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) (Nealon 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,
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

Table 1. The basic structure and name of HSLs.

| Basic structure | Remarks |
|-----------------|---------|
| ![Structure 1](image1.png) | When R is CH₃, the substance referred to as C₄-HSL |
| ![Structure 2](image2.png) | When R is CH₃, the substance referred to as C₄-hydroxy-HSL |
| ![Structure 3](image3.png) | When R is CH₃, the substance referred to as C₄-oxo-HSL |
| ![Structure 4](image4.png) | The putative new class of HSL, pC-HSL |
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 (McClell 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 C4-hydroxy-HSL (see Table 1). The RI (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 cviI 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 C6-HSL, the natural C. violaceum HSL; other HSLs that are reasonably well induced include C8-3-hydroxy-HSL and C10-HSL (both sixfold less active than C6-HSL), C8-3-oxo-HSL (11-fold less active) and C4-HSL (30-fold less active) (Steindle and Venturi, 2007).

The plasmid pSB401, based on E. coli harbouring the luxCDABE operon of Photorhabdus luminescens, produces bioluminescence, which can be used to differentiate structure of HSLs. The plasmid construct is based on LuxR of Vibrio fischeri and cognates luxl promoter controlling luxCDABE expression (Winson et al., 1998b). They are most sensitive to cognate C10-3-oxo-HSL while display relatively good sensitivity towards other HSLs, such as C6-HSL, C8-3-oxo-HSL and C10-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 C2 to C10 in length. Plasmid sensor pSB1075 (Winson et al., 1998a) based on E. coli can detect C10-HSL, C12-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 C4 to C12, 3-unsubstituted-HSLs with side-chains from C6 to C12 and 3-hydroxy-HSLs with side-chains from C6 to C12. 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 hydrophila and the cognate ahyl gene promoter fused to luxCDABE (Swift et al., 1997), is sensitive to C4-HSL. Another specific sensor, which contains two plasmids: pSF105 and pSF107, is developed based on the Phz/R HSL QS system of Pseudomonas fluorescens 2–79 (Khan et al., 2005). It can be used for detecting 3-hydroxy-HSLs, such as C6-3-hydroxy-HSL, C8-3-hydroxy-HSL and C10-3-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 quantitation 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-Marinol and Eulogio, 2001).

The operational procedure is described in the following. HSLs extracted from pure bacterium cultures and
standard samples are loaded onto a TLC plate. The plate is often a C\textsubscript{18} reversed-phase one (Steindle and Venturi, 2007). After the solvents in the HSLs samples are completely 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., 1998b). 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. 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**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) test bacterium is first grown in the methionine-free 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.

**HPLC**

The extracted samples obtained from LLE or SPE are resolved in ACN or methanol. The samples are then applied to a C8 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 C18 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 frequently 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 characterize 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). EI, ESI and CI 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 Fromberger 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 (Fromberger 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$^{-1}$.© 2010 The Authors
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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 (Fromberger et al., 2003; Schmitt-Kopplin and Kettrup, 2003). The temperature-dependent 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-electrospray-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 C18 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 (Fromberger 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, C7-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; Brellés-Maríno 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). 1H-NMR (90 and 400 MHz) (Chhabra et al., 1992; Winson et al., 1995) and 13C-NMR (100 MHz) (Moon, 2004) spectra are measured on a Varian EM390 or Bruker AM-400 spectrometer and are recorded in CDC13 or CDCl3 with tetramethylsilane as the internal standard except for the compounds dissolved in D2O 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 (C8-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 \(\gamma\)-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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