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A Cell-Free Biosensor for Detecting Quorum Sensing Molecules in *P. aeruginosa*-Infected Respiratory Samples

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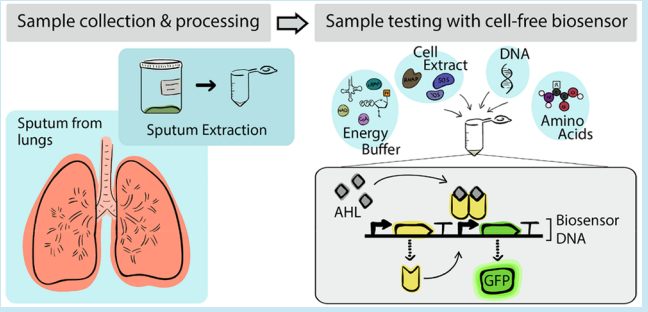
Supporting Information

ABSTRACT: Synthetic biology designed cell-free biosensors are a promising new tool for the detection of clinically relevant biomarkers in infectious diseases. Here, we report that a modular DNA-encoded biosensor in cell-free protein expression systems can be used to measure a bacterial biomarker of *Pseudomonas aeruginosa* infection from human sputum samples. By optimizing the cell-free system and sample extraction, we demonstrate that the quorum sensing molecule 3-oxo-C12-HSL in sputum samples from cystic fibrosis lungs can be quantitatively measured at nanomolar levels using our cell-free biosensor system, and is comparable to LC−MS measurements of the same samples. This study further illustrates the potential of modular cell-free biosensors as rapid, low-cost detection assays that can inform clinical practice.



KEYWORDS: *Pseudomonas aeruginosa, cystic fibrosis, quorum sensing, cell-free synthetic biology, biosensor*

Cystic fibrosis (CF) is an autosomal recessive genetic disease characterized by the buildup of thick mucus secretions in the lung and the occurrence of numerous respiratory infections, which cause significant pulmonary decline and increased mortality.[1] *Pseudomonas aeruginosa*, a Gram-negative bacterium that can opportunistically infect humans, is the dominant pathogen affecting persons with CF by their late teens − 60% of persons with CF aged 16 or above in the UK have *P. aeruginosa* infections.[2]

Many of these infections are defined as chronic, whereby the pathogen continually evades eradication by the host immune system and cannot be completely eliminated by aggressive antibiotic treatment regimes. A better understanding of the mechanisms by which these bacteria invade, persist, and respond to treatment in the CF lung is desperately needed to target treatments more effectively. Quorum sensing (QS), the production and sensing of small extracellular molecules that enables bacteria to coordinate their behavior in a density-dependent manner, is one system employed by many bacterial species,[3−6] including *P. aeruginosa*,[7−10] to regulate cooperative and pathogenic behaviors such as biofilm formation and the production of virulence factors.[11] Acyl homoserine lactones (AHLs) are a subset of QS molecules which enable species-specific communication due to different bacterial species producing AHLs of varying acyl chain length.[12] *P. aeruginosa* produces two AHL molecules, *N*-butyryl-homoserine lactone (C4-HSL) and *N*-3-oxo-dodecanoyl-homoserine lactone (3OC12-HSL).[8,13] *P. aeruginosa*-specific QS molecules have been detected in a variety of CF patient samples including sputum, urine and blood,[14−17] and some show clinical relevance with links to infection state.[18−20] Thus, the rapid detection and monitoring of QS molecules in patient samples may prove useful in the future for identifying infection and directing treatment.

Within the field of synthetic biology, many biologically engineered systems have been designed to create affordable, easy-to-use tools for the detection of clinically relevant biomarkers.[21−25] However, only a few have demonstrated functionality with actual clinical samples,[26,27] since this requires taking into account the potentially complex composition of the test sample and the suboptimal setting in which such a biosensor may be implemented. Cell-free transcription and

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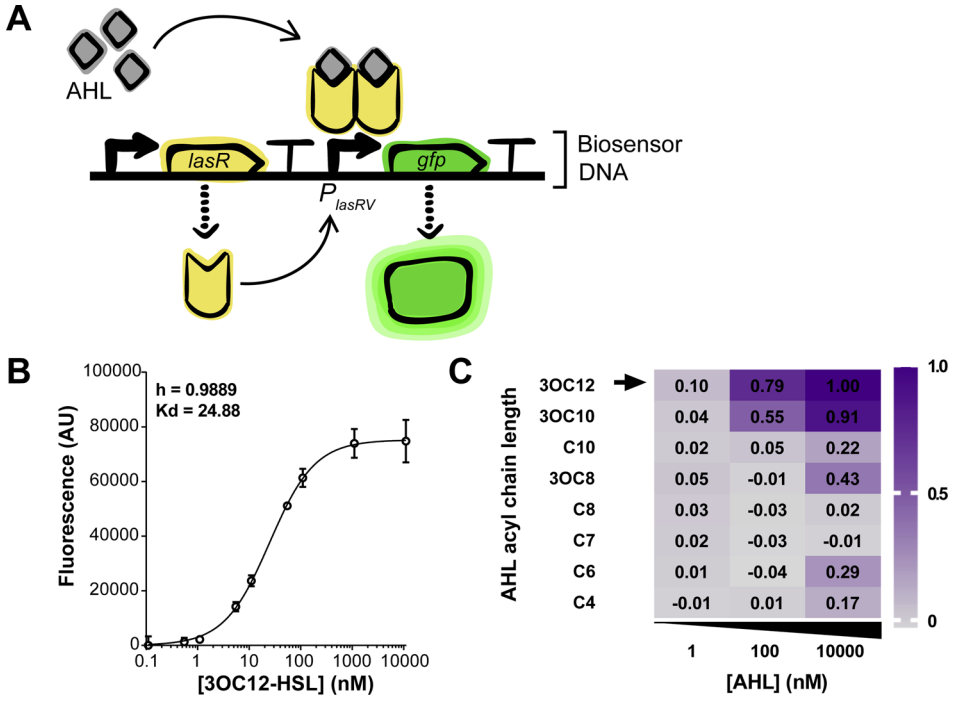

Figure 1. Characterization of the LasRV cell-free biosensor. (A) Outline of the AHL-responsive LasRV biosensor construct. LasR is under the control of constitutive promoter BBa_J23101. (B) Sensitivity of LasRV in commercial cell-free. After induction with 3OC12-HSL, fluorescence gain from 30 to 240 min was measured (Arbitrary Units, A.U.), shown as the mean and SD of $n = 3$ independent repeats. A response curve was fitted with a Hill-slope. Hill coefficient (h) and K_d are shown. (C) Specificity toward the target AHL for LasRV in cell-free. Commercial cell-free reactions were induced with eight AHLs at 1, 100, and 10 000 nM. Fluorescence gain from 30 to 240 min was taken and normalized to the response of LasRV induced with 10 μM 3OC12-HSL. The mean of $n = 3$ independent repeats is shown.

translation reactions have recently emerged as an alternative "chassis", or host, for a range of synthetic biology applications[28−35] such as genetic parts prototyping and metabolic engineering. They are composed of a buffered cell lysate containing all the cellular machinery, energy and resources to support transcription and translation of a user-supplied DNA template[36] and their ease-of-use, sterility, and low cost make them particularly suitable for applications in the field of diagnostics.[21,26] They have also been used to develop QS biosensors,[37,38] although these earlier systems focused on their use as screening tools for QS molecules binding or production rather than for the application of clinical testing.

Here, we aimed to apply our *P. aeruginosa* AHL-sensing circuit implemented in an *E. coli*-based cell-free system[35] to analyze CF patient sputum samples. Our results demonstrate that the cell-free biosensor system can quantitatively measure the presence of 3OC12-HSL within these samples, suggesting it has potential as a platform for detecting and monitoring pathogen biomarkers from human respiratory samples.

RESULTS AND DISCUSSION

Characterization of the AHL-Responsive LasRV Biosensor. To successfully develop a biosensing system for testing clinical samples, we needed to consider both the functionality of the biosensor, and the handling of the patient sample. The 3OC12-HSL-responsive DNA-based biosensor, LasRV, was previously developed by Chappell *et al.* (2013)[35] using genetic elements originating from the genome of *P. aeruginosa* strain PAO1 coupled with standard synthetic biology parts (Figure 1A).

First, it is important that the biosensor can detect the target at clinically relevant concentrations with minimal cross-activation by AHLs from different bacterial species. To assess the functionality of our AHL biosensor, we therefore sought to determine the sensitivity and specificity of LasRV implemented in a cell-free transcription−translation system. LasRV was previously shown to be inducible by addition of 3OC12-HSL in both whole-cell and cell-free systems.[35] Figure 1B shows the LasRV response range to 3OC12-HSL in cell-free; reactions were prepared using a commercial *E. coli*-based cell-free system (Promega S30), 3OC12-HSL was added at final concentrations varying from 0.11 nM to 11 μM, and GFP fluorescence was measured at 4 h after incubation at 30 °C (Figure S1). LasRV displayed sensitivity to 3OC12-HSL at nanomolar concentrations, with a linear dynamic range between approximately 5 and 100 nM. A Hill coefficient of around 1 suggests there is noncooperative binding and the lower limit of detection (LLOD), defined as the concentration at which 95% of samples would have a detected signal above background level, was calculated as 4.9 nM (see Methods for details).

To evaluate specificity, LasRV response was tested in cell-free reactions with a broad panel of eight AHL inducers that varied in acyl chain length and the presence of a 3-oxo group (Figure 1C and Figure S2). LasRV displayed the strongest activation when induced by 3OC12-HSL at all three concentrations tested. At 1 and 100 nM, LasRV showed strong preferential activation by 3OC12-HSL over other AHLs; at 10 μM there was a greater occurrence of nonspecific activation, particularly in the presence of 3OC10-HSL and 3OC8-HSL. Together with the results from Figure 1B this suggests that the optimal 3OC12-HSL response range lies between 5 nM and 100 nM AHL. Interestingly, when LasRV was tested in a whole-cell *E. coli* chassis, there was more nonspecific activation by noncognate AHLs at the 100 nM and 10 μM concentrations

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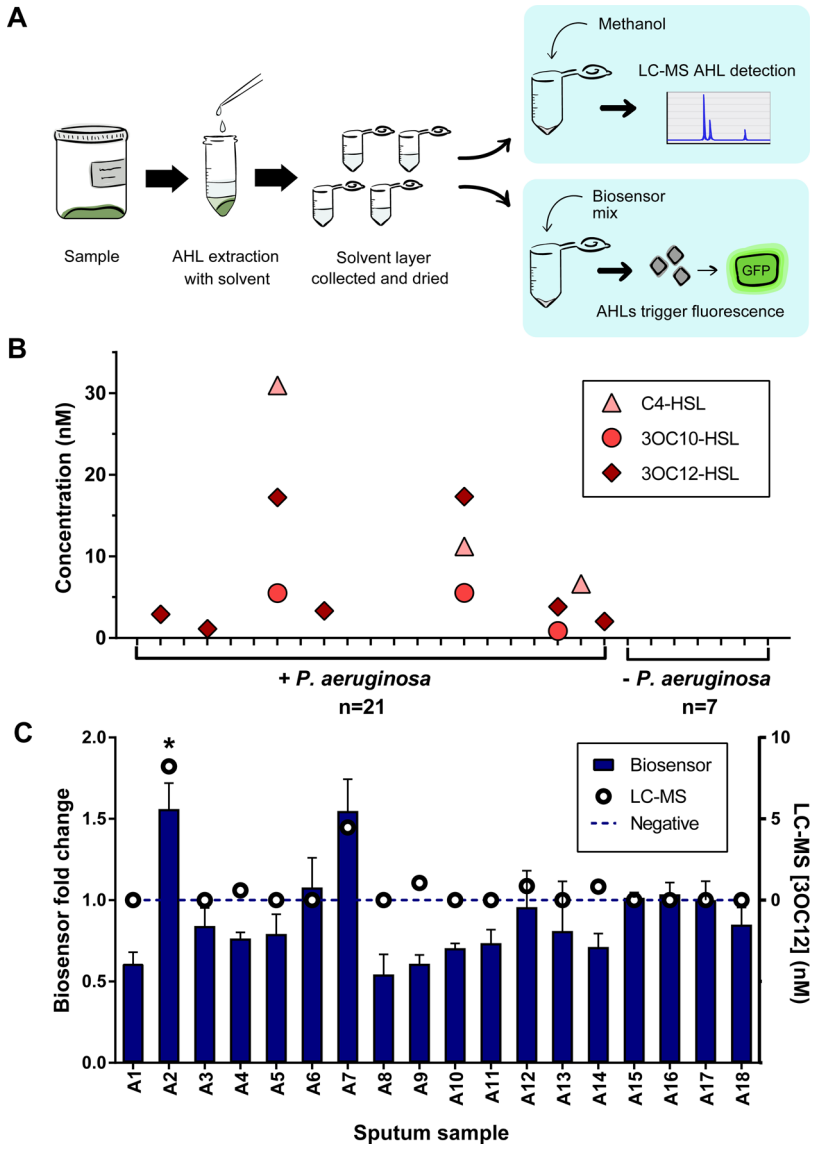

Figure 2. Investigation of AHLs in sputum samples. (A) Workflow of solvent extraction to concentrate AHLs from sputum followed by concurrent analysis by cell-free biosensor and LC−MS/MS. (B) LC−MS/MS analysis of AHLs within *P. aeruginosa*-positive and -negative sputum samples from CF patients. (C) Analysis of 18 CF sputum samples by LasRV cell-free biosensor and LC−MS/MS. One aliquot of sample extract was resuspended in methanol for LC−MS/MS and one was resuspended in cell-free master mix of 3× reaction volume. Fluorescence increase between 30 and 240 min was converted to fold change against a negative control without AHL. The mean and SD of $n = 3$ biosensor reactions per sample is shown. One sample t tests (two-tailed) were used to compare biosensor fold change against a hypothetical mean of 1, $p < 0.05$ (*).

than when implemented in cell-free systems (Figure S3), providing another potential advantage for using a cell-free chassis over *in vivo* systems.

Investigation of AHLs in CF Patient Sputum Samples. A preliminary processing step is necessary to prepare sputum samples for testing with the LasRV biosensor, in order to concentrate any 3OC12-HSL and reduce sample complexity. Since the cell-free system is membrane-less, it is exposed to added components and thus vulnerable to interference or inhibition. The sample processing step is depicted in Figure 2A. AHLs can be extracted through the mixing of organic solvent with the specimen, followed by separation and collection of the organic phase which is then dried to concentrate any AHLs within the sample. For validation of the biosensor, collected solvent can be divided into aliquots, each of which can be directed either toward chemical analysis using liquid chromatography tandem mass spectrometry (LC−MS/MS) or cell-free biosensor testing. This allows for accurate parallel quantification of any AHLs present.

An initial screen of 28 sputum samples from CF individuals with positive or negative *P. aeruginosa* cultures was performed, using quantitative LC−MS/MS to directly determine the type and concentration of AHLs present. As shown in Figure 2B, minimal diversity of AHL species was observed overall, with only 3OC12-HSL, C4-HSL and 3OC10-HSL detected in the 28 samples. 3OC12-HSL and C4-HSL are the most frequently detected AHLs within *P. aeruginosa*-positive CF clinical samples,[15,18,19,39] and some studies report detection of additional AHLs including C6-HSL, C8-HSL and C10-HSL.[14,16,17] Here, 3OC12-HSL was the most abundant AHL species, present in 7/21 *P. aeruginosa*-positive sputum samples tested. Three samples contained detectable C4-HSL and three contained 3OC10-HSL. No AHL species were detected in any of the seven *P. aeruginosa*-negative sputum samples. Since

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3OC10-HSL was always found in conjunction with 3OC12-HSL but at lower concentrations, it is possible that it is a byproduct of the 3OC12-HSL synthase LasI from *P. aeruginosa* or a degradation product of 3OC12-HSL, which has been observed in previous studies of *P. aeruginosa* AHL production.[40,41] Encouragingly, the concentrations of 3OC12-HSL detected after solvent-based extraction generally fell within the previously determined response range of LasRV. Considering the specificity of LasRV (Figure 1C), C4-HSL and 3OC10-HSL concentrations in the sputum samples would not induce a significant response.

The number of sputum samples which did not contain any AHL species was higher than expected. Therefore, the extraction process was investigated to establish if AHL recovery was inefficient (Figure S4). Ethyl acetate (EA), the solvent used for processing the samples in Figure 2B, yielded a mean AHL recovery of 70% from sputum samples, which was significantly higher than dichloromethane, another commonly used solvent.[14,16−18,42]

Having determined that EA extraction can recover the majority of AHLs from sputum samples, a further 18 sputum samples (A1−18) from *P. aeruginosa*-infected CF patients were processed by EA extraction and analyzed in parallel by LC−MS/MS and the LasRV cell-free biosensor (Figure 2C and Figure S5). LasRV response was measured as fluorescence fold change over background expression of GFP after 4 h, while LC−MS/MS provided accurate 3OC12-HSL quantitation. Only samples from *P. aeruginosa*-infected patients were tested since they were found to be both AHL negative and positive (Figure 2B), and therefore they can be used to assess false positives and false negatives, respectively. From the panel of 18 sputum samples, A2 and A7 resulted in the two highest detectable outputs from the cell-free biosensor, which corresponded to the two samples with the highest LC−MS/MS-determined 3OC12-HSL concentrations, 8.2 nM and 4.5 nM respectively. LasRV output was significantly above background for A2 (p = 0.038, one sample t test) but not significant for A7 (p = 0.059). However, while successfully able to detect AHL from clinical samples we observed that both samples resulted in relatively low fluorescence expression output with around 1.5-fold activation.

Optimizing the Cell-Free Platform for Measurement of AHLs from CF Sputum Samples. In order to boost the output of the biosensor assay, we sought to optimize the cell-free system for expression of the AHL biosensor. Cell-free extracts were prepared using an adapted protocol for making highly active cell-free transcription and translation (TX-TL) reactions.[36,43] The activity of cell-free extract from three different *E. coli* strains were compared against the commercial kit (Promega S30) using LasRV biosensor induced with 110 nM 3OC12-HSL (Figure 3A). Compared to the commercial system which is BL21-derived, the activity of cell extracts made from the BL21-Gold (DE3) was not significantly different, while the MG1655 strain produced significantly lower GFP output. Rosetta cell-free displayed a 3-fold improvement in GFP output. This is in agreement with previous findings showing Rosetta-based cell extracts are high performing,[29,30,36,43] and suggested that AHLs extracted from sputum would be able to induce a greater measurable response in Rosetta cell-free than in the commercial system.

To provide a means of converting biosensor fluorescence output directly into AHL concentration, a biosensor response curve was constructed. Since extraction efficiency is below

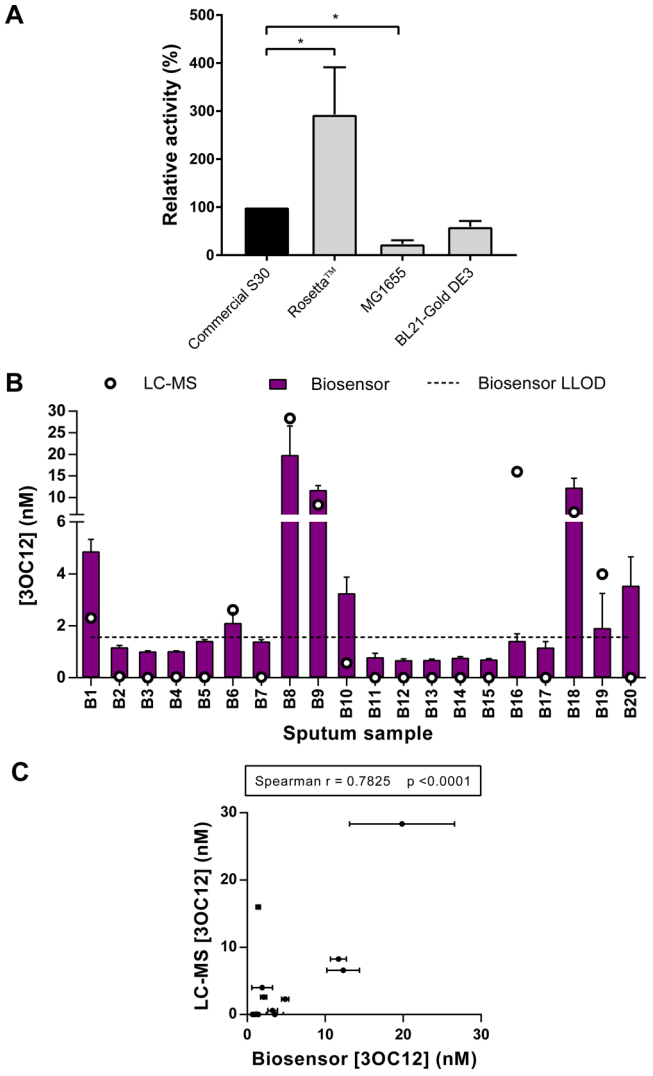

Figure 3. Optimization of the cell-free LasRV biosensor for sputum sample analysis. (A) TX-TL cell-free extracts from three *E. coli* strains were compared to commercial cell-free extracts in reactions containing LasRV and 110 nM 3OC12-HSL. Fluorescence increase between 30 and 150 min is represented as a percentage relative to the commercial cell-free. Mean and SD is shown for each TX-TL group (Rosetta n = 4, MG1655 n = 2, BL21-Gold DE3 n = 2). One sample t tests (two-tailed) were used to compare TX-TL extracts against a hypothetical mean of 100% relative activity, $p < 0.05$ (*). (B) Analysis of 20 CF sputum samples by LasRV in Rosetta cell-free and by LC−MS/MS. Biosensor fluorescence output between 30 and 300 min was converted to 3OC12-HSL concentration using the calibration curve. The mean and SD of n = 3 biosensor reactions per sample is shown. (C) Correlation between biosensor-predicted and LC−MS/MS concentration of 3OC12-HSL, using Spearman's rank correlation coefficient.

100% (Figure S4), water samples were spiked with different concentrations of 3OC12-HSL prior to EA extraction and then measured with the LasRV Rosetta cell-free system (Figure S6). This demonstrated that the concentration at which expression was equal to background fluorescence was 1.55 nM (SD = 0.00124), and the LLOD for 3OC12-HSL in sample extracts was calculated as 1.56 nM. The response curve in Rosetta cell-free showed increased sensitivity compared to the commercial cell-free system (Figure 1B), where the LLOD was near to 5 nM. This is in part because the output of background (AHL-negative) samples, relative to 110 nM 3OC12-HSL standards,

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was much lower in the Rosetta system than in the commercial system (Figures S6 and S1, respectively), resulting in a higher signal-to-noise ratio. In addition to this, the lower variation in background fluorescence from AHL-negative samples after they were subjected to solvent extraction contributes to the lower LLOD observed.

An additional 20 *P. aeruginosa*-positive CF sputum samples (B1−20) were subsequently analyzed, using both LC−MS/MS and the Rosetta-based cell-free LasRV biosensor. A positive PBS control, prespiked with 110 nM 3OC12-HSL before extraction, was run alongside sputum extracts to calculate relative biosensor activity and estimate 3OC12-HSL concentration in the clinical samples using the standard curve.

The biosensor-predicted concentrations were then compared to the LC−MS/MS-quantified values (Figure 3B). There was good agreement between LC−MS/MS and the biosensor results across the samples, with one false negative biosensor outcome (B16) and one false positive (B20) based on a 1.56 nM biosensor cutoff. To assess the agreement between levels of 3OC12-HSL that were determined by LC−MS/MS and biosensor, Spearman's rank correlation analysis was performed (Figure 3C) and showed a positive correlation between both methods of detection ($p < 0.0001$, Spearman correlation 0.7825). In terms of the absolute values, 3OC12-HSL concentrations predicted by the biosensor were within 3 nM of the LC−MS/MS value (mean difference 2.7 ± 3.3 nM).

Clinical Relevance of QS Molecules. Despite the plethora of biosensing systems which have been proposed in recent years, the process of translation from laboratory conditions to detection in real-world samples remains a significant challenge. Here, we have presented a proof-of-concept biosensor for the detection of a *P. aeruginosa*-specific quorum sensing molecule in clinical lung-derived samples. Using a DNA-encoded biosensor and *E. coli* cell-free extracts, we could measure 3OC12-HSL concentrations in CF sputum samples with a comparable accuracy to LC−MS/MS methods.

While cell-free reactions were run up to 6 h to allow full expression of GFP, signal differences between samples could be seen as early as 2.5 h (Figure S7), making it a potentially competitive system to other whole-cell and cell-free QS systems in terms of rapidity.[37,38,44]

Previous reports of QS molecules in CF lung samples show that 3OC12-HSL levels range from picomolar to low micromolar concentrations.[14,15,17−19] In this study we observed 3OC12-HSL levels of around 0.1−2.0 nM in sputum, after accounting for an approximate 10× concentration due to the sample processing methodology. While this is lower than some studies, it does fall within the range of reported concentrations. As seen in the work here, and reported elsewhere,[16,17,19] individuals with *P. aeruginosa* infections (determined by microbiological culturing) do not necessarily produce AHL-positive clinical samples. The role of QS in bacterial infections is still the subject of active research, and thus monitoring the presence of QS molecules in clinical samples may reveal more about the physiological state of bacteria during an infection cycle,[39,45−47] rather than acting as a direct indicator of bacterial presence. In *P. aeruginosa,* as much as 10% of the genome is under some regulation by QS,[48,49] including genes involved in biofilm maturation—a state which is 10−1000 times more resistant to antibiotics[50]—and the production of virulence factors.[13,51,52] On the other hand, *P. aeruginosa* strains isolated from CF chronic lung infections have been found to accumulate loss-of-function mutations in QS genes[53,54] which suggests QS is not an important contributor to late-stage infections. The direct clinical relevance of 3OC12-HSL has been suggested previously by Struss *et al.* (2013),[18] who found that the level of 3OC12-HSL in sputum was higher in hospitalised than stable individuals. Other clinically relevant QS targets include the proposed *P. aeruginosa* early infection biomarker 2-heptyl-4-hydroxyquinoline (HHQ).[20] Cell-free biosensors could be an attractive alternative to costly chemical analysis methods such as LC−MS/MS for monitoring QS production during CF lung infections, and thus contribute to a further understanding of the role of QS regulation in disease. DNA-encoded biosensors can also be adapted for simultaneous analysis of multiple biomarkers by coupling different biosensing modules using biological logic gates, which have been previously demonstrated in cell-free systems.[29]

Translating Lab-Based Cell-Free Biosensors to Practice. Consideration of the real conditions required for clinical sample testing is key to the successful application of genetically engineered biosensing devices in the clinic. For this, a number of hurdles still need to be overcome. First, while the cell-free expression system is nonreplicating and noninfectious, unlike whole-cell biosensors, there are as of yet no examples of the regulatory approval required for implementation in the clinic. Second, the current sample processing step before analysis would need to be streamlined further to reduce hands-on time and make routine testing possible. Furthermore, while ethyl acetate-based extraction can sufficiently recover AHLs within sputum, it is a nonselective process that is likely to also carry over additional compounds into the cell-free biosensor assay. Sputum samples from CF patients are highly complex and heterogeneous; further chemical analysis of solvent extracts could identify molecules that interfere with either the cell-free biosensor or AHLs directly. The one false negative and one false positive result seen among the blind trial of 20 sputum samples could have been the result of such interference either causing suppression or activation of the AHL-responsive biosensor, but a much larger number of samples would need to be tested to identify these potential contaminants and the frequency of such outcomes. Despite this, to our knowledge our study provides the first demonstration of utilizing a cell-free biosensor to quantitatively detect a relevant biomarker from clinical samples.

The cost of cell-free systems like the Rosetta-based one used in this work is extremely low compared to commercial systems,[43] but to improve this further it has been shown that reactions can be freeze-dried, either directly or embedded in paper discs;[21] this greatly simplifies the setup process and eliminates the need for cold-chain transport. As tools for the genetic engineering of cell-free biosensors continue to develop, we envision that it will be possible to design and build affordable biomarker tests that are suitable for clinical settings and can inform the diagnosis and treatment of disease.

METHODS

The LasRV Biosensor Device. The pSB1A2:LasRV biosensor plasmid was previously designed and constructed by Chappell *et al.* (2013).[35] In brief, the system encodes constitutive expression of transcription factor LasR from the *P. aeruginosa* strain PAO1, which recognizes its cognate AHL 3-oxo-dodecanoyl homoserine lactone (3OC12-HSL). Upon binding AHL, LasR dimerizes and can bind to sequence-specific regions of DNA, located within promoter P_{LasRV} (a section of the upstream region from the PAO1 gene *vqsR*),

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which is placed in front of the Green Fluorescence Protein (GFP) gene (see Supporting Information). Thus, presence of AHL induces expression of GFP and can be measured as an increase in fluorescence over time.

Commercial *E. coli* Cell-Free Reactions. Cell-free reactions were prepared with *E. coli* S30 Extract System for Circular DNA (Promega, Southampton UK). Each reaction was prepared following manufacturer's guidelines, up to a final volume of 25 μL containing 1 μg LasRV plasmid DNA. For characterization assays, 0.5 μL AHL stock solutions in DMSO were added as inducer (within the 25 μL total reaction volume) to give final concentrations of 0.11, 0.55, 1.1, 5.5, 11, 55, 110, 1100 and 11 000 nM unless otherwise specified. Reactions were transferred to a black clear-bottom 384-well plate (Greiner Bio-One) and measured in a CLARIOstar plate reader (BMG Labtech). Plates were incubated at 30 °C for 8 h, and fluorescence (excitation at 485 nm, emission at 520 nm) measured every 15 min, with 10 s shaking at 200 rpm before each measurement. Fluorescence output was calculated as gain in fluorescence between 30 min (the first 30 min are omitted due to fluctuations in fluorescence as the temperature of the reactions equalizes) and 240 min, when maximum fluorescence was reached (Figure S1). For characterization of biosensor specificity, eight AHLs (C4-HSL, C6-HSL, C7-HSL, C8-HSL, 3OC8-HSL, C10-HSL, 3OC10-HSL, 3OC12-HSL) were used as inducer. See Supporting Information for further information.

TX-TL Cell-Free Reactions. Cell extract was prepared following the protocol described[43] with minor modifications. A full description is provided in Supporting Information. Each reaction was prepared with 7.5 μL cell extract, 12.5 μL energy mix, 1 μg of LasRV plasmid DNA and nuclease-free water up to a final volume of 25 μL and transferred to a black clear-bottom 384-well plate (Greiner Bio-One). GFP measurements were taken following the same method described above for commercial cell-free reactions, but fluorescence output was calculated as gain in fluorescence between 30 and 300 min for TX-TL reactions alone (when maximum gain in fluorescence is reached, see Figure S6). For comparison against commercial extract, fluorescence gain was calculated between 30 and 150 min, to be within the linear range of activity for all compared extracts (Figure S8).

Collection of CF Patient Samples. CF patient sputum samples were collected by staff from the Respiratory Biobank at Royal Brompton Hospital, London SW3 6NP, UK under two ethically approved projects (Biomedical Research Unit (BRU) Advanced Lung Disease (ALD) Biobank 10/H0504/9 and Biomedical Research Unit (BRU) Respiratory Biobank 15/SC/0101). Sputum samples were stored at −80 °C and thawed before use by investigators on this project.

AHL Extraction from CF Sputum. The method for extraction of AHLs from sputum samples was adapted from Ortori *et al.* (2011) and Struss *et al.* (2013).[18,55] Sputum samples (approximately 1 mL) were added to 1 mL PBS (pH 7.4). An equal volume of ethyl acetate (acidified, 0.1% acetic acid) or dichloromethane (acidified, 0.1% formic acid) was added and gently mixed for 5 min. Samples were centrifuged at 3000*g* for 1 min, after which the organic phase was removed and collected. This was repeated three times in total. The collected ethyl acetate extracts were pooled and dried under N_2 gas. For parallel biosensor and LC−MS/MS analysis, the pooled solvent was divided into four aliquots of equal volume before drying. Per sputum sample, three aliquots of sample extract were analyzed by cell-free assay, and one aliquot was sent for LC−MS/MS analysis.

Testing of Sputum Sample Extract by LasRV Biosensor. For cell-free biosensor testing with commercial *E. coli* S30 extract, one aliquot of sample extract was resuspended in 80 μL cell-free master mix and transferred to a 384-well plate as 3 × 25 μL reactions. Fluorescence was measured as described above between 30 and 240 min. For each batch of sputum samples analyzed, a negative control extract of PBS only was run in parallel. The fold change was calculated as fluorescence output of sample extract divided by mean fluorescence output of negative control.

For cell-free biosensor testing with Rosetta TX-TL extract, three aliquots of sample extract were resuspended in 25 μL cell-free master mix each and transferred to a 384-well plate. Fluorescence was measured as described above between 30 and 300 min. For each batch of sputum samples analyzed, a positive control extract of PBS prespiked with 3OC12-HSL (to give an expected final concentration of 110 nM) and a negative control extract of PBS only was run in parallel. The percentage relative activity is calculated as the fluorescence output of sputum extract divided by mean fluorescence output of positive extract, multiplied by 100. This is converted to concentration of 3OC12-HSL using a calibration curve (Figure S6), which was prepared by prespiking samples of ddH_2O with 3OC12-HSL to give final cell-free reaction concentrations ranging from 1.1 nM to 1.1 μM.

Efficiency of AHL Recovery by Solvent Extraction. To calculate the efficiency of AHL recovery by solvent extraction, sputum samples were spiked with 20 μL 500 nM ^{13}C-labeled 3OC12-HSL. Samples were then extracted in either dichloromethane or ethyl acetate following the method described above. The collected solvent extract was dried under N_2 gas and resuspended in 100 μL methanol to achieve a final expected concentration of 100 nM labeled 3OC12-HSL (assuming complete recovery).

The concentration of ^{13}C-labeled 3OC12-HSL in sample extracts was calculated after LC−MS/MS analysis and reported as a percentage relative to the expected concentration of 100 nM. The number of sputum samples tested was: dichloromethane (n = 4), ethyl acetate (n = 4). The positive control was PBS spiked with 100 nM 3OC12-HSL and extracted with ethyl acetate (n = 7).

Comparison of Different Cell-Free Systems Activity. Reactions containing 1 μg of LasRV DNA were induced with 3OC12-HSL at a final concentration of 110 nM. The 25 μL reaction volumes were transferred into a 384-well plate and GFP fluorescence was measured as before. Activity was measured as the change in fluorescence over time during the linear dynamic range of each reaction. Results for each extract were then compared against the commercial extract to give relative activity.

Mass Spectrometry Analysis of Extracted AHLs. LC−MS/MS analysis was conducted based on the protocol described in Struss *et al.* (2013)[18] using the 1290 LC system and 6550 iFunnel Quadropole-Time of Flight mass spectrometer with electrospray ionization (Agilent Technologies, California US). A Zorbax Extend C-18 LC column (Agilent Technologies), 2.1 × 50 m and 1.8 μm particle size, was used for LC. Protonated molecules $[M + H]^+$ were subjected to collision induced disassociation (10 eV) and product ions monitored either throughout or based on retention time windows. Fragment ions (mass measured to 4 decimal places)

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were quantified by the summed peak area ±20 ppm (Table S1). Calibration curves for quantitation were generated by injecting increasing volumes of AHL standard solutions in methanol and plotting the summed peak area of fragment ions against loaded sample.

Sputum sample extracts were reconstituted in 100 μL methanol (LC−MS CHROMASOLV-grade, Sigma-Aldrich) when whole, or 25 μL when aliquoted. 0.5−12.5 μL was injected. Each sample was analyzed for the presence of eight AHLs (see Supporting Information). The concentration was calculated using the equation of the AHL standards calibration curves.

Data Analysis and Statistics. All data was plotted, and statistical analysis conducted, with GraphPad Prism software (GraphPad, California US) unless otherwise stated. A Hill equation was fitted to 3OC12-HSL induction of the LasRV biosensor using the specific binding with Hill slope function: $Y = B_{max} \times X^h/(K_d{}^h + X^h)$, where B_{max} is the maximum specific binding, K_d is the concentration of ligand needed for half-maximum binding at equilibrium, and h is the Hill slope. Correlations are reported as a Spearman's rank correlation coefficient and associated p value. Unpaired t tests were used for two group comparisons where data followed a Gaussian distribution. Lower limits of detection (LLODs) were calculated as previously described[56] from the limit of background (LOB) value ($Mean_{(blank)} + 1.645 \times SD_{(blank)}$) using the equation $LLOD = LOB + 1.645 \times SD_{(lowest\ concentration\ sample)}$. In this way, 95% of samples at a concentration equal to the LLOD would have a measured signal above the LOB.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acssynbio.7b00219.

DNA part sequences, pSB1A2:LasRV plasmid map, preparation of TX-TL extract, Acyl homoserine lactones, characterization of biosensor plasmid in whole cells; Fluorescence gain over time for biosensor LasRV in commercial S30 cell-free, The fluorescence output of the biosensor LasRV in commercial cell-free when induced with varying types and concentrations of AHLs, Fluorescence output of the LasRV biosensor in whole cells when induced with varying types and concentration of AHLs, Recovery of spiked ^{13}C-labeled 3OC12 in CF sputum following extraction protocol, Fluorescence over time for LasRV in commercial S30 cell-free induced with CF sputum extract, 3OC12-HSL response curve for LasRV in Rosetta cell-free, Fluorescence over time for sputum samples B1−20 tested with the LasRV cell-free biosensor using the TX-TL Rosetta cell-free system, Comparison of commercial and Rosetta cell-free extract run times; AHL fragment ions monitored for LC−MS/MS (PDF)

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Notes

The authors declare no competing financial interest.

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