa* exhibit high genetic and phenotypic variability.[14] In order for 2-AA to serve as an efficient biomarker for *P. aeruginosa* infections it is important that it will be produced by all *P. aeruginosa's* strains. We therefore examined 52 clinical isolates from blood and lung infections, in addition to PAO1 and PA14 strains. The isolates were analysed for production of 2-AA using both the native and the modified receptors. The assay was conducted in a manner allowing only the exchange of air-borne substances between the biosensor and *P. aeruginosa* isolates.

Results showed that out of total 54 isolates, 53 activated the biosensors (Table 1). Notably, in all these 2-AA-positive isolates, the response of the biosensor encoding the modified LuxR-M22 receptor was significantly stronger than the native LuxR biosensor (Fig S3). One isolate (R11), obtained from a pneumonia patient, did not activate neither of the two biosensors. We have previously showed that *P. aeruginosa* strains that were mutated in their LasR QS receptor did not produce 2-AA.[11] We therefore examined whether the isolate that did not activate the LuxR biosensor is QS-deficient and thus does not produce the QS-regulated signal AHL. In order to determine whether AHL is produced by this isolate we used *las* and *rhl* biosensors (details in section 1.10 of the supplementary information). Results showed that the 2-AA negative strain (R11) was indeed deficient in both *las*- and *rhl*-regulated AHL production (Fig. S4). It has been previously reported that clinical isolates of *P. aeruginosa* could sometimes be defective in their QS pathways, mainly due to a mutation in the LasR receptor or in LasI AHL-synthase.[15–17] Indeed, sequencing of the gene encoding for LasR receptor in R11 isolate indicated that this isolate possesses a receptor with three mutated amino acids, as compared to LasR from *P. aeruginosa* PA14 strain and a lung isolate (R12) that activated the LuxR biosensor (Fig. S3, Fig. S5). Such *lasR* mutants of *P. aeruginosa* are generally found in chronic infections.[18] Accordingly, the QS-deficient strain (R11) was isolated from lung infections, where a chronic phenotype is relatively common.[17] In contrast, blood infections are mainly acute and indeed all the 30 isolates from the blood samples were 2-AA positive, indicating that they harbour a functioning QS system.

In order to examine the diagnostic potential of the pSB401-M22 biosensor, we analyzed sputum samples collected from subjects suffering from lung infection. For proof of concept, 24 sputum samples were collected. All samples were analyzed for the presence of *P. aeruginosa* by culture and the modified biosensor assay. In this small-scale proof of concept study, we found that nine samples were negative in both the biosensor and culture, and 12 samples were positive according to both methods. Two samples were positive according to the biosensor assay but negative by culture and one was false negative (negative according to the biosensor assay but positive in culture). These results reflect high sensitivity and specificity (92.3% and 81.8%, respectively) with positive (PPV) and negative (NPV) predicted values of 85.7% and 90%, respectively.

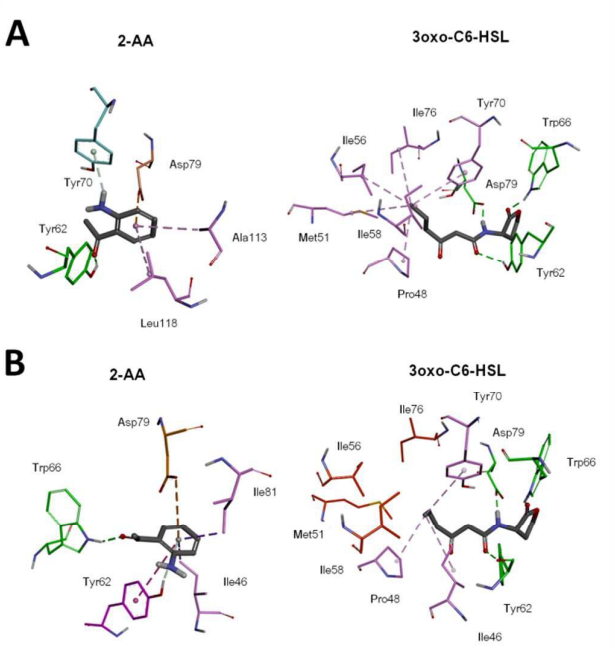

Figure 3. *In silico* docking of 3-oxo-C6-HSL and 2-AA into native (WT) (panel A) and modified (M22) (panel B) LuxR models. Interactions are shown as dotted lines and coloured according to interaction type. A) *In silico* docking of 2-AA and 3-oxo-C6-HSL into WT LuxR model. Hbonds – green; hydrophobic interactions – pink; anion-pi interaction – orange, pi-donor interaction – grey. B) *In silico* docking of 2-AA and 3-oxo-C6-HSL into modified LuxR-M22 model. Hbonds – green; anion-pi interaction – orange; pi-donor interaction – grey; pi-pi interaction – violet.

Conclusions

Detection of 2-AA has thus far been carried out by elaborate gas-chromatograph mass-spectrometry (GC-MS) or selected-ion flow-tube mass-spectrometry (SIFT-MS) analyses.[10,20] These methods are costly and require technical experience and specialized laboratories. Therefore, an efficient and low-cost method for detection of 2-AA will be of a significant advantage for diagnosis. The presented study shows that three point mutations in the binding pocket of the QS LuxR receptor increased its activation by 2-AA while decreasing the affinity towards its cognate signal AHL. Expression of this modified LuxR receptor within a luminescent bacterial biosensor provided an efficient tool for 2-AA detection. Examination of 52 clinical isolates of *P. aeruginosa* using this biosensor found that 51 of them indeed produce 2-AA while the isolate that did

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not produce it was defective in QS pathways. Quorum sensing mutants in chronic infections necessarily co-exist with QS wild type strains.[21] Thus it can be speculated that tissue samples containing such QS-mutant would still harbour strains that produce 2-AA to activate LuxR-based biosensor strain. Analyses of sputum samples with the modified biosensor suggested that this biosensor could provide an efficient diagnostic tool of *P. aeruginosa* infections. Additional studies are required for implementing the use of the modified biosensor in clinical environments such as hospitals or local clinics. Building a light-tight reading chamber with a Photo multiplier tube (PMT) detector, as well as using noble metal nanoparticles for enhancing the emitted signals and shortening the response time,[22] are noteworthy options that will be tested in future studies.

Here we show that binding specificity of a particular response regulator could be tuned to suit a specific application. Such manipulations can open up an array of novel sensors for various targets.

Conflicts of interest

There are no conflicts to declare.

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