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REVIEW

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

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Abstract *N*-Acylated homoserine lactones (AHLs) are produced by Gram-negative bacteria as communication signals and are frequently studied as mediators of the "quorum sensing" response of bacterial communities. Several reports have recently been published on the identification of AHLs from different species and attempts have been made to study their role in natural habitats, for example the surface of plant roots in the rhizosphere. In this article, different analytical methods, including bacterial biosensors and chromatographic techniques, are reviewed. A concept for assignment of the structures of AHLs is also presented. The retention behaviour of derivatives of AHLs containing β-keto or hydroxyl groups and/or double bonds has been evaluated in relation to the separation behaviour of AHLs with saturated and unsubstituted alkanoyl chains. Samples have also been analysed by high resolution mass spectrometry (Fourier-transform ion-cyclotron-resonance mass spectrometry, FTICR-MS), nano liquid chromatography–electrospray ionization ion trap mass spectrometry (nano-LC–MS) and by the aid of a biosensor. The results obtained from ultra performance liquid chromatography (UPLC), FTICR-MS, nano-LC–MS, and bioassays have been compared to attempt structural characterisation of AHL without chemical synthesis of analytical standards. The method was used to identify the major AHL compound produced by the rhizosphere bacterium *Acidovorax* sp. N35 as *N*-(3-hydroxydecanoyl)homoserine lactone.

Keywords *N*-Acylhomoserine lactones · Biosensing · Chromatography · UPLC · FTICR-MS

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Introduction

Quorum sensing mediated by AHLs

Low-molecular-weight molecules mediate cell-to-cell communication in bacteria in a sense that these signalling

molecules enable bacteria to detect each other. The signal compounds of Gram-negative bacteria are mostly *N*-acylhomoserine lactones (AHLs) [1–3]. AHL-producing bacteria synthesize AHLs constitutively at a low rate. The small molecules are thought to diffuse across the cell envelope. On reaching a threshold concentration they form enough complexes with a specific receptor (R) protein to bind to promoter binding domains, which finally activates gene expression of its own biosynthesis (autoinducer molecules) and other biosynthesis pathways. Important traits that contribute to occupation of ecological niches, e.g. biofilm development, production of virulence factors or antibiotics, and persistence and viability on colonized host surfaces [1, 2], are under the control of the AHL–receptor complex. AHL-mediated cross-talk is also reported to be effective across species borders within microbial communities [4, 5] and even between prokaryotes and eukaryotes [6–8].

The root surface and the rhizosphere are rather densely populated microbial habitats in soil and are, therefore, hot spots of quorum sensing [3]. In plant–microbe interactions many virulence factors of plant pathogens are regulated by autoinducers, for example the interaction and virulence of plant pathogenic bacteria or the establishment of rhizobial infection of roots and nodule formation [3, 9]. The bacterial traits activated by AHLs can be beneficial or harmful to hosts and thus identification of regulated functions is of great interest. It has been demonstrated in tomato plants that AHL-producing root colonizing bacteria, for example *Serratia liquefaciens* MG1, elicit a systemic response in tomato plants accompanied by increased salicylic acid content of root and shoot tissues and expression of pathogen-related proteins [8]. Initiation of a signalling cascade in the plant could also be achieved when roots of axenically grown plant seedlings were treated with 10 μmol L^{-1} AHLs such as hexanoyl homoserine lactone (C_6-HSL) or butanoyl homoserine lactone (C_4-HSL). Schuhegger et al. [8] also showed that this response was systemic, because different gene expression of pathogen related proteins was observed in leaf tissues also. This response seemed to be similar to the SAR-response pattern elicited by plant pathogenic bacteria [10]. The authors also demonstrated that attack by a fungal leave pathogen, *Alternaria alternata*, was dramatically reduced when the roots of tomato plants had been inoculated with the AHL-producing strain *S. liquefaciens* MG1 before infection, whereas inoculation with an isogenic AHL-negative mutant had no effect. Bacterial AHLs may provide an additional signal to the plant that pathogens are going to organize themselves on the root surface to attack the plant.

The chemical nature of bacterial AHL compounds and the actual in situ production and concentration of AHL molecules reached e.g. in the rhizoplane thus warrants in-depth analysis by advanced chemical and in situ biosensor methods. In this report, current knowledge of biosensors, of AHL chemical analysis, and of the most recent developments in AHL analysis using ultra-high-pressure liquid chromatography and in silico simulations of AHLs combined with ultra-high-resolution mass spectrometry is presented.

AHL biosensors

The occurrence and ecological importance of AHLs in environmental microhabitats, e.g. on the root surface, can be assessed with AHL-sensors using genes for autofluorescent proteins, for example green fluorescent protein (GFP) or red fluorescent protein/Dsred (RFP) fused to AHL-regulated promoters [11]. These biosensor strains have a deletion in their AHL biosynthesis so regulation of AHL-controlled promoters is governed entirely by extracellular AHLs. Depending on the different AHL-regulated promoters, either short or long-chain AHLs can be monitored with very high efficiency (Table 1). Steidle et al. [11] reported concentrations down to 20 nmol L^{-1} to be sufficient for induction of AHL-induced gene expression. It also became apparent, however, that these reporters are quite selective in their detection. *P. putida* F117 carrying the reporter plasmid pAS-C8 detected 3-oxo-C_{12}-HSL over 100 times more sensitively than C_{12}-HSL [11]. Other

Table 1 List of plasmids with AHL biosensor constructs

Plasmid/strain	Genotype	Source	AHL detected	Ref.
pSB403	*luxR*$^+$*luxI*::*luxCDABE*; Tc^R, pRK415	*Vibrio fisheri*	oxo-C_6 to C_{14}-HSL, C_6 to C_{12}-HSL	[41]
pSB1075	*lasR*$^+$*lasI*::*luxCDABE*; Ap^R, ColE1 origin	*Pseudomonas aeruginosa*	oxo-C_{10} to C_{14}-HSL, C_{10} to C_{12}-HSL	[41]
pSB406	*rhlR*$^+$*rhlI*::*luxCDABE*; Ap^R, pUC18	*Pseudomonas aeruginosa*	oxo-C_4 to C_{14}-HSL, C_4 to C_{12}-HSL	[41]
pSB536	*ahyR*$^+$*ahyI*::*luxCDABE*; Ap^R, pUC18	*Aeromonas hydrophyla*	oxo-C4 to C8-HSL, C_4 to C_8-HSL	[41]
pKR-C12	*lasR*$^+$*lasB*::*gfp*(ASV); Gm^R, pBBR1MCS-5	*Pseudomonas aeruginosa*	oxo-C_{10} to C_{14}-HSL, C_{10} to C_{12}-HSL	[5]
pAS-C8	*cepR*$^+$*cepI*::*gfp*(ASV); Gm^R, pBBR1MCS-5	*Burkholderia cepacia*	oxo-C_{10} to C_{12}-HSL, C_6 to C_{10}-HSL	[5]
pJBA89	*luxR*$^+$*luxI*::*gfp*(ASV); Ap^R, pME6031	*Vibrio fisheri*	oxo-C_6 to C_{14}-HSL, C_6 to C_{12}-HSL	[44]
NT1	*traR*$^+$*traG*::*lacZ*; pDSK519	*Agrobacterium tumefaciens*	most AHLs	[45]
LC13	*smaI*::miniTn*5lacZ1*, Km^R	*Serratia marcescens*	C_4 and C_6-HSL	[45]
CV026	miniTn*5* double mutant; *cviI*$^-$	*Chromobacterium violaceum*	oxo-C_4 to C_8-HSL, C_4 to C_8-HSL	[46]

biosensors express different reporter genes or produce pigments when AHLs are provided exogenously.

Using GFP-based biosensors the occurrence of AHLs could be visualized on the surface of roots [11] and could be related to single cells or microcolonies of AHL-producing cells carrying a constitutive fluorescence tag [12]. This enabled monitoring of "landscapes" of AHLs on colonized surfaces, for example rhizoplane. It became apparent that concentration gradients of AHLs are present and are affected by the AHL-producing bacterial assemblage and by diffusion. Distances of up to 70 μm to the next AHL-producing cell or cell assembly were detected [12]. In this study the powerful approach of geostatistics was used to measure the connectivity in regional variability of local cell density of the spatial distribution of AHL-producing (red fluorescent) cells, to make statistically sound predictions of AHL concentration gradients radiating out from these sources. In this respect, quorum sensing on surfaces has many features of diffusion sensing [13, 14]. In their sessile lifestyle on surfaces or in biofilms bacteria apparently regulate important ecophysiological and interactive traits—either symbiotic or parasitic—by means of signalling compounds such as AHLs. In a more comprehensive view, bacteria on surfaces are able to sense the quality of their abiotic and biotic microenvironment using these small molecules before they synthesize large and costly macromolecules excreted into the cell environment. The very efficient AHL-production and reception system can therefore also be regarded as a kind of "local efficiency sensing" [15]. In microcolonies, embedded in a polymer matrix which restricts the diffusion, or when free diffusion is limited by membranes, local concentrations of AHLs can become quite high. By use of a mathematical model to describe the auto-regulated production of AHLs and restricted diffusion [16], quite high local concentrations were calculated. For a micro-colony of ten cells of e.g. *Burkholderia cepacia,* enclosed in a 5-μm cube (125 μm^3) a local concentration in the mmol L^{-1} range can be reached (Kuttler, unpublished). This situation of local accumulation can be regarded as the inverse situation of the limited degradability of compounds in the microenvironment of degrading cells [17].

Chromatographic determination of AHLs

Although use of biosensors reveals local information about the occurrence of AHL in situ, they cannot provide information about a wide or complete spectrum of AHL compounds, because they are limited to determination of a limited range of AHL structures only, with high sensitivity. Analytical tools and separation techniques, including gas chromatography (GC), high-performance liquid chromatography (HPLC), and capillary electrophoresis (CE) have therefore been used for chemically sound quantitative and qualitative analysis of AHL (Table 2). These techniques have usually been used to verify the results of bioassay, to unambiguously identify the AHL species produced.

Pretreatment of the sample for clean-up and preconcentration before analysis is usually negligible. Bacterial supernatant has been acidified to prevent hydrolysis of the AHLs during sample treatment [18], and liquid–liquid extraction performed with ethyl acetate, chloroform, or dichloromethane. For further purification, preparative HPLC [19–26], thin-layer chromatography (TLC), or solid phase extraction (SPE) [18, 27] have been integrated in the method. For example, Chambers et al. [21] identified C_8 and C_{10}-HSL from nine cystic fibrosis patients by applying fractionation with a three-stage acetonitrile gradient on a C_{18} silica column followed by a bioassay; oxo-AHLs were also identified at trace levels. Fractions in which AHL were shown to be present were sometimes further purified with the same fractionation system in isocratic mode, with aqueous mobile phases containing 30% or 50% ACN [19, 24, 26]. Another method used to clean the matrix is SPE, which can also be used for additional pre-concentration. Testing of different solid phases for SPE resulted in improvement of method sensitivity between two and tenfold compared with liquid–liquid extraction [27].

Methods have been developed for determination of the AHLs using GC [25, 28–30]. Separations were usually performed on fused silica capillary columns 20 m long with helium as carrier gas and with temperature gradients from 100 to 300 °C. Sometimes, however, sensitivity was lower than expected. There could be several reasons for this, for example masking effects by matrix constituents, e.g. diketopiperazines [29], the polar nature of the AHLs, and/or their decomposition in the injector [31]. To increase the sensitivity of the method, splitless injection [25, 29, 30], fractionation before GC analysis [25], selective-ion monitoring (SIM) in MS detection, or derivatisation of the β-oxo group to an oxime, resulting two isomers of each AHL [30], were also applied.

Most analytical procedures have been based on use of reversed-phase HPLC coupled with mass spectrometry (MS) for selective detection. The targets were usually separated on C_{18} silica columns with water–methanol or water–acetonitrile mobile phases and isocratic and/or gradient elution. AHLs and 3-oxo-AHLs have been separated on a C_{18} column in isocratic mode, by use of 50:50 methanol–water as mobile phase, followed by a gradient to elute non-polar compounds [32]. Different quantification techniques, for example use of external and internal standards, were studied to improve the reliability of the quantification. The internal standard selected was C_7-HSL, which is rarely produced in nature. Other standards, for example deuterium-labelled C_6-HSL, had also been

Table 2 Summary of the methods developed for determination of AHL compounds

Method	Matrix	Targets	Sample treatment	Aim	Ref
RP-HPLC–MS	*E. Coli, P. stewartii, P. Aeruginosa*	oxo-C_6–. C_6–, C_{12}-HSL	SPE (Sep Pak Plus Silica)	Specification of AHL synthases, identification of other AHL derivatives	[18]
Bioassay	cystic fibrosis patients	C_4–, C_6–,C_8–, C_{10}–, C_{12}–, oxo-C_6–, 3-oxo-C_8–, 3-oxo-C_{10}–HSL	LLE (dichloromethane) Fractionation with LC	Quantification of AHLs for different patients	[20, 21]
RP-HPLC–MS	*V. Anguillarum,* infected rainbow trout	OH-C_6–, C_6–, oxo-C_{10}–HSL	LLE (dichloromethane), fractionation with HPLC	Study of the synthesis of AHLs in V. *anguillarum*	[24, 47]
GC–MS, ESI-MS–MS	*Halomonas* species *Sinorhizobium meliloti*	C_4–, C_6–, C_8–, C_{12}–, oxo-C_6-HSL	LLE then fractionation with HPLC	Identification of AHLs as signal molecules	[23, 31]
GC–LIF, GC–MS	Bacterial supernatant	C_6-HSL	LLE (ethyl acetate) then fractionation with LC	Chiral separation of AHLs	[25]
RP-HPLC–PDA and MS	Bacterial supernatant	C_4–, C_6-HSL	LLE (dichloromethane), fractionation with HPLC	Study of in-vitro biosynthesis of *P. aeruginosa*	[26]
Bioassay	*Agrobacterium tumefaciens*	C_6–, oxo-C_6–HSL	SPE	Comparison of different SPE methods	[27]
GC–MS	*Burkholderia cepacia*	C_4–, C_6–, C_7–, C_8–, C_{10}–, C_{12}–, C_{14}-HSL	LLE (chloroform)	Method development and identification	[28]
GC–MS	Marine isolate	C_4–, oxo-C_6–, C_8–, C_{12}-HSL		Identification of AHLs in marine bacteria	[29]
GC–MS	Flow cell cultures of *P. aeruginosa* (biofilm)	oxo-C_6–, oxo-C_8–, oxo-C_{10}–, oxo-C_{12}–, oxo-C_{14}-HSL	Derivatisation with pentafluorobenzyloxime	Quantification of AHLs produced during biofilm production	[30]
HPLC–MS	*Vibrio vulnificus P. aeruginosa*	C_4–, C_6–, C_8–, C_{10}–, C_{12}–, C_{14}–, oxo-C_6–, oxo-C_8–, oxo-C_{10}–, oxo-C_{12}–, oxo-C_{14}–	LLE (chloroform)	Method development	[32]
RP-HPLC–HRMS	Fillets of cod	hydroxy-C_8–, C_4–, C_6–, C_8–, oxo-$C_{8,}$ oxo-C_{10}	LLE (ethyl acetate)	Study of bioluminescence in marine bacteria	[48]
RP-HPLC–HRMS pH control	Spoiled meat	oxo-C_6–, C_6–, oxo-C_8–, C_8-HSL	LLE (ethyl acetate) then TLC	Identification of AHLs during spoilage with combination of microorganism identification	[49]
RP-HPLC–APCI-MS	Soil and bacterial supernatant	C_4–, C_6–, C_7–, C_8–, C_{10}–, C_{12}–, C_{14}–, oxo-C_6–, oxo-C_{12}-HSL		Study of degradation of AHLs by pseudomonas	[33]
RP-HPLC–ESI-MS	Bacterial biofilm	oxo-C10–,	LLE (ethyl acetate)	Study of AHL acylase, derivatisation with dansyl chloride	[50]
RP-HPLC–APCI-MS RP-HPLC–DAD	Standard solution	C_6–, oxo-C_6–, oxo-C_8–HSL		Study of degradation pathway of AHLs by oxidizing halogenated antimicrobials	[34]
nanoLC–ESI-MS	Nano-LC	C_4–, C_6–, C_7–, C_8–, C_{10}–, C_{12}–, C_{14}-HSL	LLE (chloroform)	Use of a self-packed capillary for nanoLC	[35]
HPLC–PDA	Different plant species	C_6–HSL		C_6-AHL stability during plant growth	[51]
CZE–ESI-MS	*Burkholderia cepacia*	C_4–, C_6–, C_7–, C_8–, C_{10}–, C_{12}–, C_{14}-HSL	LLE (chloroform) hydrolysis, SPE	Determination of AHLs after their hydrolysis, method development	[37]
PF–MEKC–ESI-MS	*Burkholderia cepacia*	C_4–, C_6–, C_7–, C_8–, C_{10}–, C_{12}–, C_{14}-HSL	LLE (chloroform)	Partial filling MEKC, method development	[36]
GC–MS	Standard solution	AHLs with e.g. biphenyl group		Polymer-supported synthesis of AHLs	[52]
HPLC–HRMS	Spoiled bean sprouts	oxo-C_6-HSL	SPE (Strata X)	Identification of AHLs by bioassay, study of spoilage process	[53]

used previously. In soil bacteria degradation of oxo-C_{12}-HSL and C_4-HSL as sole carbon source by AHL acylase and lactonase has been studied by HPLC on a C_{18} silica phase, with a mixture of acidified water and methanol as mobile phase, coupled to atmospheric-pressure chemical-ionisation MS [33]. To increase the separation efficiency an isocratic part before the gradient elution was integrated into the method. A more polar stationary phase (cyano column) has also been used for determination of AHLs and their degradation products resulting from the presence of chlorinated biocides; use of this column resulted in the need for less organic solvent in the mobile phase [34]. The results revealed rapid reaction of the halogenated biocides with oxo-AHL containing β-keto amide group but not with *N*-alkanoyl homoserine lactones.

A simple microelectrospray interface to MS was developed by Frommberger et al. [35] and used after nano-LC separation of the sample in a 75 μm i.d fused-silica capillary self-packed with reversed phase silica particles. The target compounds were concentrated in the inlet of the separation capillary, as in solid-phase extraction, by increasing the polarity of the sample solvent (30% methanol in water) relative to the mobile phase (80% methanol in water). In determination of selected AHLs in different bacterial supernatant liquids the sensitivity of the method was good (1 μg L^{-1}).

A method applying micellar electrokinetic chromatography (MEKC) has also been developed for determination of AHLs [36], and retention behaviour was studied as a function of AHL structure and surfactant concentration. Because detection was based on MS, the surfactant was injected partially into the capillary to avoid loss of sensitivity of the electrospray as a result of ion suppression. Capillary zone electrophoresis (CZE) was used for analysis of the serines (HS) produced by alkaline hydrolysis of AHLs [37]. The carboxylic acid group of the serines enabled their separation at alkaline pH, in which they are fully deprotonated. Because the serines were separated as anions, the longer chain HS (i.e. the larger molecules) migrated more rapidly, resulting in an order of elution opposite to that achieved in HPLC. To improve the performance of the method, C_7-HSL was used as internal standard for analysis of AHLs produced by *Burkholderia* sp. Mmi 1537. The AHLs produced were identified as C_8-HSL, C_{10}-HSL, and C_{12}-HSL.

Experimental

Materials

N-acylhomoserine lactones: *N*-butanoyl-homoserine lactone (C_4-HSL), *N*-hexanoyl-homoserine lactone (C_6-HSL), *N*-heptanoyl-homoserine lactone (C_7-HSL), *N*-octanoyl-homoserine lactone (C_8-HSL), *N*-decanoyl-homoserine lactone (C_{10}-HSL), *N*-dodecanoyl-homoserine lactone (C_{12}-HSL), *N*-tetradecanoyl-homoserine lactone (C_{14}-HSL) *N*-(β-ketocaproyl)-homoserine lactone (oxo-C_6-HSL) and *N*-(3-Oxooctanoyl)-homoserine lactone (oxo-C_8-HSL) were obtained from Sigma–Aldrich (Steinheim, Germany). Stock solutions (1,000 mg L^{-1}) of the analytes were prepared in acetonitrile (ACN). The stock solutions were kept at −20 °C and could be stored for four weeks. ACN of “hypergrade” quality for UPLC analysis, methanol, and 2-propanol were purchased from Merck (Darmstadt, Germany), hexane was from Riedel–de Häen (Seelze, Germany). Water was purified with a Milli-Q plus system (Millipore, Billerica, USA). All chemicals used in the experiment were of analytical grade at least.

To obtain AHLs produced by pure cultures of bacteria 10 to 50 mL nutrient broth (NB no. 4; Fluka, Buchs, Switzerland) or M9 minimal medium [38] with glucose as carbon source were inoculated and grown at 30 °C and 175 rpm overnight. Bacteria were harvested at 5,000 rpm and 4 °C for 5 min in a Hettich Universal 32R centrifuge equipped with a 1620A rotor (Andreas Hettich, Tuttlingen, Germany) and the supernatant was used for AHL extraction. The bacteria used were *Acidovorax* sp. N35, derived from wheat roots.

UPLC analysis

Before UPLC analysis bacterial supernatant was extracted according to a procedure described elsewhere [39]. Briefly, 10 or 50 mL of the sample diluted with 25% acetonitrile was applied to a MegaBond Elute SPE cartridge (Varian, Palo Alto, USA), after conditioning of the cartridge with methanol then water. The cartridge was washed with methanol–water then the solutes were eluted with 85:15 (*v*/*v*) 2-propanol–hexane. The eluate was dried under a nitrogen stream and re-dissolved in water containing 30% ACN.

Analysis was performed with a Waters (Milford, USA) Aquity UPLC System equipped with a 2996 PDA detector. Reversed-phase separation was achieved on a BEH C_{18} packing with a particle diameter of 1.7 μm and column dimensions of 100 mm×2.1 mm (Waters). The column thermostat was set to 60 °C and the autosampler temperature to 27 °C. The flow rate was 0.8–0.9 mL min^{-1} and the injection volume was 20 μL, injected by use of a full loop. A linear gradient was applied with starting with water containing 10% acetonitrile as mobile phase; the ACN content was increased to 100% in 1 min. The detection wavelength was set to 197 nm (1.2 nm width) and the scan rate was 20 Hz. The log *P* (lipophilicity) values of the

analytes were calculated by use of Pallas 3.1 (CompuDrug International, Budapest, Hungary).

FTICR-MS analysis

Positive-ion FTICR mass spectra were acquired with a Bruker Daltonics (Bremen, Germany) Apex Qe 12 T system equipped with an Advion (Ithaca, NY, USA) TriVersa Nanomate nano-electrospray source operated in positive-ion mode at 2 psi and 2 kV using the standard chip. Spectra were acquired in broadband mode and were calibrated externally on clusters of arginine (ca. 10 mg L^{-1} in 50% methanol with 0.1% formic acid) in the required mass range (*m*/*z* 175.11895, *m*/*z* 349.23062, *m*/*z* 523.34230 and *m*/*z* 697.453979). This external calibration was verified with the diketopiperazines *cyclo*-(Pro-Phe), *cyclo*-(Pro-Ile), and *cyclo*-(Pro-Trp), known to be present in the NB samples (verified by GC–MS and MS–MS; results not shown). Mass errors of these peaks were always less than 0.1 ppm (relative difference of 0.0000001×10^6).

Peaks exceeding a threshold signal-to-noise ratio of 4 were exported to peak lists. The resulting text files with up to 1,300 mass–intensity pairs were searched with a software tool written in Python (http://www.python.org) for the presence of peaks from a reference file with theoretically possible protonated homoserine lactone masses or sodium cation adducts of these masses (i.e. unsubstituted 3-ketoacyl and 3-hydroxyacyl homoserine lactones with both saturated and monounsaturated side chains ranging from 4 to 16 carbon atoms; 84 possible ions in total). The window width for the search was set to 1 ppm mass accuracy.

Nano-LC–ESI ion-trap mass spectrometry

MS–MS confirmation by nano-LC–ESI ion-trap mass spectrometry was performed essentially as described else-

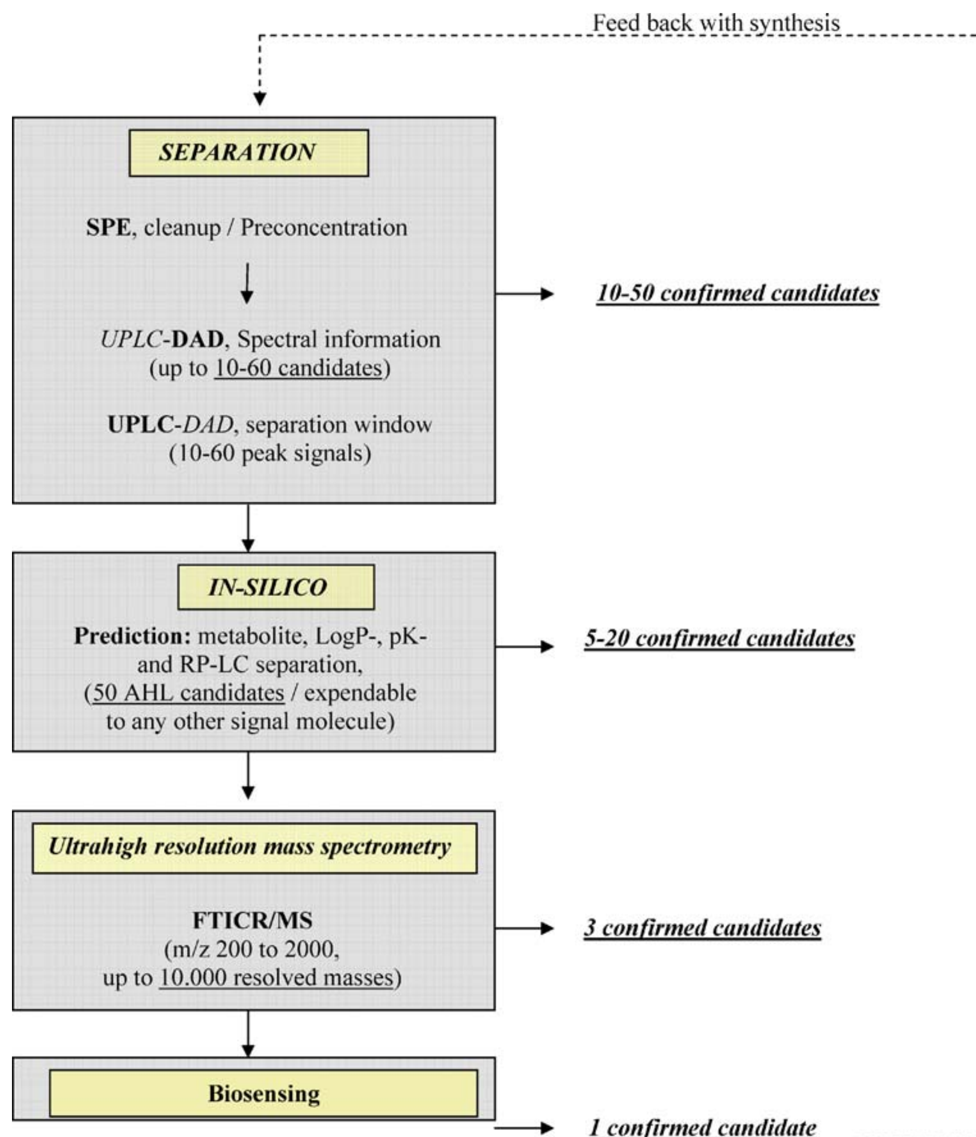

Fig. 1 Concept of process for identification of AHLs by use of different analytical techniques

where [35]. Briefly, laboratory-packed fused-silica columns (75 μm inner diameter, 20 cm length, Hypersil ODS; Grom, Herrenberg, Germany) were connected via a laboratory-constructed ESI sprayer to an LCQ Duo (Thermoquest, San José, CA, USA) ion-trap instrument. Positive-ion electrospray was performed without nebulizer gas using a sheath liquid (methanol–water–glacial acetic acid, 50:50:1, *v*/*v*) at a flow rate of 1 μL min^{-1}. A Series 200 LC pump (Perkin–Elmer, Norwalk, CT, USA) was used to deliver an isocratic flow of 80% methanol and the injection volume was 1 μL, via a full loop. For structural confirmation, the typical fragmentation pattern of the AHLs, with the common product ion at *m*/*z* 102 (corresponding top the deacylated homoserine lactone) was used [32].

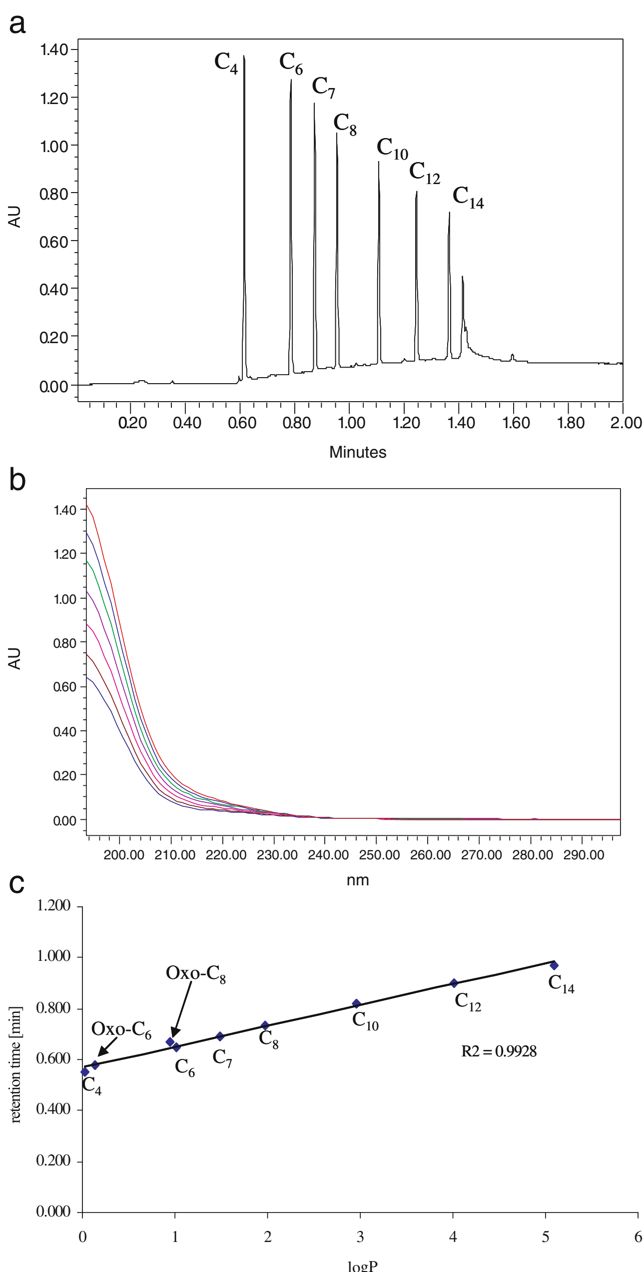

Fig. 2 Typical chromatogram obtained from the alkanoyl-AHLs (**a**), UV–visible spectra of the seven AHLs (**b**), and retention times of the AHLs as a function of their log *P* values (**c**). The compounds were separated on C_{18}-modified silica by linear gradient elution in 1 min with an initial mobile phase of 30% acetonitrile in water and a final mobile phase of acetonitrile, at flow rate of 0.9 mL min^{-1}, with a typical pressure of 850–550 bar; the detection wavelength was 197 nm. *Cx*=*N*-alkanoyl-homoserine lactone

Bioassay

This method is described in detail elsewhere [40]. Briefly, AHL extracts from pure cultures and standard dilutions of synthetic AHLs (5×10^{-10} mol) were spotted on a thin-layer chromatography plate (RP-18 F_{254s}, 20 cm×20 cm; Merck). After drying, the plate was placed upright in a glass chamber containing 300 mL 60:40 methanol–water such that the sample spots were well above the liquid surface. The AHL samples were separated, in accordance with their polarity, by ascending development of the plate for at least 4 h. After drying of plate 150 mL soft agar (3 g casein hydrolysate, 1.5 g yeast extract, 1.5 g NaCl, 1.2 g agar, 150 mL water) was inoculated with 10 mL exponentially growing *Escherichia coli* MT102 harbouring the sensor plasmid pSB403 [41]. This construct contains the *lux* gene fused to an AHL inducible promoter, so light is emitted whenever AHL is present. The TLC plate was coat with this agar and, after solidification of the agar, incubated at 30 °C for 16 h. Finally, an X-ray film (Fuji, RVW, Obernissa, Germany) was applied to the plate for 30 s, and then developed. AHL spots on the chromatography plate were apparent from black spots on the X-ray film and, by use of synthetic AHL reference compounds, approximate determination of the type of AHLs extracted was possible.

Results and discussion

The strategy for AHL analysis

Identification of signalling molecules in biological systems is often difficult because of the lack of reference material. A system was therefore developed which facilitated acquisition of structural information and identification of the compound of interest. Although the method is not compatible with the method that uses of reference material, it reduces the time and effort of analysis. For this purpose different methods with high selectivity and/or sensitivity were combined, as shown in Fig. 1.

The first approach was in-silico prediction of derivatives of AHLs based on optimized UPLC. The retention times of AHL derivatives for which no standards are available can, if they differ in the number of carbon atoms (homologous series), be estimated from their log *P* values. Thus, if a peak

in the real samples had a UV spectrum similar to that of the AHLs, its side-chain could be approximately predicted.

For further improvement of chemical characterisation, high-resolution mass spectrometry, FTICR-MS, was used to give spectra containing up to 1,300 distinct signals from *m*/*z* 200 to *m*/*z* 2000 in a single run. Because of the high mass accuracy, a few exact masses corresponding to theoretically possible components could be attributed to AHLs—further restricting the possible candidates present in the mixture. Mass spectrometry alone does not give sufficient information, however, even though it enables assignment of elemental composition (but not chemical structures) to many of the typically more than 1,000 peaks from a single measurement across a sizable mass range. As already pointed out by others [42], a mass accuracy of better than 1 ppm may not be sufficient for assignment of one unique elemental formula to an exact mass when, as in the bacterial extracts under investigation, a plethora of possible structures can be expected. Hence combination of exact mass measurement by use of ultra-high-resolution mass spectrometry with chromatographic data gives unprecedented insight into the nature of the AHLs in the sample.

For final confirmation, nano-LC–MS^2 and thin layer chromatography with the detection of the compounds by use of a reporter strain were applied. Taken together, complete information from all the approaches can be reduced to intersection of the confirmed candidates; application of feedback with reference material would, however, increase the reliability of identification.

Screening by UPLC

A standard solution of unsubstituted and saturated alkanoyl AHLs with side chains from butanoyl (C_4) to tetradecanoyl (C_{14}) were successfully separated by linear gradient elution with water–acetonitrile mixtures as mobile phase (Fig. 2a) [39]. Other AHL derivatives may also be present in the samples of supernatant, however, so the retention patterns of AHLs had to be evaluated if the method was to be applied to real samples, e.g. for extracts of bacterial supernatant. For selection of AHL possibly present in a sample the following approach was used. First, peaks with UV spectra similar to those of AHLs (Fig. 2b) were selected. To characterise the possible side-chain of AHLs, in-silico methods were used, because measured retention times of the target AHLs were proportional to their log *P* (the linear regression coefficient was 0.996, as shown in Fig. 2c). According to the Martin rule [43] in isocratic elution, the net retention time of compounds with a homologously increasing number of carbon atoms depends exponentially on this number. If a linear gradient is used, the dependence becomes linear. If a new group is introduced into the mother compounds this rule may be true, but retention will be shifted according to the polarity of the group introduced. In this case, two oxo-derivatives were used to check this hypothesis before other AHLs were used (Fig. 2c). Because differences between predicted and the measured retention times of 3-oxo-AHLs were less than 2%, a detection window of 0.05 min was set for real applications. The objective of this procedure was to reduce the number of candidates, and not exact identification, as in the next steps; by improving the selectivity of the analytical method, the number of candidates will further be reduced.

To show how this system works in practical application, extracts of bacterial supernatant of *Acidovorax* sp. N35, a rhizosphere bacterium, grown in NB medium were investigated (Fig. 3). Peaks between 0.55 min and 1.00 min had spectra different from those of AHLs but similarities were observed for the small peaks at 0.33, 0.36, 0.39, 0.41, and 0.51 min; these peaks were therefore taken for further

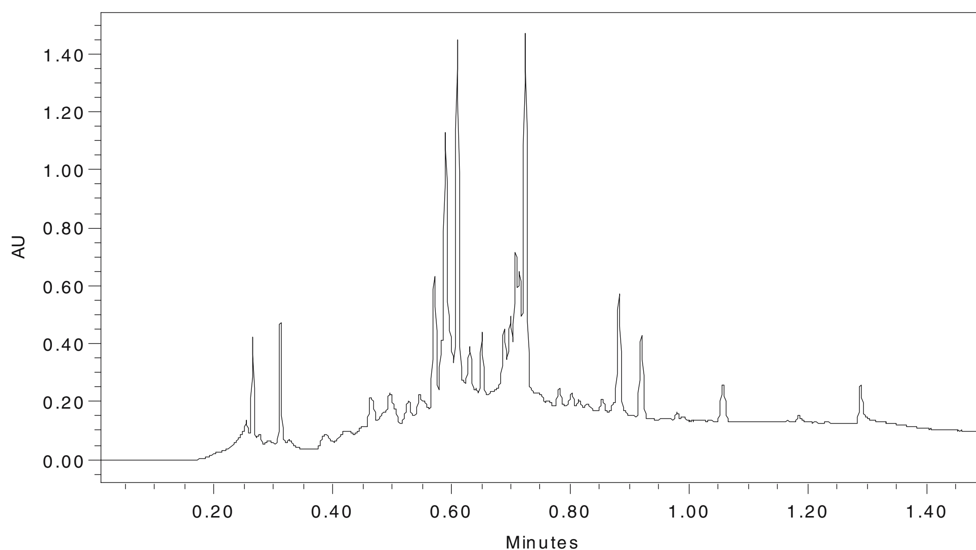

Fig. 3 Chromatogram obtained from extract of 10 mL supernatant from culture of *Acidovorax* sp. N35 in NB medium. Treatment was based on solid-phase extraction (see section entitled *UPLC analysis*) and the extract was separated as described in the caption to Fig. 2

prediction. These retention times might match the retention of fifteen derivatives of AHLs that have mainly hexanoyl and octanoyl side-chains (Table 3a, marked in bold). To confirm these assumptions, the same samples were measured by FTICR-MS but the masses detected did not match those of the predicted AHLs. Presumably, matrix constituents with no chromophor were observed.

To eliminate matrix effects from the NB medium, *Acidovorax* sp. N35 was grown in a minimal medium and the supernatant was used for subsequent analysis. The volume of the sample had to be increased, thus the concentration factor was changed from 10 to 100 by passing a larger volume of sample through the SPE cartridge. Compared to analysis with the NB medium, fewer possible candidates with different retention times were determined. The peaks with matching spectra were at retention times of 0.84, 0.86, 0.90, 0.97, and 0.99 min (Table 3b), obviously different from results for the NB

Table 3 Predicted retention times of AHL derivatives and measured peaks of *Acidovorax* sp. N35

Table A	logP	Mass protonated	Mass Na adduct	tr(pred) [min]	tr(E), NB,10 ml [min]	Table B	tr(pred) [min]	tr(E) M9, 50 ml [min]
OH-C4-HSL	−0.98	188.091735	210.07368	0.32		OH-C4-HSL	0.47	
OXO-C4-HSL	−0.79	186.076085	208.05803	0.21		OXO-C4-HSL	0.50	
OH-C6:1-HSL	−0.46	214.107385	236.08933	0.27		OH-C6:1-HSL	0.55	
OXO-C6:1-HSL	**−0.13**	**212.091735**	**234.07368**	**0.32**	**0.32**	OXO-C6:1-HSL	0.59	
OH-C6-HSL	**−0.05**	**216.123035**	**238.10498**	**0.34**	**0.33**	OH-C6-HSL	0.60	
C4-HSL	**0.03**	**172.09682**	**194.078765**	**0.35**	**0.36**	C4-HSL	0.61	
OXO-C6-HSL	**0.14**	**214.107385**	**236.08933**	**0.37**		OXO-C6-HSL	0.63	
C4:1-HSL	**0.28**	**170.08117**	**192.063115**	**0.39**	**0.37**	C4:1-HSL	0.65	
OH-C8:1-HSL	**0.33**	**242.138685**	**264.12063**	**0.40**	**0.39**	OH-C8:1-HSL	0.66	
OXO-C8:1-HSL	**0.36**	**240.123035**	**262.10498**	**0.41**	**0.41**	OXO-C8:1-HSL	0.66	
OH-C8-HSL	**0.89**	**244.154335**	**266.13628**	**0.50**	**0.51**	OH-C8-HSL	0.74	
OXO-C8-HSL	**0.94**	**242.138685**	**264.12063**	**0.50**		OXO-C8-HSL	0.74	
C6-HSL	**1.02**	**200.12812**	**222.110065**	**0.52**		C6-HSL	0.75	
C6:1-HSL	**1.15**	**198.11247**	**220.094415**	**0.54**		C6:1-HSL	0.77	
OXO-C10:1-HSL	**1.17**	**268.154335**	**290.13628**	**0.54**		OXO-C10:1-HSL	0.77	
OH-C10:1-HSL	**1.2**	**270.169985**	**292.15193**	**0.55**		OH-C10:1-HSL	0.78	
OXO-C10-HSL	1.7	270.169985	292.15193	0.63		**OXO-C10-HSL**	**0.85**	**0.841**
OH-C10-HSL	1.76	272.185635	294.16758	0.64		**OH-C10-HSL**	**0.86**	**0.860**
OXO-C12:1-HSL	1.9	296.185635	318.16758	0.67		**OXO-C12:1-HSL**	**0.88**	
C8-HSL	1.97	228.15942	250.141365	0.68		**C8-HSL**	**0.89**	
OH-C12:1-HSL	1.97	298.201285	320.18323	0.68		**OH-C12:1-HSL**	**0.89**	
C8:1-HSL	2.05	226.14377	248.125715	0.69		**C8:1-HSL**	**0.90**	**0.904**
OXO-C12-HSL	2.49	298.201285	320.18323	0.77		**OXO-C12-HSL**	**0.96**	
OXO-C14:1-HSL	2.63	324.216935	346.19888	0.79		**OXO-C14:1-HSL**	**0.98**	**0.971**
OH-C12-HSL	2.65	300.216935	322.19888	0.79		**OH-C12-HSL**	**0.98**	**0.990**
OH-C14:1-HSL	2.75	326.232585	348.21453	0.81		**OH-C14:1-HSL**	**0.99**	**0.993**
C10:1-HSL	2.88	254.17507	276.157015	0.83		**C10:1-HSL**	**1.01**	
C10-HSL	2.96	256.19072	278.172665	0.85		**C10-HSL**	**1.02**	
OXO-C14-HSL	3.35	326.232585	348.21453	0.91		OXO-C14-HSL	1.08	
OXO-C16:1-HSL	3.46	352.248235	374.23018	0.93		OXO-C16:1-HSL	1.09	
OH-C14-HSL	3.57	328.248235	350.23018	0.95		OH-C14-HSL	1.11	
OH-C16:1-HSL	3.63	354.263885	376.24583	0.96		OH-C16:1-HSL	1.12	
C12:1-HSL	3.74	282.20637	304.188315	0.98		C12:1-HSL	1.13	
C12-HSL	**4.02**	**284.22202**	**306.203965**	**1.03**		C12-HSL	1.17	
OXO-C16-HSL	**4.27**	**354.263885**	**376.24583**	**1.07**	**1.062**	OXO-C16-HSL	1.21	
OH-C16-HSL	4.55	356.279535	378.26148	1.12		OH-C16-HSL	1.25	
C14-HSL	5.09	312.25332	334.235265	1.21		C14-HSL	1.32	

Compounds in the extract of bacteria grown in NB full medium were separated at a flow rate of 0.9 mL min^{-1} (A); those in the extract of bacteria grown in M9 minimal medium were separated at 0.8 mL min^{-1} (B). Other conditions were as described in the section *UPLC analysis*. Abbreviations: log *P*: octanol–water partition coefficient; $t_{r(pred)}$: predicted retention time; $t_{r(E)}$, NB, 10 mL: retention times of peaks with UV spectra similar to those of AHL from 10 mL supernatant from bacteria grown in NB full medium; $t_{r(E)}$, M9, 50 mL: retention time of peaks with UV spectra similar to those of AHL from 50 mL supernatant from bacteria grown in M9 minimal medium, OH-Cx-HSL: 3-hydroxy-alkanoyl-homoserine lactone, OXO-Cx-HSL: 3-oxo-alkanoyl-homoserine lactone; Cx-HSL: alkanoyl-homoserine lactone. Bold letters indicate possible structures of AHL in the extracts

medium. AHLs with log *P* values from 1.70 to 2.96, for example 3-(hydroxydecanoyl)-HSL (OH-C_{10}-HSL), 3-(oxodecanoyl)-HSL (OXO-C_{10}-HSL), C_{10}-HSL with ($C_{10:1}$-HSL) and without (C_{10}-HSL) a double bond, 3-(hydroxydodecanyl)-HSL with (OH-$C_{12:1}$-HSL) and without (OH-C_{12}-HSL) a double bond, 3-(oxododecanoyl)-HSL with (OXO-$C_{12:1}$-HSL) and without (OXO-C_{12}-HSL) a double bond, 3-(hydroxytetradecanyl)-HSL with (OH-$C_{14:1}$-HSL) and without (OH-C_{14}-HSL) a double bond, and 3-(oxotetradecanoyl)-HSL with (OXO-$C_{14:1}$-HSL) and without (oxo-C_{14}-HSL) a double bond might be possible candidates, as shown in Table 3b marked in bold. For confirmation of the identities of the AHLs the samples were analysed by FTICR-MS.

Confirmation of UPLC results by FTICR-MS analysis

Because of its ultra-high resolution, in excess of 200,000 FWHM in broad-band measurements or up to 1,000,000 FWHM in high-resolution mode, and mass accuracy routinely better than 0.5 ppm, especially at high-fields (12 Tesla) Fourier-transform ion-cyclotron-resonance mass spectrometry (FTICR-MS) enables advanced chemical characterization of metabolites of known and hitherto unknown structures in complex and heterogeneous samples of biological origin. The use of newly available on-chip nanoelectrospray ionization systems in this context enables another significant increase in sensitivity, a substantial reduction in the amount of sample, and more efficient ionization of low-abundance ions in the presence of highly abundant (matrix) species. Figure 4 shows the spectra acquired from the extract of the supernatant of *Acidovorax* grown in NB full and M9 minimal medium. The M9 extracts generally produced less noise and thus should enable better detection of putatively formed *N*-acylhomoserine lactones, because of less signal suppression during the electrospray ionisation and because fewer ions accumulate in the FTICR cell and contribute to, e.g., peak coalescence. After use of the minimal medium the number of false positives was lower, because all compounds detected were actually derived from bacterial metabolism

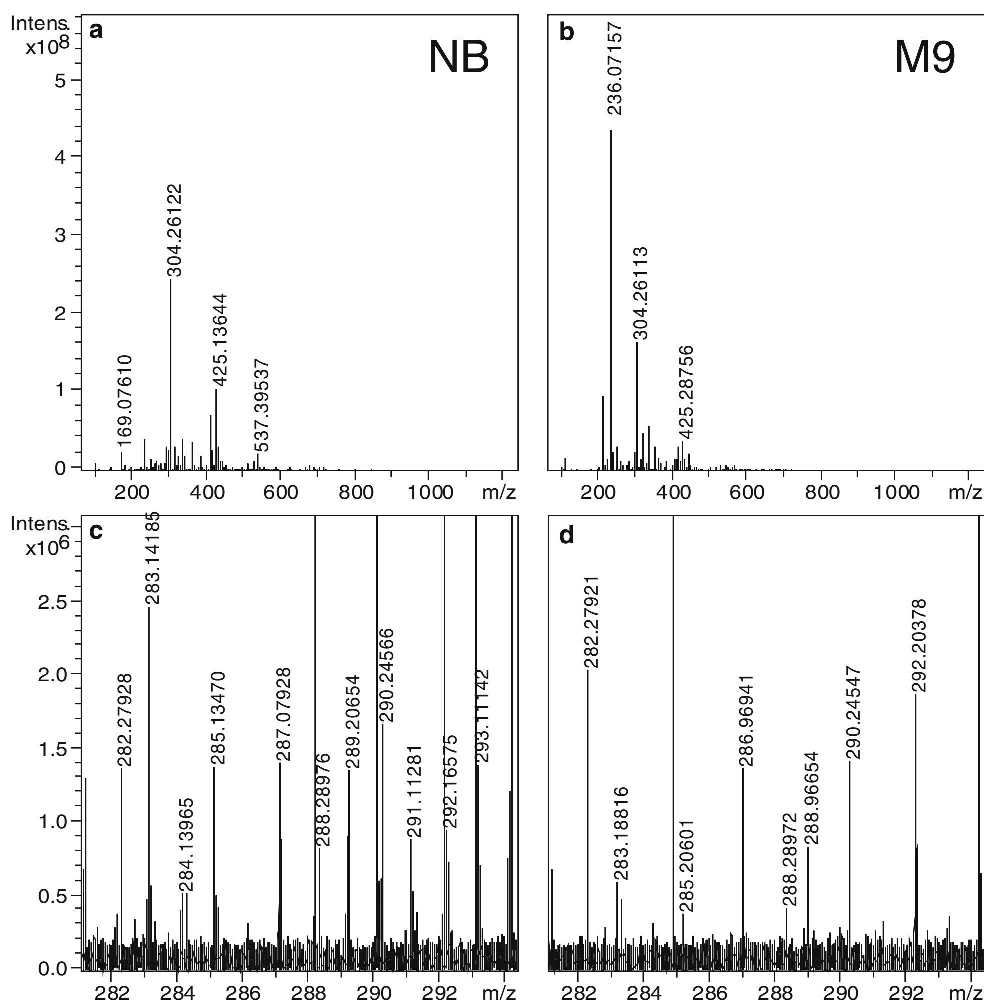

Fig. 4 Overview scans from *m*/*z* 200 to *m*/*z* 2000 (**a**) and details of the FTICR-MS spectra from *m*/*z* 280 to *m*/*z* 295 (**c**) obtained from extract of 10 mL supernatant from culture of *Acidovorax* in NB medium. Overview scans from *m*/*z* 200 to *m*/*z* 2000 (**b**) and details of spectra from *m*/*z* 280 to *m*/*z* 295 (**d**) obtained from extract of 10 mL supernatant from culture of *Acidovorax* sp. N35 in M9 minimal media. A nanoelectrospray source was used via nanomate operated in positive-ion mode at 2 psi and 2 kV using the standard chip

Table 4 Putative *N*-acylhomoserine lactone masses, determined by FTICR-MS, from *Acidovorax* sp. N35 grown in M9 minimal medium with extraction volumes of 10 mL and 50 mL, and from *Acidovorax* sp. N35 grown in NB medium with an extraction volume of 10 mL

Structure	Theor. mass	Exp. mass	Intensity	Error (ppm)
10 mL extract, minimal medium				
$C_{10:1}$-HSL+Na	276.15701	276.15695	357148	−0.22
OH-C_{10}-HSL+Na	294.16757	294.16766	5701376	0.31
C_{14}-HSL+Na	334.23526	334.23514	424368	−0.37
10 mL extract, NB medium				
OH-C_6-HSL+H	216.12303	216.12299	498296	−0.21
OH-C_{10}-HSL+Na	294.16757	294.16779	1323232	0.73
C_{14}-HSL+Na	334.23526	334.23495	459664	−0.91
50 mL extract, minimal medium				
C_6-HSL+Na	222.11006	222.11015	312476	0.41
C_8-HSL+Na	250.14136	250.14156	340420	0.79
OH-C_{10}-HSL+H	272.18564	272.18561	323524	−0.11
OH-C_{10}-HSL+Na	294.16757	294.16769	1839808	0.41
OH-C_{12}-HSL+Na	322.19888	322.19858	409440	−0.95
C_{14}-HSL+Na	334.23526	334.23541	587696	0.46
OH-C_{14}-HSL+Na	350.23019	350.23019	475240	0.00

The conditions used for determination are described in the section *FTICR-MS analysis*. Abbreviations: Theor. mass: theoretical mass, Exp. mass: mass of compound detected in the extract, Intensity: intensity of a mass peak, error (ppm): difference between the measured and calculated peaks in ppm, OH-Cx-HSL+Na: sodium adduct of 3-hydroxy-alkanoyl-homoserine lactone, OH-Cx-HSL+H: protonated 3-hydroxy-alkanoyl-homoserine lactone, Cx-HSL+Na: sodium adduct of alkanoyl-homoserine lactone, Cx-HSL+H: protonated alkanoyl-homoserine lactone

and were not artefacts from the medium (for example, formation of diketopiperazines).

The theoretical masses of the possible AHLs in the protonated form and as the sodium adducts were calculated and compared with masses measured in the extracts with mass differences less than 1 ppm. Both, protonated ions and sodium adducts had to be taken into consideration, because the mass spectra obtained from the standard solution of alkanoyl-AHLs had both forms, although the intensity of the peaks of the sodium adducts was always higher. Possible *N*-acylhomoserine lactones in extracts of *Acidovorax* supernatant detected by the FTICR-MS are sum-

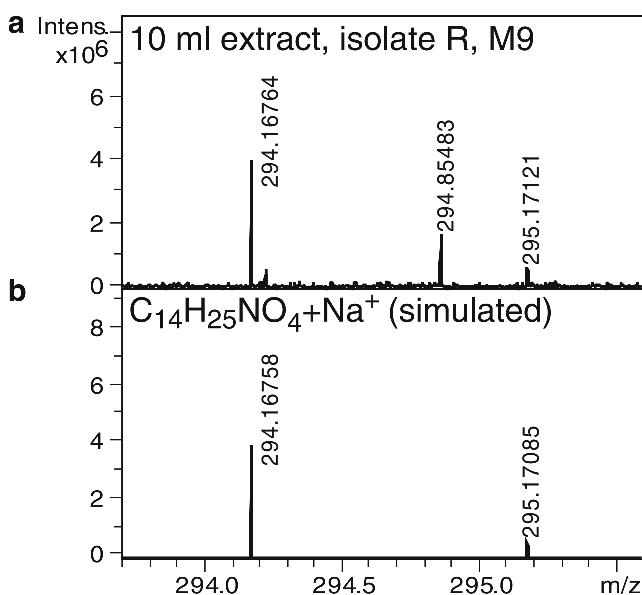

Fig. 5 (**a**) Detection, by FTICR-MS, of the Na adduct of OH-C_{10}-HSL in the extract obtained from 10 mL supernatant from culture of *Acidovorax* sp. N35 in M9 medium. (**b**) Calculated spectrum for OH-C_{10}-HSL. Analytical conditions were as for Fig. 4

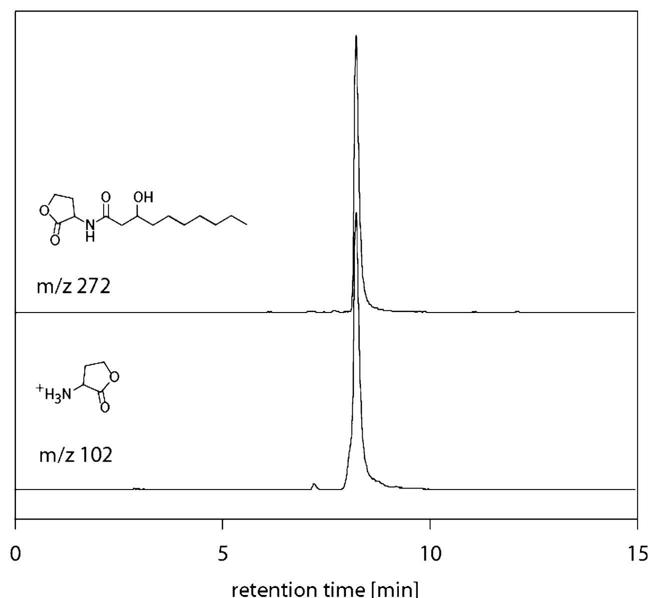

Fig. 6 Nano-LC–MS^2 chromatogram obtained from an *Acidovorax* sp. N35 extract: *top*, reconstructed ion chromatogram of *m/z* 272 (protonated OH-C_{10}-HSL); *bottom*, reconstructed ion chromatogram of *m/z* 102 (characteristic homoserine lactone fragment [32]) from the same run. The fragmentation energy was 22.5%. Experimental details are given in the section *Nano-LC–ESI ion-trap mass spectrometry*

marised in Table 4. Except for the sodium-adduct ion of OH-C_{10}-HSL, all peaks of other putative AHLs listed in Table 4 were at the limit of detection with an intensity of 5,000,000 or less, and were thus too small for examination of the isotope peaks, so these compounds may have a completely different elemental composition. These peaks also seem to appear randomly and cannot be correlated with one of the isolates, or one type of medium. OH-C_{10}-HSL was detected in all samples as its Na adduct, with its isotope peak (Fig. 5); it was found irrespective of medium type or of isolation procedure, albeit in different amounts.

This finding satisfactorily corroborates results from UPLC analysis, in which OH-C_{10}-HSL was predicted by the retention time simulation and was further confirmed by nano-LC–ESI ion-trap mass spectrometry. Figure 6 shows the reconstructed ion chromatograms of *m/z* 272 (protonated OH-C_{10}-HSL) and *m/z* 102 (characteristic homoserine lactone fragment [32]) from a nano-LC–MS^2 run at 22.5% fragmentation energy.

AHL Bioassay

With TLC it was not possible to detect any AHLs after solid-phase extraction. By use of liquid-liquid extraction and ten times more of the bacterial culture supernatant a weak spot could be detected between references C_8-HSL and C_6-HSL corresponding to a molecule of intermediate log *P*, for example OH-C_{10}-HSL. Further characterization of this signal was not possible because synthetic reference substances were not available. The weak signal produced by the reporter strain used was probably because of a lack of sensitivity for the rather atypical OH-C_{10}-HSL. Another bioreporter would be necessary to characterize this homoserine lactone.

Conclusion

Analytical procedures including bioassay and chromatographic techniques for the structural assignment of AHLs are summarized in this paper. Although several methods with either high sensitivity or high selectivity have been developed for analysis of AHLs in supernatant from spent culture media, few studies have investigated the role of matrix effects and the performance of a method. In addition to the overview of the different methods published for detection of AHL, a conceptual framework is presented that enables identification of AHL species produced by bacteria. By use of UPLC, FTICR-MS, nano-LC–MS, and a biosensor, 3-(hydroxydecanoyl)-homoserine lactone was unambiguously identified in supernatant from culture of *Acidovorax* sp. N35. The effect of the growth medium on UPLC and FTICR-MS analysis was also studied. Co-elution of the target compounds and matrix constituents was observed in UPLC. This interference could, however, be eliminated by changing the separation conditions. Ionisation in the MS was not affected by the medium. Coupling of UPLC to TOFMS is planned, and is needed in the future for direct on-line assignment of the structures of AHLs (by MS^2). A similar analytical strategy has been developed for hydrolysis products of AHLs and other quorum-sensing molecules.

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