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Minireview

Extraction, purification and identification of bacterial signal molecules based on *N*-acyl homoserine lactones

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Summary

Bacteria possess an extraordinary repertoire for intercellular communication and social behaviour. This repertoire for bacterial communication, termed as quorum sensing (QS), depends on specific diffusible signal molecules. There are many different kinds of signal molecules in the bacterial community. Among those signal molecules, *N*-acyl homoserine lactones (HSLs, in other publications also referred to as AHLs, acy-HSLs etc.) are often employed as QS signal molecules for many Gram-negative bacteria. Due to the specific structure and tiny amount of those HSL signal molecules, the characterization of HSLs has been the subject of extensive investigations in the last decades and has become a paradigm for bacteria intercellular signalling. In this article, different methods, including extraction, purification and characterization of HSLs, are reviewed. The review provides an insight into identification and characterization of new HSLs and other signal molecules for bacterial intercellular communication.

Introduction

Quorum sensing (QS) (Nealson and Hastings, 1979; Fuqua and Winans, 1994) is a term coined to describe an environmental sensing system that allows bacteria to monitor their own population density. The monitoring function is achieved by cell-to-cell communication via small signal molecules (Camilli and Bassler, 2006). *N*-acyl homoserine lactones (HSLs) (Christopher and Bassler, 2005), known as autoinducers, are important signal molecules widely recognized in QS systems of many Gram-negative bacteria. Different types of HSLs have been identified and characterized in the last decades. The structure and concentration of HSLs play significant roles in intercellular signalling system.

HSLs secreted by bacteria are different in the length of the acyl-chain moiety, saturation and substitution on the third carbon by either oxo or hydroxyl group (Table 1) (Salmond *et al.*, 1995; Wagner-Döbler *et al.*, 2005). In recent years, a putative new class of HSL signal molecule called p-coumaroyl-HSL (pC-HSL) has been characterized (Palmer and Blackwell, 2008; Schaefer *et al.*, 2008). This new class of HSL is found with a p-coumaric group other than fatty acyl group (Table 1). Another new class of signal molecule called CAI-1, which regulated gene expression through a new HapR-independent pathway, has been found in *Vibrio cholerae* (Hammer and Bassler, 2007). However, the specific structure of the new signal molecule remains unknown.

HSLs signal molecules are synthesized and regulated by two proteins belonging to the LuxI/R protein families (Lithgow *et al.*, 2000; Williams, 2007). In this HSLs-QS system, LuxI is responsible for the synthesis of HSLs. When the concentration of HSLs reaches a critical threshold, HSLs will bind to the transcriptional activator LuxR at its amino terminus. While the carboxyl terminus of the transcriptional activator binds to specific gene promoter sequences termed as lux boxes. The lux boxes contain the *luxI* gene, which is responsible for the synthesis of the protein LuxI, so the signal molecules are synthesized automatically. That is why the type of signal molecule also is called as autoinducer. These lux boxes may induce or repress the expression of QS target genes, including virulence genes expression (Winson *et al.*, 1995), antibiotic production (Bainton *et al.*, 1992; Thomson *et al.*, 2000; Houdt *et al.*, 2007), swarming motility (Eberl *et al.*, 1996) and biofilm formation (Davies *et al.*, 1998). The above biological functions regulated by HSLs are of considerable scientific, economic and medical importance. However,

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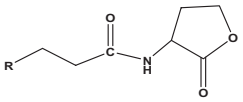
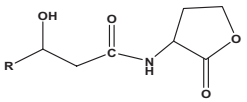
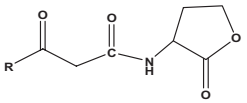
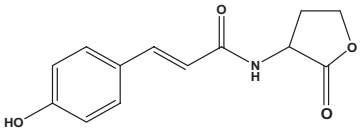
Table 1. The basic structure and name of HSLs.

Basic structure	Remarks
	When R is CH_3-, the substance referred to as C_4-HSL
	When R is CH_3-, the substance referred to as C_4-hydroxy-HSL
	When R is CH_3-, the substance referred to as C_4-oxo-HSL
	The putative new class of HSL, pC-HSL

these functions are specifically related to the structure of HSLs.

To elucidate the structure of HSLs, HSLs have to be isolated and purified from other molecules. Scientists have successfully developed a variety of analytical methods to characterize HSLs as well. Recently, two papers (Fekete *et al*., 2007; Kumari *et al*., 2008) have reviewed some methods used in their experiments. However, none of them reviewed the methods as an actual process. In this paper, we review most of the methods that have been developed to isolate, purify and characterize HSLs one by one, and a flow chart of these methods is shown in Fig. 1.

Extraction of HSLs

To obtain enough material to perform characterization studies, signal molecules should be extracted from bacterial culture supernatants. The difficulties in extraction of HSLs arise from the fact that there are many components present in the cell culture supernatants. The components are from the growth media and extracellular products produced by bacterial cells. To reduce the concentration of extracellular products, stationary cell growth phase is recommended for extraction. Liquid–liquid extraction (LLE) and solid-phase extraction (SPE) are the two most commonly used methods to isolate HSLs.

LLE

Liquid–liquid extraction is the most mature and commonly used method for extraction of HSLs (Brelles-Marino and Eulogio, 2001), which uses organic solvents as extraction reagents. After extraction, these solvents will be removed by rotary evaporation, and the residues will be dissolved in high-performance liquid chromatography (HPLC)-grade acetonitrile (ACN) for further analysis. The solvents commonly used include dichloromethane (McClean *et al*., 1997; Chambers *et al*., 2005), chloroform (Schripsema *et al*., 1996), ethyl acetate (Pearson *et al*., 1995), ethyl ether (Pearson *et al*., 1994) and hexane. Previous studies show that the first two solvents produce the best extraction results with nearly the same yields (Morin *et al*., 2003), and the extraction yield is not dependent on the medium used but, as expected, the yield decreases with an increase in the polarity of the HSLs (Morin *et al*., 2003).

In general, cell supernatants (1 l) in the stationary-phase cultures are extracted by two equal volumes of acidified ethyl acetate (0.5% acetic acid) for three times. The solvent is removed by rotary evaporation (40–45°C) and the residue is resuspended in 1 ml 20% ACN. The HSLs samples are stored at −20°C before analysis.

SPE

Another robust extraction method is based on SPE (Gould *et al*., 2006), which can also be used for additional pre-concentration. SPE is not common method, but Li and colleagues (2006) highlighted that some solid phases may improve the sensitivity by twofold to 10-fold comparing with LLE. Before application to the SPE column, the tested samples are often extracted with organic solvents, such as methanol, and acidified ethyl acetate (0.1% acetic acid) (Schupp *et al*., 2005). The extract then is evaporated to dryness. The residue is then redissolved in hexane–ethyl acetate (95:5) (Schupp *et al*., 2005) or acid acetonitrile (2% formic acid) (Frommberger *et al*., 2005). SPE column with different solid phases: silica, basic

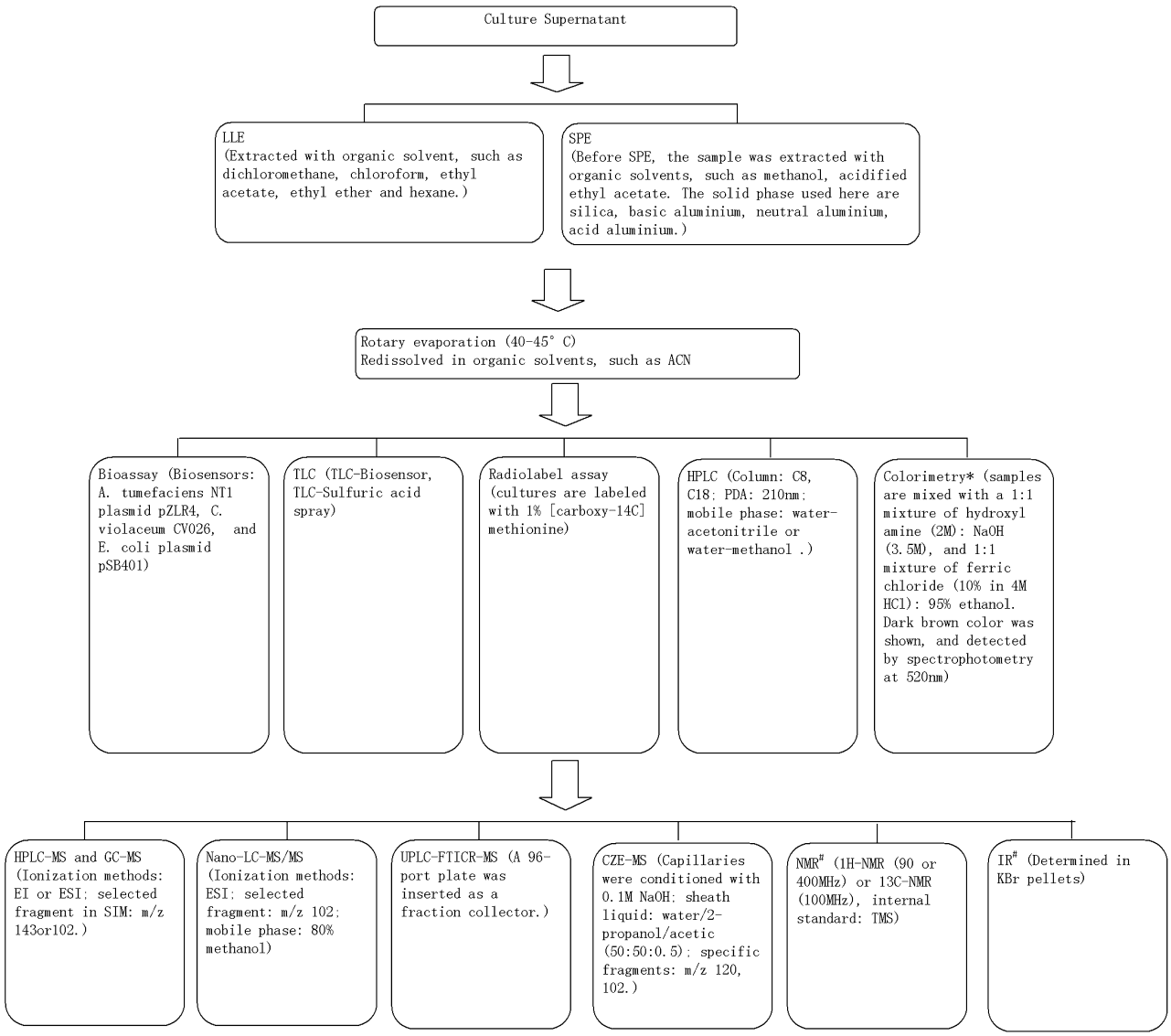


Fig. 1. The flow chart of extraction, detection, purification and identification of quorum sensing molecules. The methods with * can not be used for purification, and the methods with # are usually used to detect pure chemically synthesized HSLs.

aluminium, neutral aluminium, acid aluminium, has been used for extraction of HSLs. The column will be washed first with one column volume of 50 mM ammonium acetate buffer in 10% methanol at pH 7 and then with one column volume absolute methanol before loading the samples. Fractions are sequentially eluted from the column with additional organic solvents, such as ACN, which are used for redissolving samples.

Purification, detection and quantification of HSLs

Several methods have been developed for purification, detection and quantification of the extracts. Each method has its own advantages and disadvantages. A display of methods are discussed in the following text.

Biosensors for detection of HSLs

Bacterial sensors have been widely used to detect the presence of HSLs in the QS system (Steindle and Venturi, 2007). These biosensors can not produce purified HSLs but usually have detectable phenotypes (Andersen *et al.*, 2001) – such as light emission, expression of β-galactosidase activity or production of pigments (Milton *et al.*, 1997) upon addition of exogenous active HSLs.

The representative biosensors are *Agrobacterium tumefaciens* NT1 plasmid pZLR4 (Farrand *et al.*, 2002),

Chromobacterium violaceum CV026 (McClean *et al*., 1997; Milton *et al*., 2001) and *Escherichia coli* plasmid pSB401 (Winson *et al*., 1998a). *Agrobacterium tumefaciens* NT1 strain harbours a plasmid with a *lacZ* fusion to *traG*, a gene that requires the transcriptional activator TraR and a HSL for expression. This strain does not produce its own signal molecule, but it can induce the *traG::lacZ* reporter when supplied with an exogenous active HSL. A blue zone will be yielded at the locations to which the HSL migrates on the plate, resulting from hydrolysis of the Xgal in the medium by the β-galactosidase expressed from the *traG::lacZ* reporter fusion. The reporter can detect HSLs with 3-oxo-, 3-hydroxy- and 3-unsubstituted side-chains of all lengths except of C_4-hydroxy-HSL (see Table 1). The Rf (rate of flow) value and shape of spots in thin-layer chromatography (TLC) can be used to determine the length of side-chain and the substitution of carbon 3 of HSL molecules. The 3-oxo derivatives characteristically produce tailing spots with diffuse edges, whereas the 3-unsubstituted forms produce circular spots with sharp edges. The 3-hydroxy-substituted compounds migrate with the same mobility as their 3-oxo analogues, but the spots do not tail (Shaw *et al*., 1997).

Chromobacterium violaceum CV026 is a violacein and HSLs-negative double miniTn5 mutant. One transposon is inserted into the *cvil* HSL synthase gene, and the other is inserted into a putative violacein repressor locus. Exposure of strain CV026 to exogenous HSLs, which are able to interact with CviR, results in rapid production of a visually clear purple pigmentation. Unsurprisingly, the most active agonist HSL for CV026 is C_6-HSL, the natural *C. violaceum* HSL; other HSLs that are reasonably well induced include C_6-3-oxo-HSL and C_8-HSL (both sixfold less active than C_6-HSL), C_8-3-oxo-HSL (11-fold less active) and C_4-HSL (30-fold less active) (Steindle and Venturi, 2007).

The plasmid pSB401, based on *E. coli* harbouring the *luxCDABE* operon of *Photorhabdus luminescen*, produces bioluminescence, which can be used to differentiate structure of HSLs. The plasmid construct is based on LuxR of *Vibrio fischeri* and cognates *luxI* promoter controlling *luxCDABE* expression (Winson *et al*., 1998b). They are most sensitive to cognate C_6-3-oxo-HSL while display relatively good sensitivity towards other HSLs, such as C_6-HSL, C_8-3-oxo-HSL and C_8-HSL.

As one biosensor can only detect a narrow range of HSLs, more than one kind of biosensors will be needed to test a spectrum of HSLs from one bacterium. *Chromobacterium violaceum* CV026 is used to detect HSLs with acyl chains of C_4 to C_8 in length. Plasmid sensor pSB1075 (Winson *et al*., 1998a) based on *E. coli* can detect C_{10}-HSL, C_{12}-HSL and their 3-oxo derivatives. *Agrobacterium tumefaciens* NT1 (pZLR4) can, however, detect a broad range of HSLs, including 3-oxo-HSLs with side-chains ranging from C_4 to C_{12}, 3-unsubstituted-HSLs with side-chains from C_6 to C_{12} and 3-hydroxy-HSLs with side-chains from C_8 to C_{10}. The plasmid pHV2001$^-$ based on LuxR of *V. fischeri* has the same detective capacity as pSB401, but it can be quite sensitive to HSLs at very low concentrations (Ralling *et al*., 1985). The plasmid pSB536, which is constructed by the *ahyR* of *Aeromonas hydrophyla* and the cognate *ahyI* gene promoter fused to luxCDABE (Swift *et al*., 1997), is sensitive to C_4-HSL. Another specific sensor, which contains two plasmids: pSF105 and pSF107, is developed based on the PhzI/R HSL QS system of *Pseudomonas fluorescens* 2–79 (Khan *et al*., 2005). It can be used for detecting 3-hydroxy-HSLs, such as C_6-3-hydroxy-HSL, C_8-3-hydroxy-HSL and C_{10}-3-hydroxy-HSL. Except for the above-mentioned biosensors, new biosensors have been developed for the detection of HSLs. The biosensors developed so far are summarized and listed in Table 2.

There are two ways of using biosensors: by spotting colonies (Yan *et al*., 2007) and TLC, which will be introduced in details below. The first method is often carried out as follows: culture supernatants or sample extracts are added onto an overlay of the sensor grown on a suitable medium (usually a soft agar). After overnight incubation, a coloured zone around the site of the spot will appear due to the presence of HSLs.

Biosensor technique is a convenient, fast and effective tool for detecting the presence of HSLs. However, it can not determine the precise structure and the concentration of HSLs. In addition, biosensors are not always effective when working *in vivo*. For example, several bacterial strains have been shown to produce HSL molecules, while normal biosensors yield negative results.

TLC

For isolation and purification of the extracts, two types of TLC have been frequently used. One is coupled with biosensors, and the other is done by using sulfuric acid as chromic agent.

TLC biosensors. The theory of this method has been described previously (Shaw *et al*., 1997). TLC coupled with biosensors produces a straightforward visual index of the HSLs secreted by the test bacteria. This index can be used for detection and quantification of signal molecules. The active spot on TLC can be scraped off and extracted with dichloromethane or ethyl acetate. The extract is concentrated for further analysis, for example, most often mass spectrometry (MS) (Brelles-Marino and Eulogio, 2001).

The operational procedure is described in the following. HSLs extracted from pure bacterium cultures and

Table 2. HSL biosensors.

Biosensor	Source	Based on QS system	Reporter system	HSL(s) detected	Reference
CV026	*C. violaceum*	CviI/R	Violacein pigment	C_6-3-oxo-HSL C_8-HSL C_8-3-oxo-HSL C_4-HSL	McClean *et al*., 1997
pSB401	*E. coli*	LuxI/R	*luxCDABE*	C_6-HSL C_8-3-oxo-HSL C_8-HSL	Winson *et al*., 1998b
pHV2001–	*E. coli*	LuxI/R	*luxCDABE*	C_6-AHL C_8-3-oxo-HSL C_8-HSL	Ralling *et al*., 1985
pZLR4	*A. tumefaciens* NT1	TraI/R	β-galactosidase	All 3-oxo-HSL(s) C_6~C_{14}-HSL C_6~C_{10}-3-hydroxy-HSL	Farrand *et al*., 2002
pCF218+ pCF372	*tumefaciens* WCF47	TraI/R	β-galactosidase	As above with more sensitivity	Yan *et al*., 2007
pJZ384+ pJZ410+ pJZ372	*A. tumefaciens* KYC55	TraI/R	β-galactosidase	As above with more sensitivity	Huang *et al*., 2003
pSB403	*V. fisheri*	LuxI/R	*luxCDABE*	C_6-AHL C_8-3-oxo-HSL C_8-HSL	Riedel *et al*., 2001
pSB536	*E. coli*	AhyI/R	*luxCDABE*	C_4-HSL	Swift *et al*., 1997
pAL101	*E. coli*	RhlI/R	*luxCDABE*	C_4-HSL	Kuo *et al*., 1996
pSB1075	*E. coli*	LasI/R	*luxCDABE*	C_{10}-3-oxo-HSL C_{12}-HSL	Winson *et al*., 1998a
pSB406	*P. aeruginosa*	RhlI/R	*luxCDABE*	C_4~C_{14}-3-oxo-HSL C_4~C_{12}-3-HSL	Winson *et al*., 1998a
pKR-C12	*P. aeruginosa*	LasI/R	*gfp*	C_{10}~C_{14}-3-oxo-HSL C_{10}~C_{12}-HSL	Shaefer *et al*., 1996
pAS-C8	*B. cepacia*	CepI/R	*gfp*	C_{10}~C_{12}-3-oxo-HSL C_6~C_{12}-HSL	Shaefer *et al*., 1996
pJBA89	*V. fisheri*	LuxI/R	*gfp*	C_6~C_{14}-3-oxo-HSL C_6~C_{12}-HSL	Scott, 2007
pJBA-132	*V. fisheri*	LuxI/R	*gfp*	C_6~C_{10}-HSL	Scott, 2007

standard samples are loaded onto a TLC plate. The plate is often a C_{18} reversed-phase one (Steindle and Venturi, 2007). After the solvents in the HSLs samples are completed removed, the plate is placed upright in a glass chamber containing a developing liquid mixture: methanol–water. The sample spots are normally well above the liquid surface (Llamas *et al*., 2005; Yang *et al*., 2006). The HSLs samples migrate at different rates due to the difference in their polarity. Therefore, a range of HSLs can be separated along the migration path on the TLC plate. The TLC plate is then overlaid with a soft agar containing a HSL biosensor strain, and is incubated at 30°C for 16 h (Winson *et al*., 1998a). The light or colour will be emitted on the spot where HSL is present. Quantification is performed by the measurement of violacein production or β-galactosidase activity. The area of spots is converted into residual concentration of HSLs via a calibration curve obtained from the standard synthetic HSLs. A good linear relationship has been found for a range of HSLs and the reproducibility is also demonstrated.

Thin-layer chromatography coupled with biosensor produces a direct and visual image of the HSL signal molecules presented in supernatants of bacterial cultures. The assay method is quite sensitive, generally requiring no more than 5 ml extracts from cultures, although the volume of culture broth for HSL isolation should be more than 500 ml. Moreover, it is simple, reasonably fast, amenable to process many samples in one run and does not require elaborate or expensive instrumentation. It takes only 2–3 days to assay HSL signal molecules secreted from a bacterium. However, the assay technique must be used with caution. First, the technique can not reveal specific side-chain lengths or substitutions of HSLs. Second, the signal molecules must be present at a level detectable by the reporter (Kuo *et al*., 1996; Shaefer *et al*., 1996). Finally, the conclusion that the tested bacterium does not produce one or more HSLs can not be drawn, basing only on the absence of positive responses by the biosensors. The absence can be either credited to lower concentration of signal molecules from such organisms that are not detectable by the reporter. There also exists a high possibility on cross-talking by the matrices causing false positive identification. Therefore, the assay can be used for a minimum estimate of the complement of signal molecules produced by a given microorganism.

Sulfuric acid spray method. Thin-layer chromatography can also be used detection of HSLs via simply modified sulfuric acid method (Scott, 2007), which can detect most of involatile organic compounds. The TLC plate is loaded with sample extracts and different standards. After leaving a few minutes to dry the samples and standards on the plate, the plate is sprayed with a 5% w/v potassium dichromate solution in a 40% v/v aqueous sulfuric acid solution. The plate is then heated for about 10 min at around 200°C. The solutes are partially oxidized and a distinguished charred deposit of black carbon is left intact. However, this detection method is destructive to HSL products and HSLs cannot be recovered after detection, so it cannot be used for separation or purification of HSLs.

The sulfuric acid spray technique can allow a complete separation of a range of HSLs with chains in different lengths in a very short time. The chromatographic property of HSLs, Rf, can be used to reveal preliminary information about the kinds of HSLs and structure of the compounds present in the supernatant of bacterial cultures. By comparing Rf values for the samples with those of HSL standards, the structure information of HSLs can be determined preliminarily. However, structures of HSLs can not be determined based on this chromatographic property alone.

Radiolabelled assay

The above biosensor-based assay methods can not be applied for detecting HSLs produced by biofilm or non-planktonic cultures because the concentration of HSLs usually is low and the biofilm makes the extraction of HSLs difficult. A new radiolabelled assay is developed (Schaefer *et al*., 2000). The method is based on the uptake of radiolabelled methionine by living cells and the incorporation of the radiolabelled molecule into HSLs. The test bacterium is first grown in the methionine-free medium to the early stationary phase, and then 1% (v/v) [carboxy-^{14}C] methionine is incubated with cells for a duration of 10–30 min (Schaefer *et al*., 2001). Radiolabelled methionine will be bound onto HSLs. The newly formed HSL–methionine is extracted from the supernatant as described previously. The final sample is placed in scintillation vials, spiked with 3a70b (too specifically, may need some explanation) scintillation cocktail and counted with a scintillation detector. The counting values for the sample will be compared with those from HSL standards, and the HSL molecules in the sample can be determined.

HPLC

The extracted samples obtained from LLE or SPE are resolved in ACN or methanol. The samples are then applied to a C_8 reverse-phase preparative HPLC column (250 mm × 8 mm) eluted with an isocratic mobile phase of 70% (v/v) ACN in water (Delalande *et al*., 2005) at a flow rate of 2 ml min^{-1} and monitored at 210 nm by a PDA (photo diode-array) detector (McClean *et al*., 1997). There are other columns used in HPLC to separate HSLs as well, such as C_{18} reverse-phase semi-preparative or preparative column (250 mm × 4.6 mm) (Lewenza and Sokol, 2001; Morin *et al*., 2003). The mobile phases usually are water–ACN or water–methanol. Morin and colleagues (2003) have compared the two eluents and found that with methanol, the earliest-eluted peaks are larger, but a better separation and higher signal intensities are obtained. The eluted peaks become sharp when linear gradient of water–methanol concentration is used as the mobile phase (Pearson *et al*., 1995). It is known that the side-chains of HSLs affect the polarity, and the polarity determines the retention time of HSLs in the chromatographic column. Experiments have demonstrated that the logP value (P is the retention time) is proportional to the length of the side-chain. Therefore, the residence time for an unknown HSL molecule is obtained from the HPLC column, the length of the side-chain can be determined. Fractions for each peak in the chromatograph elution profile, representing one HSL compound, can be collected and dried by rotary evaporation. Each fraction is then dissolved in ACN or methanol for further analysis.

High-performance liquid chromatography is a competitive technique in terms of feasibility and cost-effectiveness by comparing with other complicated techniques. HPLC have been available for routine analysis in analytical laboratories. Given that the analysis is based on HSL standards, the analysis exclude other signal molecules, which are not in the category of HSLs. To compensate for the shortcomings, HPLC is often followed by MS analysis.

Colorimetry

Yang and colleagues (2006) have developed a new colorimetry method, which is simple, fast and easy to be applied for HSLs samples. This method can analyse the concentration of HSLs and the activity by using a small amount of samples (approximately 20–50 μl) in a 96-well plate. It can trace the approximately 1 nmol HSLs, which is quite comparable with the HPLC method.

This method is modified from one for analysing ester molecules (Goddu *et al*., 1955). Each sample is prepared in a 96-well plate (Yang *et al*., 2006), and then mixed with a mixture of hydroxyl amine (2 M): NaOH (3.5 M) (1:1 v/v). After mixing for a few minutes, the same amount of 1:1 (v/v) mixture of ferric chloride (10% in 4 M HCl): 95% ethanol is added into the above sample solution. A dark brown colour is produce in the samples containing HSL compounds, but in some cases it may change to yellow

colour depending on the concentrations of the compounds. The quantities of various lactones are determined by spectrophotometry at 520 nm. There is a reproducible and reliable linearity between the optical density value and the molar concentrations of HSLs. The linearity with a R^2 greater than 0.985 has been demonstrated for all HSL compounds within a concentration of 1200 nmol. However, because the optical values can be applicable for any organic compounds with an ester group, any contamination of such components in the HSL samples will produce an overestimated molar concentration of HSLs in the sample.

In summary, biosensor-based, TLC, HPLC and colorimetry are refrequently used methods to analyse HSL compounds, but each method has its own drawbacks. Biosensor-based method is the most sensitive one, down to a femtomole detection level, while it requires tailor-made strains and plasmids for each HSL molecule and takes at least 24 h to complete the analysis. TLC has a poor resolution and can only be applicable for a sample containing abundant QS molecules (if you could find the concentration level, will be more convincing, abundant means mol l^{-1} or what). HPLC is the most often used method to determine the concentration and the structure of HSLs in the sample. However, it requires a much longer time to establish the assay method as well as to process many samples. Colorimetry is an easy and convenient method for detecting HSLs, but it cannot determine the precise structure of the molecules.

Identification of HSL(s)

Although previous detection methods can reveal local information about the presence of HSLs *in situ*, they cannot provide information for a wide or complete spectrum of HSLs compounds with high sensitivity. Spectroscopic properties have been widely used to characterization of QS molecules structures. Spectroscopic methods include MS, MS/MS (Bruhn *et al*., 2004), nuclear magnetic resonance spectroscopy (NMR) and infrared spectroscopy (IR).

HPLC-MS and GC-MS

Mass spectrometry can make a record of charge–mass ratio of ionized compound. According to the molecular ion peaks and fragments, the molecular mass and structure characteristics can be estimated. MS detects even picomoles of samples and can be combined with HPLC (Holden *et al*., 1999), gas chromatography (GC) (Zhang *et al*., 1993), ultra-high-pressure liquid chromatography (UPLC) (Li *et al*., 2007a), nano-LC and capillary zone electrophoresis (CZE). The structure of HSLs can be finally determined through further analysis tools, such as NMR and IR (Zhu *et al*., 1998).

There are many types of ionization available, including electron impact (also called electron ionization, EI-MS), fast atom bombardment (FAB-MS), chemical ionization (CI-MS) (Huang *et al*., 2003), electrospray ionization (ESI-MS) (Moon, 2004), atmospheric pressure chemical ionization (APCI) and laser desorption (LD) (Zhu *et al*., 2003). ESI, EI, CI are often used to detect signal molecules. EI and ESI are common ionization methods combined with GC and HPLC respectively. Molecular ion peaks and fragments can be obtained, which can be used for structure estimation. Many quasi-molecular ion peaks can be obtained by CI, which allows determining the mass of the compound.

Most procedures have been carried out by using a combination of reversed-phase HPLC and MS for selective detection. The targets are usually separated in C_{18} silica columns by isocratic or gradient elution with water–methanol or water–ACN as mobile phases as described previously (Teplitski *et al*., 2003).

Mass spectrometry coupled with GC has been developed for determination of HSLs. Detail of the operational conditions of GC-MS has been described elsewhere (Cataldi *et al*., 2004; Cataldi *et al*., 2007). Separations are usually performed in a 20 m long fused silica capillary column with helium (He) as the carrier gas and with a temperature gradient from 100 to 300°C. Detection is performed in selected ion monitoring mode by using the prominent fragment at m/z 143 or 102 upon electron impact (EI) ionization. Therefore, quantitative characterization on HSLs can be done. Charlton and colleagues (2000) has developed a novel and sensitive method for the quantification of 3-oxo-HSLs. They convert 3-oxo-HSLs to the pentafluorobenzyloxime derivatives followed by detection and analysis by GC-MS (electron capture-negative ion). However, due to the low volatility and instability of the injector, it is more difficult to make quantification of HSLs by GC-MS than HPLC-MS.

Nano-LC-MS/MS

Nano-LC separation followed by MS-MS confirmation was firstly performed by Frommberger and colleagues (2004). Fekete and colleagues (2007) have reviewed the method in details. Briefly, fused-silica columns are connected to an ion-trap instrument via ESI sprayer. For structural confirmation, the typical fragmentation m/z 102 is used (Colon *et al*., 2000).

The separation method based on nano-LC combines characteristics of SPE and LLE on a single column (Frommberger *et al*., 2004). Gradient elution is not available for the experimental setup, so 80% methanol is found optimal for the separation of the HSLs, but the addition of acetic or formic acid does not improve the resolution. The pressure is adjusted to 15 MPa, yielding a flow rate of 300 nl min^{-1}.

The sample matrix composition is optimized, and the addition of acetic acid or formic acid to the sample improves peak sharpness, but also causes unwanted degradation of the molecules. One microlitre of sample is sufficient for analysis.

The MS parameters are also optimized. The maximum intensity of the signals in electrospray ionization MS is often a trade-off between thermal stability and adducts formation (Frommberger *et al*., 2003; Schmitt-Kopplin and Kettrup, 2003). The temperature-dependant cluster formation and degradation behaviours of the HSLs have been examined, and the results have shown that the lowest value (50°C) would be the optimum analysis temperature for HSLs with different side-chains. However, at low temperatures (< 100°C), the mass spectra show a dramatic loss in resolution due to the incomplete water removal during the electrospray ionization process. Hence, 100°C should be chosen as the capillary temperature.

These optimization procedures lead to a better analysis result of the HSLs samples. Compared with the classical HSLs analysis of bacterial cultures with biosensors, this method increases selectivity and sensitivity, and has a substantial improvement in qualitative and quantitative study on HSL molecules.

At-line coupling of UPLC to chip-elctrospray-FTICR-MS

Ultra-high-pressure liquid chromatography is based on the principle of small porous particles and ultra-high pressure. UPLC has been applied to analyse HSLs (Li *et al*., 2006), which can achieve higher resolution, better column efficiency, narrower peaks and higher speed (Neue, 1997). Fourier-transform ion-cyclotron resonance mass spectrometry (FTICR-MS) has been used to determine the HSL structure (Li *et al*., 2007b). A combination of UPLC and FTICR-MS may complete the analysis for a range of HSLs present in cell culture supernatant (Li *et al*., 2007a). However, on-line coupling of UPLC with FTICR-MS is technically infeasible, because the half-peak width in UPLC could be as low as less than 1 s, which demands a much higher detection scan rate that FTICR-MS can not offer due to the longer processing time required to achieve high sensitivity. To be able to make use of the speed efficiency of UPLC and the high resolution and mass accuracy of FTICR-MS, a 96-port plate is inserted into the autosampler of the chip-based nanoelectrospray ionization system as a fraction collector. This method buffers the time difference between the separation rate of UPLC and the data acquiring speed of FTICR-MS, which couples the two analysis methods together.

Three optimizations have been made on the conditions required for at-line coupling of UPLC to FTICR-MS. The first optimization is to insert an appropriate delay loop between the detector cell and the collector, and the delay loop length has to be optimized. The delay loop is used to compensate for the time delay, which results from the target product passing through the detector cell, and acquiring and processing data in the detector. The second optimization is to employ a gradient mode to achieve an efficient separation of the HSLs with a wide polarity range. Third, in order to collect the correct fraction, the delay time between the collecting start time (Cs) of the target component and the peak start time (ts) must be obtained firstly. The optimization enables highly efficient 'one-peak' collection, and the sampling could be automated by use of a robot.

Before UPLC analysis, the bacterial samples are extracted according to a protocol described earlier (Li *et al*., 2006). The samples are resolved in 30% ACN. The reversed-phase separation is achieved on a C_{18} column. A linear gradient of 10% ACN to 100% ACN in 1 min is used. The detection wavelength is set to 197 nm (1.2 nm width) with a scan rate of 20 Hz.

A FTICR-MS system equipped with an ESI source and a chip-based nanoelectrospray ionization system has been explored by Li and colleagues (2007a). Ionization is performed in a positive-ion mode using the standard chip. The ionization voltage is set to 1.6 kV and the backpressure is 0.5 psi. The MS electrodes, including the spray shield and the inlet end of the glass capillary, are both set to 0 V. The drying gas flow is set to 4 l s^{-1} at 200°C, and the nebulizer gas is turned off. Spectra are acquired in a broadband mode and calibrated externally on clusters of arginine. The MS settings are tuned at m/z 200–300. Broadscan spectra are acquired with a mass range of 150–2000 and with a time domain size of 1 megaword. Ions are stored in the collision cell after the quadrupole for 100 ms. Five hundred and twelve scans are accumulated for one spectrum. The resolving power is around 200 000 ppi in a relevant mass range around m/z 250.

CZE–MS

Capillary zone electrophoresis mass spectrometry is usually employed for analysis of the homoserines (HSs), which is produced by alkaline hydrolysis of HSLs (Frommberger *et al*., 2005). The carboxylic acid group of the serines enables the separation at alkaline pH because they are fully deprotonated under this pH condition. In a general procedure, the test sample containing HSLs is hydrolysed by addition of 1 M NaOH and incubated for 15 min at room temperature. The hydrolysed product is loaded onto CZE. CZE is performed with untreated fused-silica capillaries, and the capillaries are conditioned before use and before/after each day for 30 min with 0.1 M NaOH. To improve the performance of the method, C_7-HSL is used as an internal standard. Between runs, the

capillary is flushed for 3 min with 0.1 M NaOH and 3 min with running buffer (containing 20 mM ammonium carbonate and 10% 2-propanol). The capillary is coupled with an ion trap mass spectrometer, and ionization is performed with in a positive mode. To obtain a stable result, a mixture of water/2-propanol/acetic (50:50:0.5 v/v/v) is used as the sheath liquid. The specific fragments for HSLs are m/z 120 and 102. This CZE-MS method has advantages over recently published capillary separation techniques (nano-LC-MS, GC-MS) and provides a way to quantitatively differentiate between the HSs (natural degradation products of HSLs) and the intact homoserine lactones.

NMR

Nuclear magnetic resonance spectroscopy is very useful to elucidate organic structures. The hydrogen and the carbon in an organic structure resonate at different chemical shifts, depending upon their environment, and further appear as singlet, doublet, triplet etc. by coupling to the neighbouring nuclei. NMR has been used to determine many kinds of signal molecules, such as HSL (Bainton *et al*., 1992; Pearson *et al*., 1994; Brelles-Marino and Eulogio, 2001), DKP (Holden *et al*., 1999) etc. However, the detection limit of NMR is much higher than the concentration of signal molecule in the bacteria supernatant, and NMR used to detect chemically synthesized pure signal molecules. This greatly limits the application of NMR in the identification of HSLs from bacterial supernatant. In recent years, advances in the analytical methods have allowed NMR spectroscopy in combination with MS to be used in an expanding field (Pan and Raftery, 2007). ^{1}H-NMR (90 and 400 MHz) (Chhabra *et al*., 1992; Winson *et al*., 1995) and ^{13}C-NMR (100 MHz) (Moon, 2004) spectra are measured on a Varian EM390 or Bruker AM-400 spectrometer and are recorded in C^2HCl_3 or $CDCl_3$ with tetramethylsilane as the internal standard except for the compounds dissolved in D_2O with sodium 3-(trimethylsilyl)propane sulfonate as an external standard. The chemical shifts and integrated peak areas of the natural HSLs are compared with the synthetic chemicals.

IR

The IR spectrometry is useful for the identification of the functional groups in a molecule and can be used in combination with other techniques to precisely determine chemical structures. IR spectra can be obtained in KBr pellets on a Perkin-Elmer 257 spectrophotometer. IR spectrometry has been used to identify functional groups in the conjugation factor (C_8-oxo-HSL) from *A. tumefaciens* (Zhang *et al*., 1993). The result shows amide I and II carbonyl absorptions at 1646 and 1540 cm^{-1} as well as characteristic absorptions near 1780 and 1714 cm^{-1} assignable to the presence of γ-lactone and ketonic carbonyls. IR has been commonly used for identifying synthetic HSLs.

Concluding remarks

Detection and characterization of HSL signal molecules are of vital importance for understanding cell-to-cell communication. Subtle differences in molecular structure and composition as well as ultra-low concentration of HSLs in the cell culture supernatant pose significant challenges for traditional analysis techniques. Analytical procedures including biosensor-based and chromatographic techniques for the structural determination of HSLs are reviewed in this paper. Although several methods with either high sensitivity or high selectivity have been developed for analysis of HSLs, few studies have investigated the matrix effects and the performance of a method. Our group is currently working in this niche area. In addition to the traditional methods for detection of HSLs, new techniques have been developed, including FTICR-MS, nano-LC-MS, MS-MS and so on. These new techniques will be further improved and novel techniques will be explored as well. The efforts will greatly contribute to help unveil the mechanisms of QS in the bacterial community.

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References

Andersen, J.B., Heydorn, A., Hentzer, M., Eberl, L., Geisenberger, O., Christensen, B.B., *et al*. (2001) Gfp-based *N*-acyl homoserine-lactone sensor systems for detection of bacterial communication. *Appl Environ Microbiol* **67:** 575–585.

Bainton, N.J., Bycroft, B.W., Chhabra, S.R., Stead, P., Gledhill, L., Hill, P.J., *et al*. (1992) A general role for the *lux* autoinducer in bacterial cell signaling control of antibiotic biosynthesis in *Erwinia*. *Gene* **116:** 87–91.

Brelles-Marino, G., and Eulogio, J.B. (2001) Detection, purification and characterisation of quorum-sensing signal molecules in plant-associated bacteria. *J Biotechnol* **91:** 197–209.

Bruhn, J.B., Christensen, A.B., Flodgaard, L.R., Nielsen, K.F., Larsen, T.O., Givskov, M., and Gram, L. (2004) Presence of acylated homoserine lactone (AHLs) and AHL-producing bacteria on meat and potential role of AHL in spoilage of meat. *Appl Environ Microbiol* **70:** 4293–4302.

Camilli, A., and Bassler, B.L. (2006) Bacterial small-molecule signaling pathways. *Science* **311:** 1113–1116.

Cataldi, T.R.I., Bianco, G., Frommberger, M., and Schmitt-Kopplin, P. (2004) Direct analysis of selected *N*-acyl-L-homoserine lactones by gas chromatography/mass spectrometry. *Rapid Commun Mass Spectrom* **18:** 1341–1344.

Cataldi, T.R.I., Bianco, G., Palazzo, L., and Quaranta, V. (2007) Occurrence of *N*-acyl-L-homoserine lactones in extracts of some Gram-negative bacteria evaluated by gas chromatography-mass spectrometry. *Anal Biochem* **361:** 226–235.

Chambers, C.E., Visser, M.B., Schwab, U., and Sokol, P.A. (2005) Identification of *N*-acylhomoserine lactones in mucopuru respiratory secretions from cystic fibrosis patients. *FEMS Microbiol Lett* **244:** 297–304.

Charlton, T.S., Nys, R., Netting, A., Kumar, N., Hentzer, M., Givskov, M., and Kjelleberg, S. (2000) A novel and sensitive method for the quantification of *N*-3-oxoacyl homoserine lactones using gas chromatography-mass spectrometry: application to a model bacterial biofilm. *Environ Microbiol* **2:** 530–541.

Chhabra, S.R., Stead, P., Bainton, N.J., Salmond, G.P.C., Stewart, G.S.A.B., Williams, P., and Bycroft, B.W. (1992) Autoregulation of carbapenem biosynthesis in *Erwinia carotovora* by analogues of *N*-(3-oxo-hexanoyl)-L-homoserine lactone. *J Antibiot* **46:** 441–454.

Christopher, M., and Bassler, B.L. (2005) Quorum sensing: cell-to-cell communication in bacteria. *Annu Rev Cell Dev Biol* **21:** 319–346.

Colon, L.A., Maloney, T.D., and Fermier, A.M. (2000) Packing columns for capillary electrochromatography. *J Chromatogr A* **887:** 43–53.

Davies, D.G., Parsek, M.R., Pearson, J.P., Iglewski, B.H., Costerton, J.W., and Greenberg, E.P. (1998) The involvement of cell-to-cell signals in the development of a bacterial biofilm. *Science* **280:** 295–298.

Delalande, L., Faure, D., Raffoux, A., Uroz, S., D'Angelo-Picard, C., Elasri, M., *et al*. (2005) *N*-hexanoyl-L-homoserine lactones, a mediator of bacterial quorum-sensing regulation, exhibits plant-dependent stability and may be inactivated by germinating *Lotus corniculatus* seedlings. *FEMS Microbiol Ecol* **52:** 13–20.

Eberl, L., Winson, M.K., Sternberg, C., Stewart, G.S.A., Christiansen, G., Chhabra, S.R., *et al*. (1996) Involvement of *N*-acyl-homoserine lactone autoinducers in controlling the multicellular behaviour of *Serratia liquefaciens*. *Mol Microbiol* **20:** 127–136.

Farrand, S.K., Qin, Y., and Oger, P. (2002) Quorum-sensing system of *Agrobacterium* plasmids: analysis and utility. *Methods Enzymol* **358:** 452–484.

Fekete, A., Frommberger, M., Rothballer, M., Li, X.J., Englmann, M., Fekete, J., *et al*. (2007) Identification of bacterial *N*-acylhomoserine lactones (AHLs) with a combination of ultra-performance liquid chromatography (UPLC), ultra-high-resolution mass spectrometry, and in-situ biosensors. *Anal Bioanal Chem* **387:** 455–467.

Frommberger, M., Schmitt-Kopplin, P., Menzinger, F., Albrecht, V., Schmid, M., Eberl, L., *et al*. (2003) Analysis of *N*-acyl-L-homoserine lactones produced by *Burkholderia cepacia* with partial filling micellar electrokinetic chromatography-electrospray ionization-ion trap mass spectrometry. *Electrophoresis* **24:** 3067–3074.

Frommberger, M., Schmitt-Kopplin, P., Ping, G., Frisch, H., Schmid, M., Zhang, Y., *et al*. (2004) A simple and robust set-up for on-column sample preconcentration-nano-liquid chromatography-electrospray ionization mass spectrometry for the analysis of *N*-acylhomoserine lactones. *Anal Bioanal Chem* **378:** 1014–1020.

Frommberger, M., Hertkorn, N., Englmann, M., Jakoby, S., Hartmann, A., Kettrup, A., and Schmitt-Kopplin, P. (2005) Analysis of *N*-acylhomoserine lactones after alkaline hydrolysis and anion-exchange solid-phase extraction by capillary zone electrophoresis-mass spectrometry. *Electrophoresis* **26:** 1523–1532.

Fuqua, W.C., and Winans, S.C. (1994) A LuxR-LuxI type regulatory system activates *Agrobacterium* Ti plasmid conjugal transfer in the presence of a plant tumour metabolite. *J Bacteriol* **176:** 2796–2806.

Goddu, R.F., Leblanc, N.F., and Wright, C.M. (1955) Spectrophotometric determination of festers and anhydrides by hydroxamic acid reaction. *Anal Chem* **27:** 1251–1255.

Gould, T.A., Herman, J., Krank, J., Murphy, R.C., and Churchill, M.E.A. (2006) Specificity of acyl-homoserine lactone synthases examined by mass spectrometry. *J Bacteriol* **188:** 773–783.

Hammer, B.K., and Bassler, B.L. (2007) Regulatory small RNAs circumvent the conventional quorum sensing pathway in pandemic *Vibrio cholerae*. *Proc Natl Acad Sci USA* **104:** 11145–11147.

Holden, M.T.G., Chhabra, S.R., Nys, R., Stead, P., Bainton, N.J., Hill, P.J., *et al*. (1999) Quorum sensing cross talk: isolation and Chemical characterization of cyclic dipeptides from *Pseudomonas aeruginosaand* other Gram-negative bacteria. *Mol Microbiol* **33:** 1254–1266.

Houdt, R.V., Moons, P., Aertsen, A., Jansen, A., Vanoirbeek, K., Daykin, M., *et al*. (2007) Characterization of a luxI/luxR-type quorum sensing system and *N*-acyl-homoserine lactone-dependent regulation of exo-enzyme and antibacterial component production in *Serratia plymuthica* RVH1. *Res Microbiol* **158:** 150–158.

Huang, J.J., Han, J.I., Zhang, L.H., and Leadbetter, J.R. (2003) Utilization of acyl-homoserine lactone quorum signals for growth by a soil *Pseudomonad* and *Pseudomonas aeruginosa* PAO1. *Appl Environ Microbiol* **69:** 5941–5949.

Khan, S.R., Mavrodi, D.V., Jog, G.J., Suga, H., Thomashow, L.S., and Farrand, S.K. (2005) Activation of the phz operon of *Pseudomonas fluorescens* 2–79 requires the LuxR homolog PhzR, N-(3-OH-hexanoyl)-L-homoserine lactone produced by the LuxI homolog PhzI, and a *cis*-acting *phz* box. *J Bacteriol* **187:** 6517–6527.

Kumari, A., Pasini, P., and Daunert, S. (2008) Detection of bacterial quorum sensing *N*-acyl homoserine lactones in clinical samples. *Anal Bioanal Chem* **391:** 1619–1627.

Kuo, A., Callahan, S.M., and Dunlap, P.V. (1996) Modulation of luminescence operon expression by *N*-octanoyl-L-homoserine lactone in *ainS* mutants of *Vibrio fischeri*. *J Bacteriol* **178:** 971–976.

Lewenza, S., and Sokol, P.A. (2001) Regulation of ornibactin biosynthesis and *N*-acyl-L-homoserine lactone production by CepR in *Burkholderia cepacia*. *J Bacteriol* **183:** 2212–2218.

Li, X.J., Fekete, A., Englmann, M., Götz, C., Rothballer, M., Frommberger, M., *et al*. (2006) Development and application of a method for the analysis of *N*-acylhomoserine lactones by solid-phase extraction and ultra high pressure liquid chromatography. *J Chromatogr A* **1134:** 186–193.

Li, X.J., Fekete, A., Englmann, M., Frommberger, M., Lv, S., Chen, G., and Schmitt-Kopplin, P. (2007a) At-line coupling of UPLC to chip-electrospray-FTICR-MS. *Anal Bioanal Chem* **389:** 1439–1446.

Li, X.J., Fekete, J., Fekete, A., Englmann, M., Yang, F., Chen, G., and Schmitt-Kopplin, P. (2007b) Optimization of gradient elution in UPLC: a core study on the separation of homoserine lactones produced by *Bukholderia ubonensis* and structure confirmation with ultra high resolution mass spectrometry. *J Liq Chromatogr Relat Technol* **30:** 2515–2531.

Lithgow, J.K., Wilkinson, A., Hardman, A., Rodelas, B., Wisniewski-Dye, F., Williams, P., and Downie, J.A. (2000) The regulatory locus *cinRI* in *Rhizobium leguminosarum* controls a network of quorum-sensing loci. *Mol Microbiol* **37:** 81–97.

Llamas, I., Quesada, E., Martinez-Canovas, M., Gronquist, M., Eberhard, A., and Gonzalez, J.E. (2005) Quorum sensing in halophilic bacteria: detection of *N*-acylhomoserine lactones in the exopolysaccharide-producing species of *Halomonas*. *Extremophiles* **9:** 333–341.

McClean, K.H., Winson, M.K., Fish, L., Taylor, A., Chhabra, S.R., Camara, M., *et al*. (1997) Quorum sensing and *Chromobacterium violaceum*: exploitation of violacein production and inhibition for the detection of *N*-acylhomoserine lactones. *Microbiology* **143:** 3703–3711.

Milton, D.L., Hardman, A., Camara, M., Chhabra, S.R., Bycroft, B.W., Stewart, G.S.A., and Williams, P. (1997) Quorum sensing in *Vibrio anguillarum:* characterization of the *vanI/vanR* locus and identification of the autoinducer N-(3-oxodecanoyl)-L-homoserine lactone. *J Bacteriol* **179:** 3004–3012.

Milton, D.L., Chalker, V.J., Kirke, D., Hardman, A., Camara, M., and Williams, P. (2001) The LuxM homoserine VanM from *Vibrio anguillarum* directs the synthesis of N-(3-hydroxyhexanoyl) homoserine lactone and N-*h*exanoylhomoserine lactone. *J Bacteriol* **183:** 3537–3547.

Moon, H.S. (2004) Identification of homoserine lactone derivatives using the methionine functionalized soild phase synthesis by gas chromatography/mass spectrometry. *Arch Pharm Res* **27:** 25–30.

Morin, D., Grasland, B., Vallee-Rehel, K., Dufau, C., and Haras, D. (2003) On-line high-performance liquid chromatography-mass spectrometric detection and quantification of *N*-acylhomoserine lactones, quorum sensing signal molecules, in the presence of biological matrices. *J Chromatogr A* **1002:** 79–92.

Nealson, K.H., and Hastings, J.W. (1979) Bacterial bioluminescence: its control and ecological significance. *Microbiol Rev* **43:** 496–518.

Neue, U.D. (1997) *HPLC Columns: Theory, Technology, and Practice*. New York, NY, USA: Wiley–VCH.

Palmer, A.G., and Blackwell, H.E. (2008) Deciphering a protolanguage for bacteria-host communication. *Nat Chem Biol* **4:** 452–454.

Pan, Z.Z., and Raftery, D. (2007) Comparing and combining NMR spectroscopy and mass spectrometry in metabolomics. *Anal Bioanal Chem* **387:** 525–527.

Pearson, J.P., Gray, K.M., Passador, L., Tucker, K.D., Eberhard, A., Iglewski, B.H., and Greenberg, E.P. (1994) Structure of the autoinducer required for expression of *Pseudomonas aeruginosa* virulence genes. *Proc Natl Acad Sci USA* **91:** 197–201.

Pearson, J.P., Passador, L., Iglewski, B.H., and Greenberg, E.P. (1995) A second *N*-acylhomoserine lactone signal produced by *Pseudomonas aeruginosa*. *Proc Natl Acad Sci USA* **92:** 1490–1494.

Ralling, G., Bodrug, S., and Linn, T. (1985) Growth rate-dependent regulation of RNA polymerase synthesis in *Escherichia coli*. *Mol Gen Genet* **201:** 379–386.

Riedel, K., Hentzer, M., Geisenberger, O., Huber, B., Steidle, A., Wu, H., *et al*. (2001) *N*-acylhomoserine-lactone-mediated communication between *Pseudomonas aeruginosa* and *Burkholderia cepacia* in mixed biofilms. *Microbiology* **147:** 3249–3262.

Salmond, G.P.C., Bycroft, B.W., Stewart, G.S.A.B., and Williams, P. (1995) The bacterial 'enigma': cracking the code of cell-cell communication. *Mol Microbiol* **16:** 615–624.

Schaefer, A.L., Hanzelka, B.L., Parsek, M.R., and Greenberg, E.P. (2000) Detection, purification, and structural elucidation of the acylhomoserine lactone inducer of *Vibrio fischeri* luminescence and other related molecules. *Methods Enzymol* **305:** 288–301.

Schaefer, A.L., Greenberg, E.P., and Parsek, M.R. (2001) Acylated homoserine lactone detection in *Pseudomonas aeruginosa* biofilms by radiolabel assay. *Methods Enzymol* **336:** 41–47.

Schaefer, A.L., Greenberg, E.P., Oliver, C.M., Oda, Y., Huang, J.J., Banin, G.B., *et al*. (2008) A new class of homoserine lactone quorum-sensing signals. *Nature* **454:** 595–599.

Schmitt-Kopplin, P., and Kettrup, A. (2003) Capillary electrophoresis-electrospray ionization-mass spectrometry for the characterization of natural organic matter: an evaluation with free flow electrophoresis-off-line flow injection electrospray ionization-mass spectrometry. *Electrophoresis* **24:** 3057–3066.

Schripsema, J., Rudder, K.E.E., Vliet, T.B.V., Lankhorst, P.P., Vroom, E.D., Kijne, J.W., and Brussel, A.A.N.V. (1996) Bacteriocin small of *Rhizobium leguminosarum* belongs to the class of *N*-acyl-L-homoserine lactone molecules, known as autoinducers and as quorum sensing co-transcription factors. *J Bacteriol* **178:** 366–371.

Schupp, P.J., Charlton, T.S., Taylor, M.W., Kjelleberg, S., and Steinberg, P.D. (2005) Use of solid-phase extraction to enable enhanced detection of acyl homoserine lactones (AHLs) in environmental samples. *Anal Bioanal Chem* **383:** 132–137.

Scott, R.P.W. (2007) *Thin layer chromatography* [WWW document]. URL http://www.chromatography-online.org/TLC/Detection-and-Quantitatitation/Sulfuric-Acid-Spray.html

Shaefer, A.L., Hanzelka, B.L., Eberhard, A., and Greenberg, E.P. (1996) Quorum sensing in *Vibrio fischeri*: probing autoinducer-LuxR interaction with autoinducer analogs. *J Bacteriol* **178:** 2897–2901.

Shaw, P.D., Ping, G., Daly, S.L., Cha, C., Cronan, J.E., Rinehart, K.L., and Farrand, S.K. (1997) Detecting and characterizing *N*-acyl-homoserine lactone signal molecules by thin layer chromatography. *Biochem J* **94:** 6036–6041.

Steindle, L., and Venturi, V. (2007) Detection of quorum-sensing *N*-acyl homoserine lactone signal molecules by bacterial biosensors. *FEMS Microbiol Lett* **266:** 1–9.

Swift, S., Karlyshev, A.V., Fish, L., Durant, E.L., Winson, M.K., Chhabra, S.R., *et al*. (1997) Quorum sensing in *Aeromonas hydrophila* and *Aeromonas salmonicida*: identification of the LuxRI homologs AhyRI and AsaRI and their cognate *N*-acylhomoserine lactone signal molecules. *J Bacteriol* **179:** 5271–5281.

Teplitski, M., Eberhard, A., Gronquist, M.R., Gao, M., Robinson, J.B., and Bauer, W.D. (2003) Chemical identification of *N*-acyl homoserine lactone quorum-sensing signals produced by *Sinorhizobium meliloti* strains in defined medium. *Arch Microbiol* **180:** 494–497.

Thomson, N.R., Crow, M.A., McGrowan, S.J., Cox, A., and Salmond, G.P.C. (2000) Biosynthesis of carbapenem antibiotic and prodignisin pigment in *serratia* is under quorum sensing control. *Mol Microbiol* **36:** 539–556.

Wagner-Döbler, I., Thiel, V., Eberl, L., Allgaier, M., Bodor, A., Meyer, S., *et al*. (2005) Discovery of complex mixtures of novel long-chain quorum sensing signals in free-living and host-associated marine alphaproteo bacteria. *Chembiochem* **6:** 2195–2206.

Williams, P. (2007) Quorum sensing, communication and cross-kingdom signaling in the bacterial world. *Microbiology* **153:** 3923–3938.

Winson, M.K., Camara, M., Latifi, A., Foglino, M., Chhabra, S.R., Daykin, M., *et al*. (1995) Multiple *N*-acyl-L-homoserine lactone signal molecules regulate production of virulence determinants and secondary metabolites in *Pseudomonas aeruginosa*. *Proc Natl Acad Sci USA* **92:** 9427–9431.

Winson, M.K., Swift, S., Fish, L., Throup, J.P., Jorgensen, F., Chhabra, S.R., *et al*. (1998a) Construction and analysis of *luxCDABE*-based plasmid sensors for investigating *N*-acyl homoserine lactone-mediated quorum sensing. *FEMS Microbiol Lett* **163:** 185–192.

Winson, M.K., Swift, S., Hill, P.J., Sims, C.M., Griesmayr, G., Bycroft, B.W., *et al*. (1998b) Engineering the *luxCDABE* genes from *Photorhabdus luminescens* to provide a bioluminescent reporter for constitutive and promoter probe plasmids and mini-Tn5 constructs. *FEMS Microbiol Lett* **163:** 193–202.

Yan, L., Allen, M.S., Simpson, M.L., Sayler, G.S., and Cox, C.D. (2007) Direct quantification of N-(3-oxohexanoyl)-L-homoserine lactone in culture supernatant using a whole-cell bioreporter. *J Microbiol Methods* **68:** 40–45.

Yang, Y.H., Lee, T.H., Kim, J.H., Kim, E.J., Joo, H.S., Lee, C.S., and Kim, B.G. (2006) High-throughput detection method of quorum-sensing molecules by colorimetry and its applications. *Anal Biochem* **356:** 297–299.

Zhang, L., Murphy, P.J., Kerr, A., and Tate, M.E. (1993) *Agrobacterium* conjugation and gene regulation by *N*-acyl-L-homoserine lactones. *Nature* **362:** 446–448.

Zhu, J., Beaber, J.W., More, M.I., Fuqua, C., Eberhard, A., and Winans, S.C. (1998) Analogs of the autoinducer 3-oxooctanoyl-homoserine lactone strongly inhibit activity of the TraR protein of *Agrobacterium tumefaciens*. *J Bacteriol* **180:** 5398–5405.

Zhu, J., Chai, Y., Zhong, Z., Li, S., and Winans, S.C. (2003) Agrobacterium bioassay strain for ultrasensitive detection of *N*-acylhomoserine lactone-type quorum-sensing molecules: detection of autoinducers in Mesorhizobium huakuii. *Appl Environ Microbiol* **69:** 6949–6953.

