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Detection and characterization of *N*-acyl-L-homoserine lactones using GFP-based biosensors in conjunction with thin-layer chromatography

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ABSTRACT

Many microorganisms use quorum sensing to regulate several complex phenotypes, and this is accomplished by the release of a signal molecule(s) into the environment. *N*-acyl-homoserine lactones (AHLs) are a common class of signalling molecule utilized by a range of microorganisms (primarily Gram negative bacteria but most recently also archaea) and are often detected through the use of bacterial biosensors. Biosensors can be limited by both their specificity and sensitivity, and the aim of this study was to modify and improve current AHL detection strategies. The biosensor employed in the present study was *Escherichia coli* MT102 harbouring a plasmid containing a LuxR based biosensor, which produces green fluorescent protein (GFP) as a reporting mechanism. A new method of visualizing the GFP based biosensor overlaid on silica sheets for the purpose of thin-layer chromatography (TLC) is presented. This new method vastly improves sensitivity of AHL detection by a GFP biosensor than previously reported and as such represents a powerful new tool in AHL research.

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1. Introduction

Acyl homoserine lactones (AHLs) are signalling molecules commonly used by a range of bacteria and archaea in order to co-ordinate phenotypes that are more effectively expressed *en mass* in a process termed quorum sensing (Williams, 2007; Waters and Bassler, 2005). Such phenotypes include the production of extracellular enzymes (Chernin et al., 1998), genetic competence (Li et al., 2002), bioluminescence (Freeman et al., 2000), antimicrobial production (Chandler et al., 2012) as well as many others. A variety of quorum sensing phenotypes are important to industry and medicine such as biofouling driven by biofilm formation and virulence of pathogenic organisms (Erickson et al., 2002).

AHLs are a diverse group of molecules with a range of chemical modifications and differences in chain length allowing for specificity in signal. The typical structure of an AHL consists of a lactone ring with an acyl chain of varying length attached. Typically the most common modification is the addition of an oxo group to C-3 of the chain, and hydroxyl modifications have also been seen in a range of species also at the C3 position (Fuqua et al., 2001). Carboxylated AHLs have been identified in the domain Archaea (Zhang et al., 2012). There have been a variety of techniques developed to identify and quantify AHLs used by microorganisms, ranging from analytical techniques such as high-performance liquid chromatography, mass spectrometry, and nuclear magnetic resonance (Ortori et al., 2007), to other techniques employing bioassays using specifically designed biosensors (Steindler and Venturi, 2007).

Routinely used AHL biosensors consist of bacteria engineered to respond to an AHL stimulus with a reportable phenotype such as beta-galactosidase production (Shaw et al., 1997) or production of a pigment (Poulter et al., 2010; McClean et al., 1997). There are many types of AHL biosensors available, differing in levels of sensitivity and specificity as well as their reporting phenotype; some biosensors are also more effective at detecting oxo-substituted AHLs or shorter chain AHLs (Steindler and Venturi, 2007). Due to the variety of biosensors available, different assays are used for different applications, for example GFP responsive strains have primarily been utilized in microscopy or well-based quantification techniques (Steindler and Venturi, 2007).

To further characterize AHL molecules, biosensors can be combined with various separation techniques. Thin-layer chromatography (TLC) is a commonly used technique to separate molecules based on polarity. TLCs have been used in conjunction with a range of AHL biosensors – which increases the sensitivity of the assay compared to TLC alone – to assess the activity of several organisms at once, in addition to determining whether an organism can produce multiple signal molecules (Shaw et al., 1997; Chang et al., 2012; Chu et al., 2011). However not all biosensors are suited for work with TLCs, and the most commonly used biosensor strains for TLC work rely upon either pigment production or beta-galactosidase reporting systems (Steindler and Venturi, 2007). Whilst one study has used GFP reporting biosensors in combination with TLC (Andersen et al., 2001), they are not commonly used (Steindler and Venturi, 2007), possibly due to the lower sensitivities

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compared to other options (Shaw et al., 1997). Previously the only reported methods for visualizing GFP in conjunction with TLC relied upon dark-boxes with an appropriate light source (Andersen et al., 2001). However, this method cannot compete in terms of sensitivity with other commonly used strains mentioned above.

The aim of the present study was to modify and develop current screening protocols in conjunction with AHL separation methods. A novel method of TLC screening for use with GFP-based biosensors is reported here, with sensitivity levels exceeding pigment and beta-galactosidase based strains in some cases. In addition we also compare the sensitivities of the TLC system with a well-based assay to further quantify signal strength. Furthermore, an improved methodology for visualizing GFP using fluorescence detection from a phosphorimager is presented. A new technique is described for visualization of TLC sheets overlaid with an AHL GFP based biosensor (*Escherichia coli* MT102), with vastly improved sensitivity of AHL detection than previously reported.

2. Materials and methods

2.1. Strains and growth conditions

E. coli strain MT102 harbouring the plasmid pJBA132 was used as a reporter strain for detection of AHL activity (Andersen et al., 2001). *E. coli* MT102 was a kind gift from the Centre for Marine Bioinnovation. The plasmid encodes the *Vibrio fischeri lux* machinery with an unstable variant of the green fluorescent protein reporter gene fused to the AHL inducible P_{luxI} promoter (Andersen et al., 2001). The reporter strain was routinely cultured aerobically in LB_{10} broth (unless otherwise specified) supplemented with 100 μg/ml of ampicillin at 30 °C with shaking at 150 rpm. A range from C4 to oxo-C12 *N*-acyl-L-homoserine lactones were purchased from Sigma-Aldrich and stored at −20 °C as 20 mM stocks dissolved in MeOH acidified with 0.1% formic acid. For a list of AHLs used in this study please see Table 1.

2.2. Thin layer chromatography and well-plate assays

Overnight cultures of *E. coli* MT102 (pJBA132) were diluted 10 times in AB minimal medium (Clark and Maaløe, 1967) for 96-well plate assays. Diluted reporter strain was dispensed in 200 μl aliquots into flat-bottomed 96-well plates (Corning, Costar). AHL standards were air-dried prior to addition of the reporter strain. Equal volumes of acidified methanol were used for negative controls. Plates were incubated at 30 °C with shaking at 150 rpm for 4 h. Fluorescence (excitation, 485 nm; emission, 535 nm) and OD_{600} of cultures were measured by a fluorometer (CLARIOstar, BMG LABTECH). Specific fluorescence activity was expressed per unit of optical density (600 nm).

Thin-layer chromatography in combination with biosensor overlays were prepared for phosphorimager readings. Culture extracts and AHL standards were spotted on non-fluorescent reverse-phase C18 TLC glass plates coated with silica gel (Analtech TLC uniplates, Sigma-Aldrich). It is important to use TLC plates with no fluorescence indicator in order to eliminate background fluorescence. Culture extracts and serial dilutions of AHL standards were prepared in acidified methanol and 2 μl and 20 μl of each standard and culture extracts, respectively, were spotted onto TLC plates. Plates were developed with a methanol and MilliQ water mixture (60:40 *v*/*v*) and allowed to dry at room temperature for 15 min before being overlaid. Overnight *E. coli* MT102 (pJBA132) cultures were diluted to an optical density of 2.0 and incubated at 30 °C with shaking at 150 rpm for 4 h. Equal volumes of culture and agar-enriched medium were mixed for a final agar concentration of 0.8% (*w*/*v*) before pouring over a dried silica sheet. Plates were incubated for 24 h at 30 °C.

Table 1
Limits of AHL detection observed in present study using GFP-based biosensor.

AHL	Lowest detection observed	
	Present study	Andersen et al. (2001)
C4 HSL	1710 ng	5000 ng
C6 HSL	0.2 ng	50 ng
3-oxo-C6 HSL	0.009 ng	5 ng
C7 HSL	0.43 ng	ND
C8 HSL	4.54 ng	2500 ng
3-oxo-C8 HSL	0.012 ng	ND
C10 HSL	25.54 ng	ND
C12 HSL	113.2 ng	ND
3-oxo-C12 HSL	2.97 ng	ND

ND not determined.

2.3. Environmental isolates

To test the modified detection protocol further, several known AHL-producers were selected and analysed. Wild-type *Chromobacterium violaceum* and *Serratia marcescens* M9 were both cultured in LB_{10} (Bertani, 1951) for 24 h at 30 °C. *Hyunsoonleella* sp. sw033, a marine isolate, was kindly obtained from The University of New South Wales, and cultured in Marine broth (Difco) at 30 °C for 24 h.

2.4. AHL extraction from environmental isolates

After 20 ml bacterial isolate cultures were grown overnight, AHLs were extracted from cell free supernatants using ethyl acetate (Shaw et al., 1997). After evaporating the ethyl acetate the extracts were then dissolved in 200 μl MeOH acidified with 0.1% (*v*/*v*) formic acid and stored at −20 °C for further use.

2.5. Visualization of GFP TLC

Visualization of GFP TLC was achieved utilizing a GE phosphorimager that employed the Fujifilm LPB filter set with a 473 nm laser. All images were scanned with 100 μm resolution, using the EGFP method, which has an excitation source of 489 nm and an emission of 520 nm. A photomultiplier tube (PMT) value of 250 V was selected for this phosphorimager. Further image clarification i.e. adjustments of relative light levels were carried out using the Image Quant software package provided by the manufacturer, GE. Further adjustments would depend on strains used and specific phosphorimager model.

3. Results and discussion

A novel method is presented for visualizing TLC sheets overlaid with an *E. coli* MT102 (pJBA135) LuxR based AHL biosensor, which produces GFP in response to a subset of AHLs. The goal was to create a method by which a GFP-producing AHL biosensor could be used with TLC at high sensitivity (Shaw et al., 1997; Steindler and Venturi, 2007). To achieve this, the minimum detectable concentration of a variety of AHLs had to be determined as well as demonstrating the efficacy of the approach for bacterial isolates in addition to AHL standards.

3.1. Assay development

In order to determine the minimum levels of AHLs needed to activate the *E. coli* MT102 (pJBA132) biosensor, a series of dilutions of each AHL described in Table 1 were analysed. Fig. 1 depicts descending concentrations of 3-oxo-C6 HSL spotted on a TLC plate as an illustration of output. While higher concentrations can oversaturate the image (data not shown), there is a clear AHL dose response. Contrast adjustment enabled quantification of low concentrations. The adjustments varied according to the individual image taken so values can be compared within a given plate.

Table 1 reports minimum detectable thresholds for nine different AHLs and a comparison with minimum levels reported previously. The

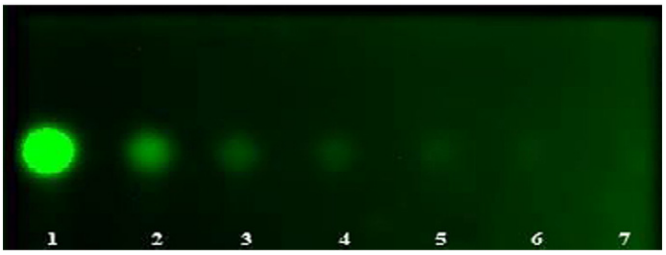

Fig. 1. Sensitivity of *E. coli* MT102 determined by TLC. Descending concentrations of 3-oxo-C6 HSL were separated on C-18 silica sheets and overlaid with *E. coli* MT102 biosensor and visualized for GFP production. Lane 1 (0.43 ng), Lane 2 (0.21 ng), Lane 3 (0.085 ng), Lane 4 (0.043 ng), Lane 5 (0.021 ng), Lane 6 (0.009 ng), and Lane 7 (0.004 ng).

values detected in this study by TLC are considerably lower than any previously reported using a GFP-based AHL biosensor (Andersen et al., 2001). In some cases, such as for 3-oxo-C6 HSL and C8 HSL, nearly a thousand fold increase in sensitivity was observed.

The *C. violaceum* (CV026) biosensor, that has been used previously in conjunction with TLC as well as being used widely in the literature (Steindler and Venturi, 2007; McClean et al., 1997), has lower sensitivity and detects fewer AHL types unless exogenous AHLs are added for an inhibition style assay (McClean et al., 1997). The *E. coli* MT102 assay presented here is able to detect more AHLs without the need to add any exogenous AHLs, thus improving both the efficacy, complexity and cost of this assay, particularly when employed in high-throughput.

In contrast to *C. violaceum* (CV026) biosensors, *Agrobacterium tumefacians* biosensors detect a broad range of different AHL types without the need to add exogenous AHLs to the assay and is commonly used for TLC overlays (Shaw et al., 1997). However *A. tumefacians* strains rely on β-galatctosidase activity as the reportable phenotype, necessitating the addition of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, which can increase both the cost per assay as well as the complexity. While biosensors that rely on pigment or colour change reporter phenotypes have been limited to agar plate well-diffusion assays for quantification (Ravn et al., 2001), GFP producing strains can be easily quantified using 96-well liquid assays. The broader utility of GFP-reporting strains in microscopy, 96 well plate assays, and now TLC shown in the present study, allows for high throughput comparisons of activity across a range of experiments.

This method can also be applied to other GFP-producing AHL biosensors. There are a number of different GFP reporting strains available each with their own range of sensitivity based upon which LuxR homologue the biosensor encodes, which explains the particular sensitivities to shorter chain AHLs observed (Steindler and Venturi, 2007). For example, it is anticipated that some strains that use GFP as a reporter could be optimized for shorter or longer chain AHLs, depending on the response element used, to build on the results obtained here for *E. coli* MT102 (Riedel et al., 2001).

3.2. Assay application using environmental isolates

To further demonstrate the utility of the optimized approach presented here, several environmental isolates were tested. Fig. 2 shows a variety of both standard AHLs as well as extracts from *S. marcescens*, *Hyunsoonleella* sp. and *C. violaceum*. A variety of AHLs were detected via the different migration rates on silica sheets, allowing for putative identification of unknown AHLs based on their location relative to standards. Both *S. marcescens* and *C. violaceum* appear to produce a signal molecule showing identical migration on TLC plates to the C6 HSL standard (Fig. 2), which is consistent with literature reporting both of these organisms producing this AHL type (Horng et al., 2002; Morohoshi et al., 2008). *Hyunsoonleella* sp. appears to produce a signal molecule that eluted more rapidly than any other standard suggesting it is likely a 3-oxo-C4 HSL. Members of the Flavobacteriaceae family

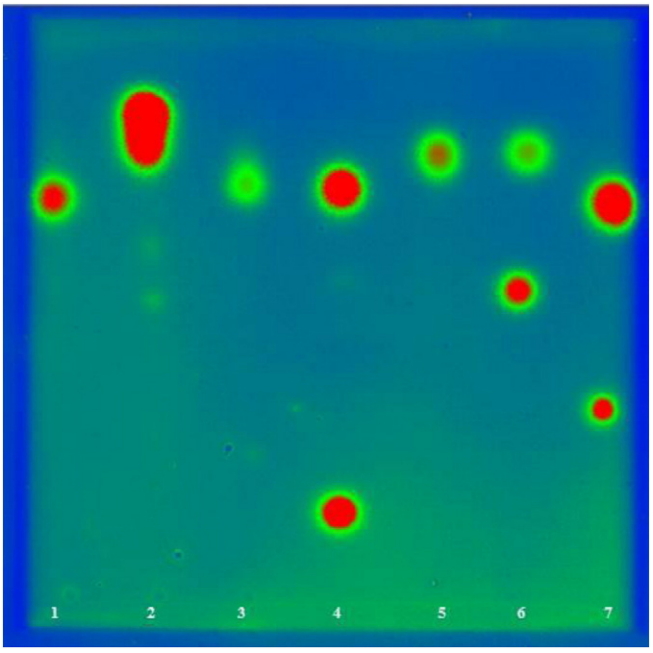

Fig. 2. Thin Layer Chromatography of environmental samples and standard AHLs. Extracts of bacterial cultures and AHL standards were chromatographed on C-18 silica sheets and overlaid with *E. coli* MT102 biosensor and visualized for GFP production. Lane 1: *S. marcescens* extracts. Lane 2: *Hyunsoonleella* sp. extracts. Lane 3: *Chromobacterium violaceum* extracts. Lane 4: 3-oxo-C8 HSL (0.24 ng) + C12 HSL (566 ng). Lane 5: 3-oxo-C6 HSL (0.21 ng,). Lane 6: C4 HSL (6840 ng) + C8 HSL (9.08 ng). Lane 7: C6 HSL (0.4 ng) + C10 HSL (102.16 ng).

that this *Hyunsoonleella* genus isolate belongs to have previously been indicated to produce short chain AHLs (Twigg et al., 2014; Romero et al., 2010). This indicates the application and robustness of this assay in the ability to detect signal molecules in environmental isolates.

3.3. Quantification

The TLC technique presented here can also be used in conjunction with a 96 well plate assay modified from Andersen et al. (2001) for quantification. The 96 well assay employed here provided a fluorescence value which can be compared to a non-induced control (Fig. 3) which allows for an estimate of activity provided by each standard (see Supplemental Fig. 1). The use of a microtitre assay is a cost-effective approach in comparison to analytical chemistry techniques (such as HPLC and GC-MS) that can also be employed for quantification (Charlton et al., 2000; Ortori et al., 2007).

4. Conclusions

This study describes the application of a GFP dependent AHL biosensor for the detection and characterization of AHLs from isolated microbes. This new method improves the sensitivity of AHL detection by a GFP biosensor and as such represents a useful new tool in the field. Given the positive findings in this study, future work could employ this same method on other GFP-based biosensor systems that could result in an even wider AHL detection range for specific purposes, such as for longer or shorter chain AHLs, or different modifications (eg. carboxylation). This method represents a cost effective, sensitive, rapid and reliable way to detect and putatively identify AHL molecules in environmental isolates.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.mimet.2015.09.012.

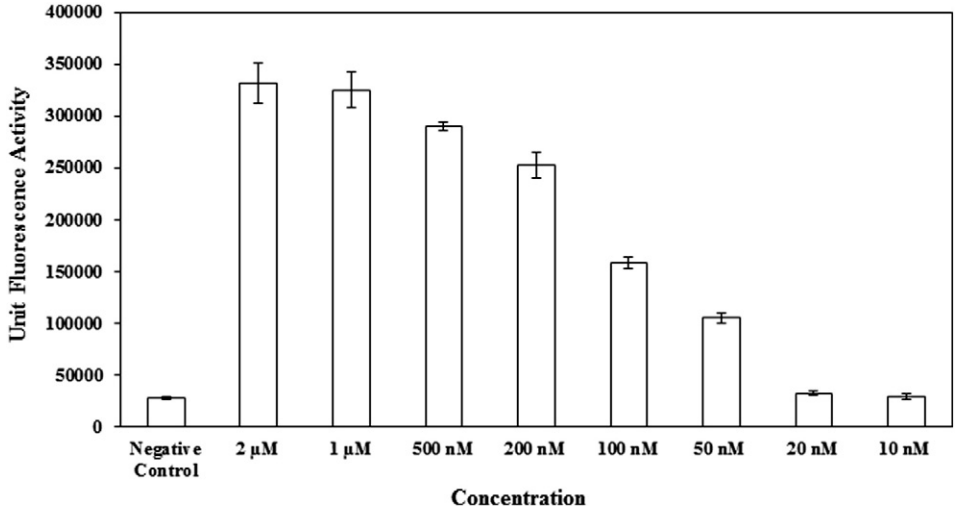

Fig. 3. Quantification of response by *E. coli* MT102 biosensor to 3-oxo-C8 HSL in 96 well assay. Fluorescence induced by 3-oxo-C8 over a range of concentrations in 96 well assay. *E. coli* MT102 was grown at 30 °C for 4 h.

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