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REVIEW

Perceiving the chemical language of Gram-negative bacteria: listening by high-resolution mass spectrometry

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Abstract Gram-negative bacteria use *N*-acylhomoserine lactones (AHLs) as their command language to coordinate population behavior during invasion and colonization of higher organisms. Although many different bacterial bioreporters are available for AHLs monitoring, in which a phenotypic response, e.g. bioluminescence, violacin production, and β-galactosidase activity, is exploited, mass spectrometry (MS) is the most versatile detector for rapid analysis of AHLs in complex microbial samples, with or without prior separation steps. In this paper we critically review recent advances in the application of high-resolution MS to analysis of the quorum sensing (QS) signaling molecules used by Gram-negative bacteria, with much emphasis on AHLs. A critical review of the use of bioreporters in the study of AHLs is followed by a short methodological survey of the capabilities of high-resolution mass spectrometry (HRMS), including Fourier-transform ion cyclotron resonance (FTICR) MS and quadrupole time-of-flight (qTOF) MS. Use of infusion electrospray ultrahigh-resolution FTICR MS (12 Tesla) enables accurate mass measurements for determination of the elemental formulas of AHLs in *Acidovorax sp. N35* and *Burkholderia ubonensis* AB030584. Results obtained by coupling liquid chromatography with a hybrid quadrupole linear ion trap-FTICR mass spectrometer (LC–LTQ-FTICRMS, 7-T) for characterization of acylated homoserine lactones in the human pathogen *Pseudomonas aeruginosa* are presented. UPLC–ESI-qTOF MS has also proved to be suitable for identification of 3O-C_{10}HSL in *Pseudomonas putida* IsoF cell culture supernatant. Aspects of sample preparation and the avoidance of analytical pitfalls are also emphasized.

Keywords *N*-Acyl-homoserine lactones · Gram-negative bacteria · Quorum sensing · FTICR MS · UPLC-ESI-qTOF MS · Mass spectrometry

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Abbreviations

ACN	Acetonitrile
AHLs	*N*-Acyl-homoserine lactones
amu	Atomic mass unit expressed in Da
DPD	4,5-Dihydroxy-2,3-pentanedione
EI	Electron impact
ESI	Electrospray ionization
FA	Formic acid
FTICR	Fourier-transform ion cyclotron resonance
HRMS	High-resolution mass spectrometry
LC	High-performance liquid chromatography
LTQ	Quadrupole linear ion trap

MeOH	Methanol
MS	Mass spectrometry
m/z	Mass over charge
QS	Quorum sensing
qTOF	Quadrupole time-of-flight
UPLC	Ultra-pressure liquid chromatography

Autoinducers and their function in quorum sensing

The chemical language of bacteria is based on signaling compounds, also known as autoinducers (AIs), which are continually released into their surrounding medium. Recognition of these signals is enabled by use of a variety of chemical receptors sensitive for intraspecies and interspecies communication. When the bacterial population density increases, or when the concentration of these compounds rises past a specific threshold, a selection of genes are turned on or off, helping the bacteria switch between solitary and group activity. This method of communicating and coordinating behavior via diffusible signaling molecules [1–3] is called quorum sensing (QS) and was first described for the bioluminescent Gram-negative bacterium *Vibrio fischeri*. Although this bacterium is non-luminescent when free-living in sea water [4], it is able to symbiotically colonize the light organ of some fishes and squids where it produces light. Production of light by *V. fischeri* can also be observed in liquid cultures and is associated with high population densities [5]. Nealson et al. attributed this phenomenon to autoinduction, a process during which an extracellular signal induces light-production genes in the bacterial population [6, 7]. When *V. fischeri* cells are free-living, i.e. when they are not associated with a host, they are present at very low concentrations and the autoinducer does not accumulate to levels that induce luciferase gene expression.

In a quorum sensing regulatory system, the bacterium produces one or more types of signaling molecule. In Gram-negative bacteria, two types of AIs have been observed: AI-1 and AI-2 (Fig. 1). AI-1 molecules are a family of *N*-acyl-homoserine lactones (AHLs) with a lactone core for each compound; AI-2 molecules, also termed a "universal" bacterial signal, are a group of interconverting molecules all derived from the common precursor 4,5-dihydroxy-2,3-pentanedione (DPD) produced and detected by a wide variety of bacteria [8–11].

The chemical structure of AHLs contains a homoserine lactone moiety, derived from amino acid metabolism, linked to a variable acyl side-chain, putatively derived from fatty acid biosynthesis. Common variations of the *N*-acyl-side-chain structure include chain length and the nature of a substituent (i.e., oxo or hydroxy group) at the third carbon indicated "R" in Fig. 1a. Several bacteria produce the same

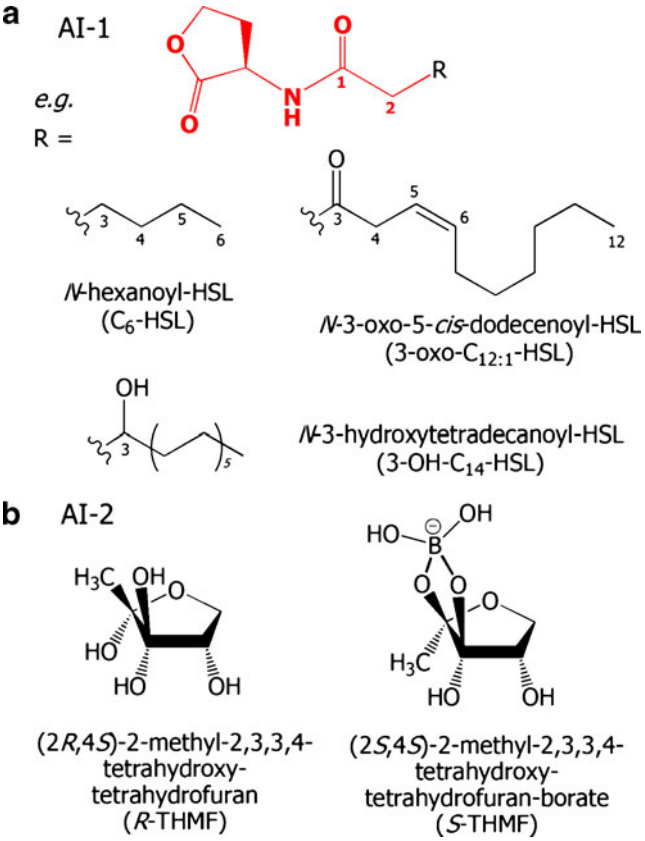

Fig. 1 Chemical structures of diffusible molecules called autoinducers 1 and 2, AI-1 and AI-2: **a** *N*-acylhomoserine lactones (AHLs) with their basic chemical structure and three examples; the lactone core of each molecule is shown in *red*. The acyl side chains may contain one or more double bonds. **b** Two examples of active AI-2 molecules, (2*R*,4*S*)-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran (*R*-THMF) and the anion furanosyl borate diester of (2*S*,4*S*)-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran (S-THMF)

signaling molecule, although, in each species such a mediator is used to regulate the expression of different target genes [12]. Other bacteria have been shown to produce multiple AHLs, each having different effects on the phenotype [13]. Numerous cellular functions that are activated by QS have been described, for example nutrient acquisition, toxin release, becoming virulent invaders, and, inter alia, giving rise to biofilm formation [14]. Insights into the molecular features required for small molecule activation and/or inhibition of these QS systems should prove to be valuable in the rational design of chemical tools, with improved activity or selectivity, to study or manipulate these cellular functions. It is believed that blockage of QS-mediated virulence in bacteria would prevent the production of virulence factors that are an immediate cause of disease in bacterial infections. Therefore, QS is an obvious target for a novel class of antimicrobial drugs which would function to efficiently block reception of the cognate QS signals in vivo, and thereby be capable of inducing chemical attenuation of virulence. Because QS is not directly involved in processes

essential for growth of the bacteria, the use of signal-molecule-based drugs to attenuate bacterial virulence rather than bacterial growth is attractive for several reasons, particularly considering the emergence of increasingly antibiotic-resistant bacteria [15, 16].

The main challenge with bacteria is to reveal the molecular basis of their communication. In this quest, Gram-negative bacteria are increasingly perceived as systems in which the dynamic interplay of a large number of components determines the output of many biological processes occurring in parallel. To characterize these processes and to reveal the underlying principles, it is necessary to evaluate the dynamic composition and location of the molecular components. Among different possible approaches used to study QS, mass spectrometry is increasingly applied to acquire the data important for understanding these processes. This technology is rapidly advancing and modern MS instruments are very important for study of bioreporters, and many MS approaches and instruments have been used in the last ten years [17–23]. Here, we will mainly focus on two particular forms of high-resolution MS that has proven robust and successful in our hands.

We are interested in increasing our knowledge of AHLs and their hydrolyzed products (e.g., open-ring forms [24–29]) in bacterial supernatants and better understanding the chemical language of bacteria by use of high resolution separation techniques and high resolution mass spectrometry, namely Fourier-transform ion cyclotron mass spectrometry (FTICR MS or FTMS) and quadrupole time-of-flight (qTOF) mass spectrometry. Here, we present a short introduction to the issue of cell–cell communication of bacteria and the quorum sensing phenomenon extended to a series of bacteria. In a critical overview of the use of bioreporters in the study of AHLs, examples are selected to illustrate how FTMS methods are used to obtain accurate-mass elemental formulas which are then used to elucidate the production of signaling molecules involved in the chemical language of two rhizosphere bacteria, *Acidovorax sp. N35* and *Burkholderia ubonensis* AB030584. Results obtained by coupling high-performance liquid chromatography (LC) with a quadrupole hybrid linear ion trap-FTICR mass spectrometer (LC-LTQ-FTMS) for analysis of acylated homoserine lactones in bacterial isolates of *Pseudomonas aeruginosa*, well known for its pathogenicity, are also presented. In our view, the extent of our understanding the molecular basis of bacterial communication will benefit from the use of accurate-mass-based data.

Quorum sensing and the cell–cell communication

In the last two decades or so there has been growing awareness that almost all bacteria are capable of multicellular behavior [1–3]. The ability of a single bacterium to communicate with its neighbors to mount a unified response that is advantageous to its survival makes much sense. Bacteria which use QS, i.e. produce and secrete signaling compounds, inhabit diverse ecological niches where survival depends on their capacity to sense the local environmental conditions and adapt by regulating the expression of specific genes. Not surprisingly, bacteria have developed highly sophisticated mechanisms to gather, process, and transduce environmental information. Bacteria using an AHL-dependent quorum sensing system have a receptor which can specifically detect the autoinducer. When the autoinducer binds to its receptor, it activates transcription of specific genes, including those for autoinducer synthesis. If only a few other bacteria of the same kind are in the vicinity, diffusion reduces the concentration of the autoinducer in the surrounding medium, so the bacteria produce little autoinducer. At high bacterial density, the concentration of the autoinducer passes a threshold, so more autoinducer is synthesized. This forms a positive feedback loop, and the receptor becomes fully activated. For example, opportunistic bacteria can grow within a host without harming it, until they reach a specific concentration. When their numbers are sufficient to overcome the host's immune system, they change their behavior and initiate disease. Gram-negative bacteria use quorum sensing to regulate a diverse array of physiological activity [3, 30, 31].

QS controls crucial functions in pathogenesis, so disrupting quorum sensing is a potential method for developing new antimicrobial therapeutics. In Gram-negative bacteria at least two chemically different AIs, AI-1 and AI-2, are involved in regulation. As already mentioned, DPD is the precursor to all AI-2 signaling molecules. It is biosynthesized by the LuxS protein in the *S*-adenosylhomocysteine pathway, and is a highly unstable molecule. It undergoes dehydration and cyclization reactions in solution to give a mixture of interconvertible furanones; initially, two epimeric furanoses are formed, (2*R*,4*S*) and (2*S*,4*S*)-2,4-dihydroxy-2-methyldihydrofuran-3-one (*R* and *S*-DHMF, respectively). Hydration of *R* and *S*-DHMF then gives rise to *R* and *S*-THMF, respectively, and complexation of *R*-THMF with borate forms a furanosyl borate diester [32]. Two examples of AI-2 are illustrated in Fig. 1b. An interesting observation is that AI-2 is conserved among many different bacterial species, including *Escherichia coli* and other enteric bacteria [33]. Apparently, AI-2 molecules are used for interspecies communication [34].

The LuxR–LuxS model system in Gram-negative bacteria: from bioluminescence in *Vibrio fischeri* to virulence in *Pseudomonas aeruginosa*

AHLs are important "messenger" molecules involved in cellular communication of Gram-negative bacteria. Use of

a bioreporter to screen spent culture supernatant led to the discovery that AHLs are produced by a plethora of unrelated bacteria. These AHL autoinducers control a variety of physiological processes, including bioluminescence, biofilm formation, and production of virulence determinants (Table 1).

Acyl-homoserine lactone-specific quorum sensing in Gram-negative bacteria generally involves two protein components, a signal synthase (LuxI homolog) that catalyzes the synthesis of acyl-HSLs signals and an acyl-HSL-responsive transcriptional factor (LuxR homolog). As the local bacterial population density increases, the acyl-HSLs accumulate to physiologically relevant threshold concentrations and interact with the cognate transcription factor [35]. In most cases, signal-bound LuxR-type proteins activate the regulated target genes, whereas others function as transcriptional repressors in a ligand-free state [36]. The overall effect is the coordinated transcription of specific gene systems in response to a bacterial quorum. These acyl-HSL-regulated gene systems are diverse and include a range of different virulence functions with involvement in plant and animal diseases, and beneficial effects. Several LuxI homolog proteins catalyze the synthesis of a range of specific acyl-HSLs [37].

The substrates for AHL synthesis by LuxI homologs (i.e., LuxI-type AHL synthases) are *S*-adenosylmethionine (SAM) and the appropriate acylated carrier protein (acyl-ACP) [38]. Figure 2 depicts the synthesis of *N*-(3-oxohexanoyl)-L-homoserine lactone. For diverse Gram-negative bacteria, many different AHL signal molecules have been characterized. The marine bacterium *V. fischeri* uses 3-oxo-C_6–HSL (*N*-(3-oxohexanoyl)-L-homoserine lactone) as a QS signaling molecule [7, 34]. The synthesis of 3-oxo-C_6-HSL is catalyzed by the LuxI-AHL-synthase, using SAM and hexanoyl-acyl carrier protein as substrates for the reaction [39]. The QS response regulator is LuxR, a DNA-binding transcriptional regulator, which becomes activated on physically binding to 3-oxo-C_6-HSL [40]. In Gram-negative bacteria AHL synthesis is usually catalyzed by enzymes that are homologous with the LuxI protein from *V. fischeri*, and the molecules are sensed by members of the LuxR protein family which contain both AHL and DNA-binding domains [41]. Many AHLs cross membranes freely and are detected in the cytoplasm by LuxR-type proteins. Upon ligand binding, the LuxR-AHL complexes bind DNA promoter elements and activate transcription of quorum sensing-controlled genes [40, 42]. The specificity of the LuxR-AHL interaction is conferred by an acyl binding pocket in the LuxR protein, which precisely accommodates the acyl chain of its cognate AHL signal [43, 44].

As mentioned earlier the AI-1 regulatory system consists of two structural genes: LuxI that encodes the AI-1 synthase and LuxR that encodes the AI-1 response regulator. LuxI and LuxR homologues are present in a wide variety of Gram-negative bacteria and control several processes ranging from virulence genes to biofilm formation (Table 1). The gene responsible for AI-2 production (LuxS) is highly conserved across many species and the ability of AI-2 from a diverse group of species to regulate gene expression in other bacterial species indicates that it may have a role in inter-species communication as opposed to intra-species communication typical of AI-1 autoinducers. The AI-2 system is particular interesting because it has been correlated with the pathogenicity of several organisms. With regard to symbiosis, multiple signaling systems may be important in a complex community structure in which bacterial species need to coordinate their activities with bacteria of the same species and with a host of other bacterial species.

The bacterium *Pseudomonas aeruginosa* is an opportunistic pathogen which proliferates within hosts as varied as

Table 1 Examples of AHL-mediated QS systems with their corresponding functions[a]

Gram-negative bacteria	QS regulatory system (i.e., LuxI/LuxR homologue)	Cognate signal	QS-regulated phenotypes	Ref.
Vibrio fischeri	LuxI/LuxR	$3OC_6$-HSL	Bioluminescence	[7, 27, 72]
Aeromonas hydrophilia	AhyI/AhyR	C_4-HSL	Production of protease	[73]
Burkholderia cepacia	CepI/CepR	C_6-HSL, C_8-HSL	Production of siderophore and exoprotease	[74]
Pseudomonas aeruginosa	LasI/LasR RhlI/RhlR	C_4-HSL $3OC_{12}$-HSL	Production of virulence factors	[46, 47]
Salmonella enterica serovar *Typhimurium*	SdiA (LuxR homologue)	$3OC_6$-HSL, $3OC_8$-HSL, C_6-HSL, C_8-HSL	Production of possible virulence determinants	[42, 75]
Serratia liquefaciens	SwrI/SwrR	C_4-HSL, C_6-HSL	Adhesion, swarming and biofilm cluster formation	[76]
Agrobacterium vitis	avsI/avsR	C_{12}-HSL, $3OC_{14}$-HSL, $3OC_{16:1}$-HSL, $C_{16:1}$-HSL, and C_{18}-HSL	Grape necrosis, hypersensitive-like response	[77]

[a] Homoserine lactone (HSL) ring

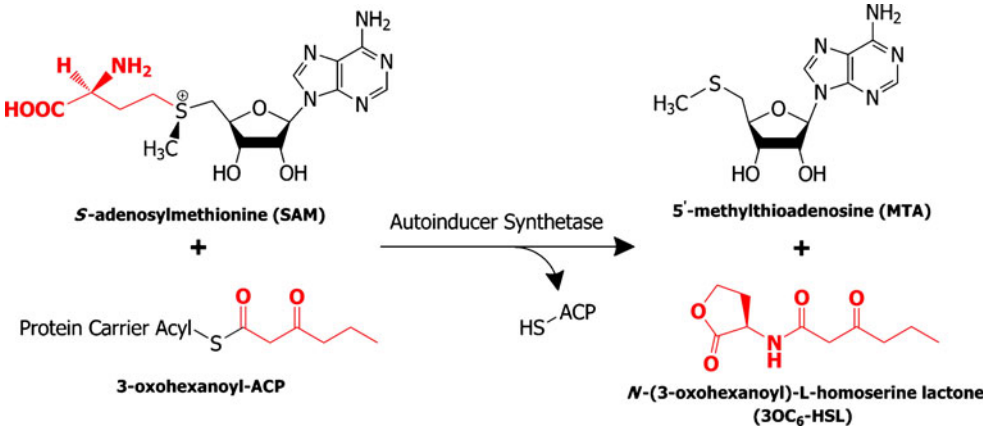

Fig. 2 Enzymatic synthesis of *N*-(3-oxohexanoyl)-L-homoserine lactone showing, *in red*, the involved groups of the resulting molecule. *S*-Adenosylmethionine (SAM) is the common substrate, with the appropriately charged acyl–acyl carrier protein (acyl-ACP) for AHL synthesis [38]

mammals, insects, nematodes, and plants, and interacts with yeasts also. In humans it causes urinary tract, respiratory system, bone, joint, gastrointestinal, and a variety of systemic infections, particularly in patients with severe burns and in cancer and AIDS patients who are immunosuppressed or have cystic fibrosis. Different *P. aeruginosa* strains have the remarkable ability to inhabit diverse environments and infect a range of organisms [45]. It is recognized that the virulence of the opportunistic human pathogen *P. aeruginosa* is controlled by an *N*-acyl-homoserine lactone (AHL)-dependent quorum-sensing system with a long and a short chain AHL, viz. *N*-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C_{12}-HSL) and *N*-butanoyl-L-homoserine lactone (C_4-HSL), respectively. *P. aeruginosa* utilizes two *N*-acyl-homoserine lactone (AHL)-dependent quorum-sensing systems, termed *Las* and *Rhl*, which together regulate an extensive set of cell population density and growth-phase-dependent virulence factors [46, 47]. The *Las* and *Rhl* quorum-sensing systems use 3-oxo-C_{12}-HSL and C_4-HSL, respectively. A previous study in our laboratory has also shown that *P. aeruginosa* (strain PAO1) produces not only 3-oxo-C_{12}-HSL and C_4-HSL, but a wider range of AHLs, i.e. C_6-HSL, C_8-HSL, and an extra keto derivative, i.e. 3-oxo-C_{10}-HSL when grown in TSB broth. It has also been observed that 3-oxo-C_{12}-HSL and 3-oxo-C_{10}-HSL are actually present also as hydrolyzed forms in the bacterial strain investigated [25].

Detection of *N*-acylhomoserine lactones by bioreporters

Genetically engineered bacterial whole-cell sensing systems are a useful tool for assessing QS-signal-molecule levels in real samples [48]. However, until now screening for AHL production from bacterial strains has typically relied on bacteriological monitor systems. These bioreporter bacteria consist of a phenotypic response, e.g. bioluminescence, violacin production, or β-galactosidase activity, activated through an AHL-receptor protein. Each receptor protein responds to a different range of AHL signaling molecules [49]. Thus, the TraR of *Agrobacterium tumefaciens* is highly sensitive to AHLs with an acyl chain length from C_6, whereas the CviR of *Chromobacterium violaceum* responds mainly to short-chain unsubstituted AHLs. Currently, several AHL bacterial bioreporters are available to test for and identify AHL production in QS systems by measuring the activity of the reporter system present in the bioreporter bacterial strain [50]. Each bioreporter relies on a particular LuxR family protein, which has enough selectivity for the cognate AHL and in some cases for closely related AHLs. Double plus signs in Table 2 indicate the most active AHLs. Because many bioreporters detect a narrow range of AHLs, each responding to AHLs with different structural features, the use of several bioreporters is needed. AHLs are purified from spent supernatant of late exponential phase cultures by liquid–liquid extraction with organic solvents. These extracts are subsequently subjected to thin-layer chromatography (TLC) on C_{18} reversed-phase plates, and detected by coating the TLC plates with genetically modified AHL-sensitive reporter strains with readily detectable physiologic reactions, for example light emission, enzymatic activity, or pigment production.

A commonly used bioreporter is based on *C. violaceum*, a Gram-negative water and soil bacterium that produces the purple pigment violacein. *C. violaceum* (strain CV026) is able to induce violacein production in response to all the AHL compounds with *N*-acyl side chains from C_4 to C_{12} in length, with varying degrees of selectivity. The most active agonist AHL for CV026 is C_6-HSL, i.e. the natural *C. violaceum* AHL; other AHLs that induce reasonably well include 3-oxo-C_6-HSL, 3-oxo-C_8-HSL, C_8-HSL, and C_4-HSL. Strain CV026 responds very poorly to AHLs with C_{10} acyl chains; longer chains have little or no agonist activity, as confirmed by recent work [26] in which the minimum signal-inducing concentration of selected AHLs was determined for *C. violaceum* (strain C026). In addition, all 3-hydroxy-AHLs are not detected by strain CV026.

Table 2 Reporter strain (or plasmid) sensitivities to common AHLs[a]

AHL[b]	*C. violaceum* (CV026)[b] [78]	*A. tumefaciens* NT1 (pZLR4) (pCF218+)[c] [79]	*E. coli* JM109 (pSB401)[d] [80]	*E. coli* JM109 (pSB536)[d] [73]	*E. coli* JM109 (pSB1075)[d] [80]
C_4-HSL	++	−	−	++	−
C_6-HSL	++	+	+	+	−
C_8-HSL	++	+	+	+	−
C_{10}-HSL	−	+	+	−	+
C_{12}-HSL	−	+	+	−	++
C_{14}-HSL	−	+	−	−	−
3-oxo-C_4-HSL	++	+	−	+	−
3-oxo-C_6-HSL	++	+	++	+	−
3-oxo-C_8-HSL	++	++	+	+	−
3-oxo-C_{10}-HSL	−	+	+	−	+
3-oxo-C_{12}-HSL	−	++	++	−	++
3-oxo-C_{14}-HSL	−	+	−	−	+
3-OH-C_4-HSL	n.e.	++	−	−	−
3-OH-C_6-HSL	n.e.	+	−	−	−
3-OH-C_8-HSL	n.e.	++	n.e.	n.e.	n.e.
3-OH-C_{10}-HSL	n.e.	n.e.	n.e.	n.e.	n.e.
3-OH-C_{12}-HSL	n.e.	n.e.	n.e.	n.e.	n.e.
3-OH-C_{14}-HSL	n.e.	n.e.	n.e.	n.e.	n.e.

[a] Double and single plus signs indicate best and normal response, respectively; minus sign indicates lack of detection, and n.e. indicates no experimental data for a given AHL by the indicated bioreporter

[b] Exposure of this strain to exogenous AHLs results in rapid production of visually clear purple pigmentation because of violacein

[c] These reporter systems are β-galactosidase-based bioreporters

[d] Exposure of these strains to exogenous AHLs induces bioluminescence

Several other bioreporters rely on a plasmid harboring the *luxCDABE* operon. These bioreporters, in the presence of AHLs, induce bioluminescence. In TLC analysis, bioluminescence can be easily detected by use of a luminometer with a TLC plate coated with the bioreporter. These plasmids are usually harbored in *Escherichia coli*, which typically does not produce AHLs. Three LuxR bioreporters, *E. coli* pSB401, *E. coli* pSB536, and E. coli pSB1075 are used for identification of AHLs. Although the bioreporter pSB536 is most sensitive to cognate C4-HSL, the bioreporter pSB1075 responds strongly to 3-oxo-C12-HSL, 3-oxo-C10-HSL, and C12-HSL. Both these systems can be conveniently used in conjunction with TLC analysis. *Agrobacterium tumefaciens* AHL bioreporters based on the TraI/R QS system detect a broad range of AHLs occurring in spent culture supernatants. AHL bioreporter *A. tumefaciens* NT1 (pZLR4) consists of strain NT1 cured of the Ti plasmid and thus unable to produce AHLs. The *A. tumefaciens* bioreporter is a β-galactosidase-based one well suited to TLC with standard β-galactosidase activity determination. It is noteworthy that AHL bioreporters are limited to the selectivity requirements of the QS family proteins; hence, most bioreporters are restricted in the range of AHLs to which they can respond. A negative result may indicate that the tested bacterial strain is not producing AHLs, but for several reasons it is not possible to conclude from the non-appearance of a signal that it does not produce AHL(s). Bacteria might produce other AHLs that are simply not detectable by the bioreporters used. For instance, no AHLs with an odd number of carbon atoms in the acyl chain have been reported by use of a bioreporter. In addition, the bacterial strain tested may produce AHLs at very low concentrations, which are below the limit of detection of the bioreporter employed [26]. In fact, AHL QS systems may themselves be regulated, requiring specific environmental conditions to be switched on [50]. False-negative results using AHL bioreporters may also occur because of the bacteriostatic effect of compounds produced by the bacteria under investigation and/or by the bacterial sensor. Additionally, the combination of liquid–liquid extraction, chromatographic behavior (TLC), and AHL bioreporters can be only used to assign tentative chemical formulas and structures. These methods cannot provide a comprehensive profile of homoserine-lactones and closely related compounds which may occur in the supernatant of bacterial isolates. AHL structures may be unequivocally established on the basis

of their mass spectra and their spectroscopic properties, including nuclear magnetic resonance (i.e., ^{1}H and ^{13}C spectra). More important is that the simultaneous use of monitor strains is required in the process of screening bacterial populations for AHL production.

Identification of AHLs by high-resolution FTICR and qTOF mass spectrometry

The chemical language of bacteria can perhaps be better heard and understood by using different analytical approaches based on mass spectrometry (MS). Although use of capillary electrophoresis coupled with MS [51] and gas chromatography with subsequent electron-impact (EI) ionization mass spectrometric analysis [52, 53] have been reported, a more reliable approach seems to be combination of high-performance liquid chromatography with MS [10–15]. It is noticeable that in MS there has been great progress owing to innovative instrumentation with ultra-high resolution and high mass accuracy operating in the ion-trapping configuration under ultra-high vacuum [54, 55]. High mass accuracy is achieved when the absolute mass error assigned to a given ion signal is low and all systematic mass errors are absent. Sub-ppm mass accuracy is routinely achieved with high-magnetic-field Fourier-transform ion cyclotron resonance (FTICR) mass spectrometers, making the assignment of molecular formula very reliable for completely unknown small molecules (ca 500 Da) [56]. The attractive combination of high sensitivity and high mass accuracy for both precursor and product ions of quadrupole with time-of-flight (qTOF) mass spectrometers could also help in the search for signaling molecules [57]. These hybrid instruments benefit from the versatile ability of quadrupoles to carry out a variety of tasks and from the high performance of TOF spectrometers in both simple mass spectrometry (MS) and tandem (MS–MS) modes [58]. Use of predicted isotopic distribution with the observed accurate mass has been used for some time as a means of more firmly establishing a proposed molecular formula [59]. Yet, in a more detailed study of the relationship between "exact-mass" and isotopic distribution, Kind and Fiehn [60] stressed that when a full array of elements are considered (C, H, N, S, O, P, and, potentially, F, Cl, Br, and Si), many possible chemical formulas can be obtained and that the additional orthogonal filter based on the isotopic abundance is useful for verification of the unique molecular formula, even when mass errors are less than 1 ppm.

As already mentioned, FTICR MS, especially at high-fields (e.g., 12 Tesla), enables reliable chemical characterization of metabolites of known and hitherto unknown structures in complex and heterogeneous samples of biological origin. Mass spectra 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 0.2 ppm are possible with such a system. The use of newly available on-chip nanoelectrospray ionization systems in this context enables another significant increase in sensitivity, substantial reduction of the amount of sample, and more efficient ionization of low-abundance ions in the presence of highly abundant (matrix) species. Use of a nanoESI source enabled the detection of putatively formed *N*-acylhomoserine lactones from an extract of the supernatant of *Acidovorax sp*. N35, a rhizosphere bacterium, grown in NB rich and M9 minimal media (Fig. 3). As expected, the M9 extracts generally cause less signal suppression during electrospray ionization, so less noise and fewer ions are accumulated in the FTICR cell, enabling better detection in terms of signal-to-noise ratio.

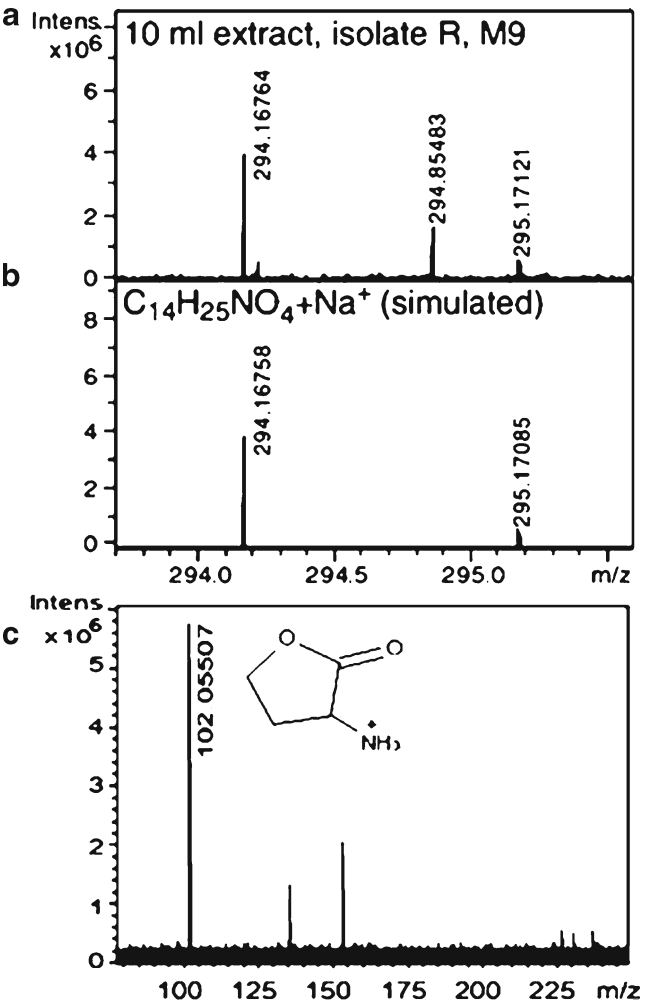

Fig. 3 **a** Detection, by FTICR MS, of OH-C_{10}-HSL as the Na^+ adduct in the extract obtained from 10 mL supernatant of cell culture of *Acidovorax sp*. N35 in M9 medium. **b** Calculated spectrum for $[OHC_{10}\text{-HSL} + Na]^+$. Reprinted from Ref. [27] with permission from Springer. **c** MS^2 fragmentation and typical HSL fragment ion

The theoretical masses of possible AHLs both in the protonated form, $[M + H]^+$, and as sodium adducts, $[M + Na]^+$, were calculated and compared with masses measured in the extracts with mass differences less than 1 ppm. Both protonated ions and sodium adducts were taken into consideration, because the mass spectra obtained from the standard solution of alkanoyl-AHLs had both forms, and the peak intensity of sodiated species was always higher. In addition to the masses of the possible AHLs in the protonated form, the isotope peak was also considered for correct identification of the elemental composition. In this way it was possible to identify OH-C_{10}-HSL (mass error −0.11 ppm), which was detected in all samples as its sodium adduct (mass error 0.31 ppm), with its isotope peak. It was found irrespective of medium type or of isolation procedure, albeit in different amounts [27].

The ultra-high resolution capability of FTMS was also used to identify homoserine lactones produced by *Bukholderia ubonensis,* AB030584, a rhizosphere colonization bacteria. In detail, C_8-AHL was recognized after comparison of the theoretically calculated mass for C_8-AHL and the measured mass (mass error 0.07 ppm) [28]. As pointed out in previous papers, MS instruments with ultra-high resolution, i.e. better than ±0.1 ppm (0.2 absolute), lead to a single elemental composition [61, 62]. Hence, accurate mass measurements are useful for study of bacterial intraspecies and interspecies communication in the rhizosphere mediated by *N*-acyl homoserine lactones [29].

However, it is also obvious that isobaric ions cannot be differentiated so easily. Moreover, FTMS alone does not yield sufficient information about chemical structure; hence a combination of exact mass measurement with LC separation is a very useful alternative giving unprecedented insight into the nature of the AHLs in bacterial extracts. This is an example of the same "*divide and conquer*" rationale that has been successfully used in proteomics [63]. The relative ease of interfacing an electrospray ionization (ESI) source in MS detection with LC separation has facilitated very intriguing applications. Such a combined system (LC–ESI–FTICR MS) is an instrument with great capabilities for identification of unknown compounds, including those occurring in complex samples. The presence of chromatographic peaks corresponding to AHLs, as putative candidates occurring as metabolites of Gram-negative bacteria, can be evaluated by accurate screening of extracted ion chromatograms (XICs) of protonated molecules with a window width of ±0.0010 (i.e., ca 7 ppm mass accuracy at *m*/*z* 300). XIC collects ion intensities falling within a given mass-to-charge-ratio window; it seems to be the method of choice for mass analysis leading to very simple chromatograms. Reduction of interferences in the XICs (*vide infra*) significantly facilitates identification of putative AHLs [24].

Figure 4 shows a typical series of XICs, with a $\Delta m/z$ setting of 0.0020, for a standard solution of eight homoserine lactones, namely C4-HSL, 3-oxo-C6-HSL, C6-HSL, 3-oxo-C8-HSL, C8-HSL, C10-HSL, C12-HSL and C14-HSL, plus the internal standard, C7-HSL, separated by reversed-phase chromatography on a C_{16}-amide narrow-bore column and detected by high-resolution FTICR MS (for details, see Electronic Supplementary Material). The XICs reveal peaks even more easily distinguished by the further added selectivity of this state-of-the-art mass analyzer, which enables acquisition of individual traces for all AHL compounds with high selectivity and sensitivity, leading to correct assignment of the corresponding signals from different LC-ESI-FTICR MS runs. The estimated value of LOD, based on an *S*/*N* ratio of 3, was found to be 1 pmol when the FTICR cell was used [24]. As biofilms typically contain high cell concentrations, AHLs could be detected by use of such a method. Charlthon et al. estimated a 600 $\mu mol L^{-1}$ concentration for 3-oxo-C12 HSL in the *P. aeruginosa* biofilm [64]. The next example explains this approach to fingerprinting the presence of AHLs in bacterial culture supernatants.

Figure 5 shows LC–ESI–FTICR MS XICs from a bacterial supernatant of *P. aeruginosa* (strain ATCC 9027) that was filter-sterilized through 0.2-μm pore-size filters. The occurrence of chromatographic peaks corresponding to 99 known and unknown AHLs [24], as putative candidates occurring as metabolites of *P. aeruginosa,* was evaluated by accurate screening of the XICs of protonated molecules. Only compounds with a given *m*/*z* value were selected and plotted, thus acting as a filter that directly reduces the number of potential false-positive assignments. At least four presumed AHLs were identified in spent culture supernatant, including the two most well known AHLs, C_4-HSL and 3-oxo-C_{12}-HSL, with two saturated acyl-side chain AHLs, C_{10}-HSL, and C_{12}-HSL. Remarkably, only by use of this method for displaying the extracted ions is it possible to greatly reduce the signal complexity of the total ion current trace (data not shown) enabling us to distinguish all the AHLs completely. Nevertheless, in the XIC plots there is no structural information about the selected ion, leaving some uncertainty about the identity of the monitored compounds. This uncertainty may be critical when XIC plots are taken from very complex samples, because several compounds in bacterial isolates may produce ions with similar *m*/*z* under electrospray ionization, making selective detection not fully reliable. Given that the occurrence of more signaling molecules is likely to reflect fundamental aspects of the biology of *P. aeruginosa*, identification of these AHLs was investigated. The signaling molecules identified in bacterial isolates of two strains of *P. aeruginosa* are listed in Table 3.

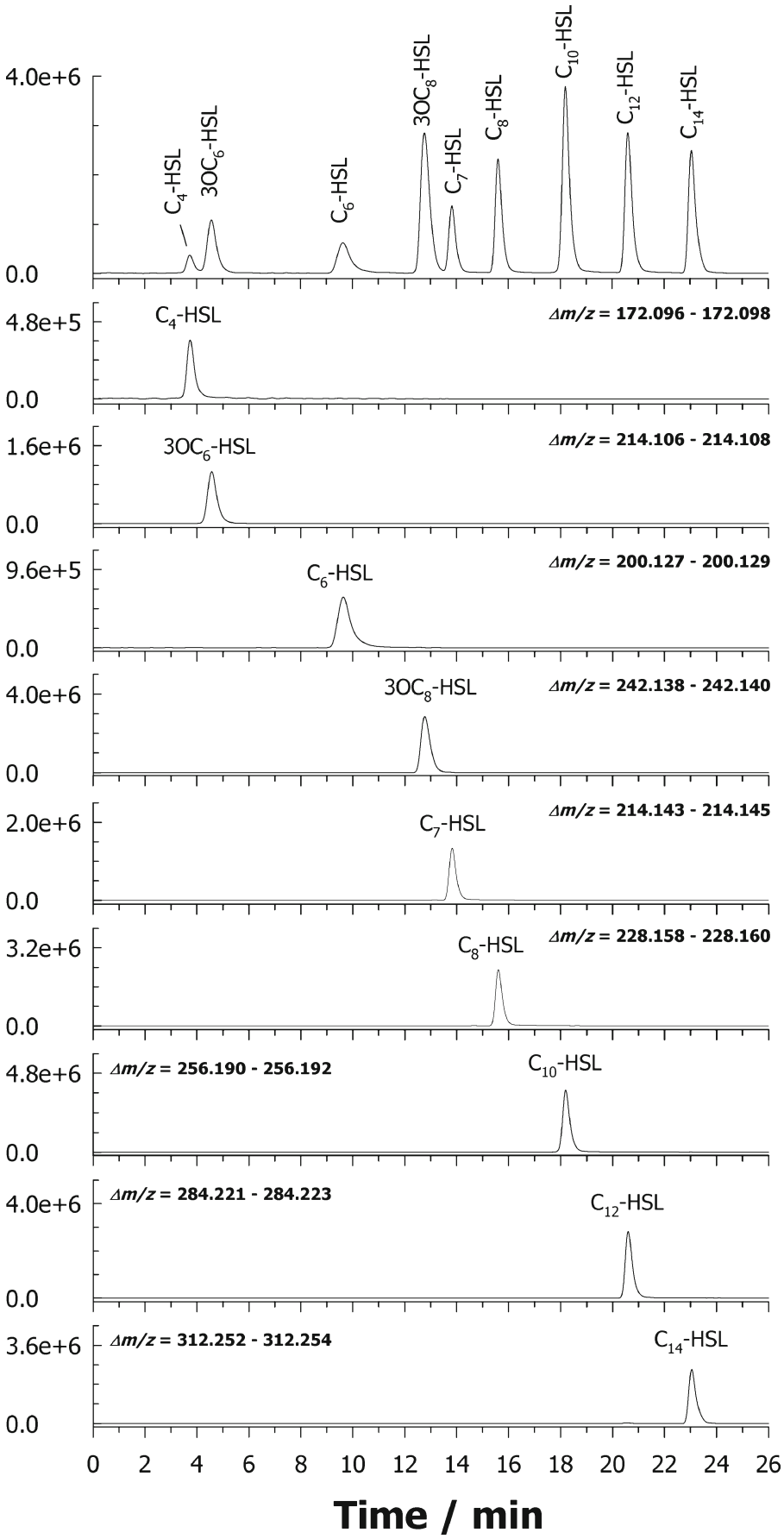

Fig. 4 Extracted-ion chromatograms obtained by LC–ESI–FTMS from a standard solution of 1 μmol L^{-1} AHLs. The ion monitored are displayed in each trace and correspond to the most abundant protonated molecules, $[M + H]^+$, using ±0.001 *m/z* unit centered around each selected ion. Chromatographic conditions: Supelcosil LC-ABZ C_{16}-amide narrow-bore column (5 mm, 250×2.1 mm i.d.); the mobile phase was a linear gradient prepared from 0.1 % HCOOH in water (component A) and 0.1 % HCOOH in acetonitrile (component B); the gradient program was from 70 % to 100 % B in 17 min, with a hold time of 3 min; the flow rate was 0.30 mL min^{-1} and 20 μL was injected into the column. A hybrid linear trap-FTICR (7-T) mass spectrometer was used. The heptanoyl-homoserine-lactone (C7-HSL, $[M + H]^+$ at *m/z* 214.14346) was used as internal standard

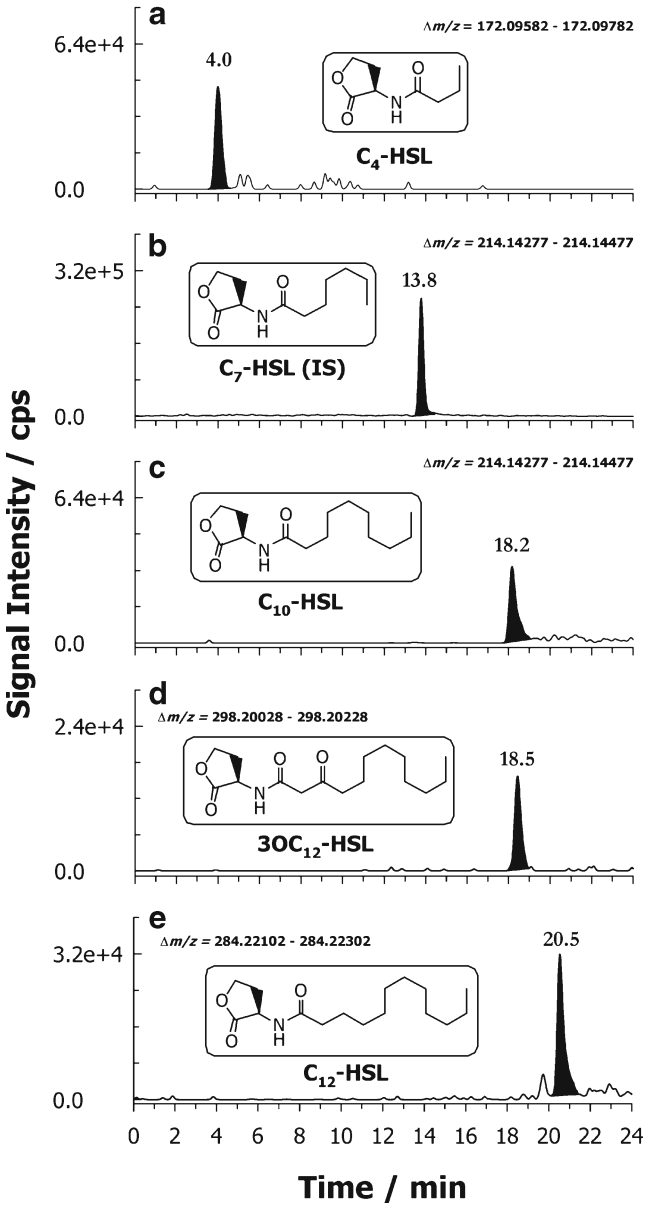

Fig. 5 LC–ESI–FTICR MS XICs, under optimized working conditions, of AHLs occurring in bacterial isolates of *P. aeruginosa* (strain ATCC 9027), with the structure and the retention time of each compound. The ions monitored are displayed in each trace and correspond to the most abundant protonated molecules, $[M + H]^+$, using a ±0.0010 *m/z* unit centered around each selected ion. The LC column was as described in Fig. 4. Bacterial cells were removed by centrifugation (13,000 rpm, 5 min), and the resulting supernatant was filter sterilized through 0.2-μm-pore-size filters (Millipore). For AHL determination, culture supernatants were first acidified with HCOOH and then incubated at 37 °C for 24 h. This was done to ensure that any AHLs hydrolyzed to the open-ring form during growth were recyclized. Other experimental conditions were as in Fig. 4. The gradient program was 100 % A for 3 min then increased to 50 % B in 17 min. All peaks were further confirmed by tandem MS in the linear ion trap analyzer

The main purpose of high-resolution LC–MS is identification and confirmation of candidate signaling molecules; to ensure correct identification of the possible new candidates, the selected mass spectra at the apex of each chromatographic peak were examined, as shown in Fig. 6. A four-step approach was used to confirm the identity of these unknown compounds as protonated molecules:

1. chromatographic behavior determined principally in terms of retention time and peak profile;
2. high mass-measurement accuracy;
3. isotope patterns; and
4. formation of characteristic fragments in tandem MS–MS, for example an ion at *m/z* 102.05 (Fig. 3).

Accurate mass measurements of the protonated molecule, $[M + H]^+$, at *m/z* 172.09692 (plot a) furnished a molecular mass, *M*, of 171.08974, which corresponds to the molecular formula $C_8H_{13}NO_3$. Likewise the ions at *m/z* 298.20098, (plot b) furnished mass values of 297.19362 ($C_{16}H_{27}NO_4$).

The ability of the FTICR trapping cell to maintain high mass accuracy at different ion intensities during elution of a chromatographic peak is also demonstrated for the $[M + H + 1]^+$ isotopic peak. Accurate mass data, chromatographic behavior, and isotopic pattern led to structure assignment for all AHLs occurring in the supernatants of *P. aeruginosa* strains. The mass accuracy for each of these compounds, using external calibration, was better than 2 ppm, irrespective of the measured ion intensity. A key observation in drawing the chemical composition conclusions is also confirmation of a typical fragment of AHLs formed in the LTQ mass analyzer. It is known that all members of the AHL family give a specific lactone ring at a nominal mass of *m/z* 102.05 (exact mass *m/z* 102.05493, Fig. 3c) [19, 24, 65]; this ion, which results from cleavage of the *N*-acyclic side chain from the homoserine lactone ring, was the focus of LTQ MS confirmation by selected reaction monitoring in collision-induced dissociation tandem MS–MS (not shown).

The main disadvantage of LC–FTMS coupling is the lengthy sample run time (not less than 20–30 min) compared with direct injection into the MS. High resolution mass measurements are possible, but the cost of instrument

Table 3 AHLS identified in bacterial strains of *P. aeruginosa* by LC–ESI–FTMS

P. aeruginosa	*N*-Acyl-homoserine lactones
Strain PA01	C_4-HSL, C_6-HSL, C_8-HSL, $3OC_{10}$-HSL, $3OC_{12}$-HSL, $3OC_{10}$-HS[a], and $3OC_{12}$-HS[a]
Strain ATCC 9027	C_4-HSL, $3OC_{12}$-HSL, $3OC_{12}$-HS,[a] C_{10}-HSL, and C_{12}-HSL

[a] *N*-Acyl-homoserine compounds (AHSs) are the open-ring forms of the corresponding AHLs

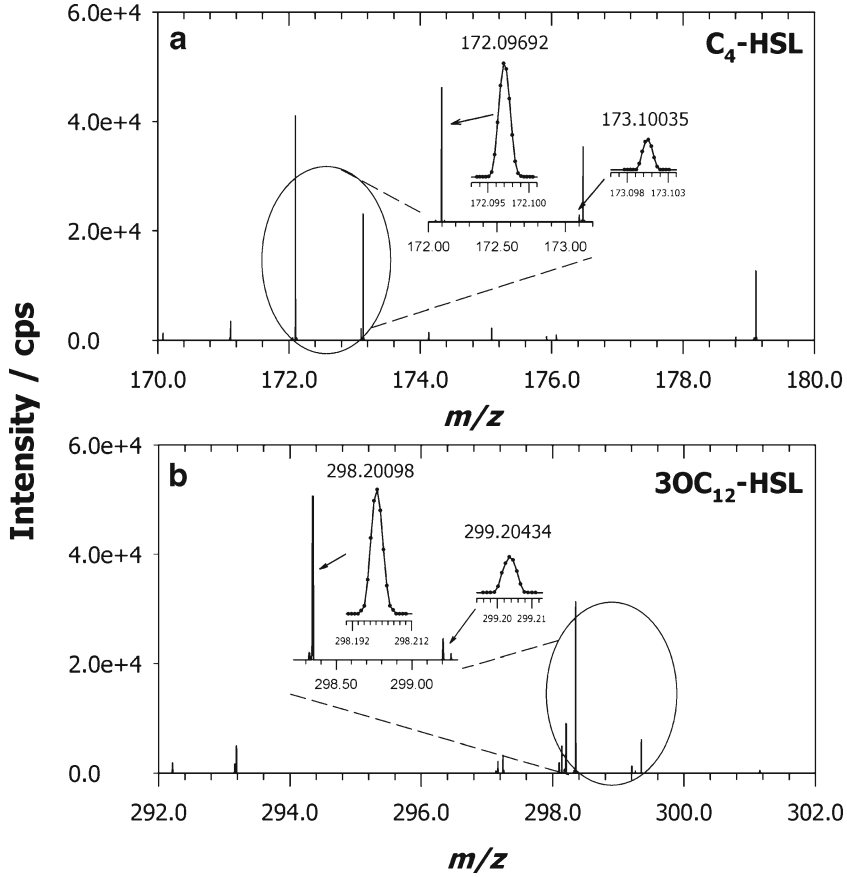

Fig. 6 High-resolution mass spectra of the two main AHLs secreted by the human opportunistic pathogen *P. aeruginosa*. Mass spectra were extracted from the chromatographic peaks at the retention times 4.0 and 18.5, min. **a** Analysis of the *m/z* 172.09702 ion gave an exact molecular mass *M* of 171.08974 Da, which corresponds to $C_8H_{13}NO_3$ (i.e., C4-HSL). **b** Analysis of the *m/z* 298.20090 ion gave an exact molecular mass *M* of 297.19362 Da, which corresponds to $C_{16}H_{27}NO_4$ (i.e., $3OC_{12}$-HSL). The experimental isotope patterns are shown as *insets* (see also Electronic Supplementary Material)

time and columns might compromises high-throughput applications. The introduction of ultra-high-pressure liquid chromatography (UPLC) dramatically reduced the total run time, enhanced chromatographic resolution, and achieved higher sensitivity by using sub-2 μm particles and smaller internal diameter columns (1–2 mm). Narrower peaks can be obtained with peak-width of 1 s and a mixture of 15 AHLs can be separated in less than 4 min (Fig. 7), on the basis of a previously described method [66]. Yet, high-resolution measurements in FTICR-MS require longer processing time and its on-line coupling with UPLC is technically unworkable. To overcome this problem, Li et al. [67] suggested a method in which fraction collection combined with on-chip nanoelectrospray ionization systems was used as UPLC–nESI–FTICR MS interface for study of signaling molecules in *Erwinia* sp. culture (Electronic Supplementary Material, Fig. S1). Their work showed how mass resolution is affected by the sample matrix leading to uncertainties and misinterpretation of results in direct infusion measurements, whereas at-line measurements of fractionated samples improve mass accuracy and signal-to-noise ratio and exhibit fewer disturbed mass peaks. Some advantages of the at-line approach include the good repeatability and fast separation provided by UPLC, UV spectra and retention time information from the PDA detector, and accurate mass spectra that can be acquired for each fraction collected, minimizing false-positive results and enabling simultaneously purification of the

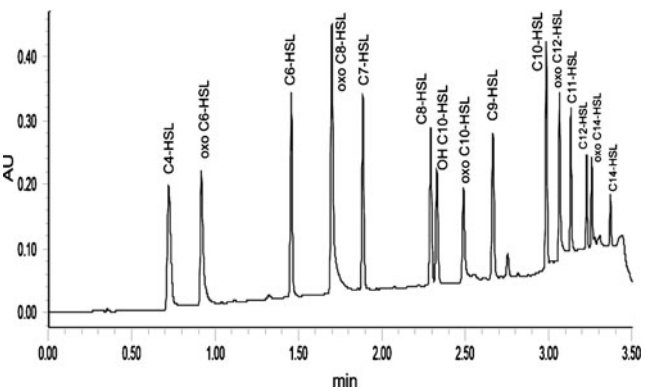

Fig. 7 UPLC–PDA chromatograms of a standard mixture of AHLs. Chromatographic conditions: 100 mm×2.1 mm, 1.7 μm particle size, BEH C_{18} column; the mobile phase was a linear gradient prepared from water containing 10 % acetonitrile (component A) and acetonitrile (component B); the gradient program was from 0 to 48 % B in 2.55 min, then to 100 % B in 3.5 min; the flow rate was 0.8 mL min^{-1}, the column temperature was 60 °C, and the autosampler temperature was 27 °C. (Reproduced from Ref. [27], with permission)

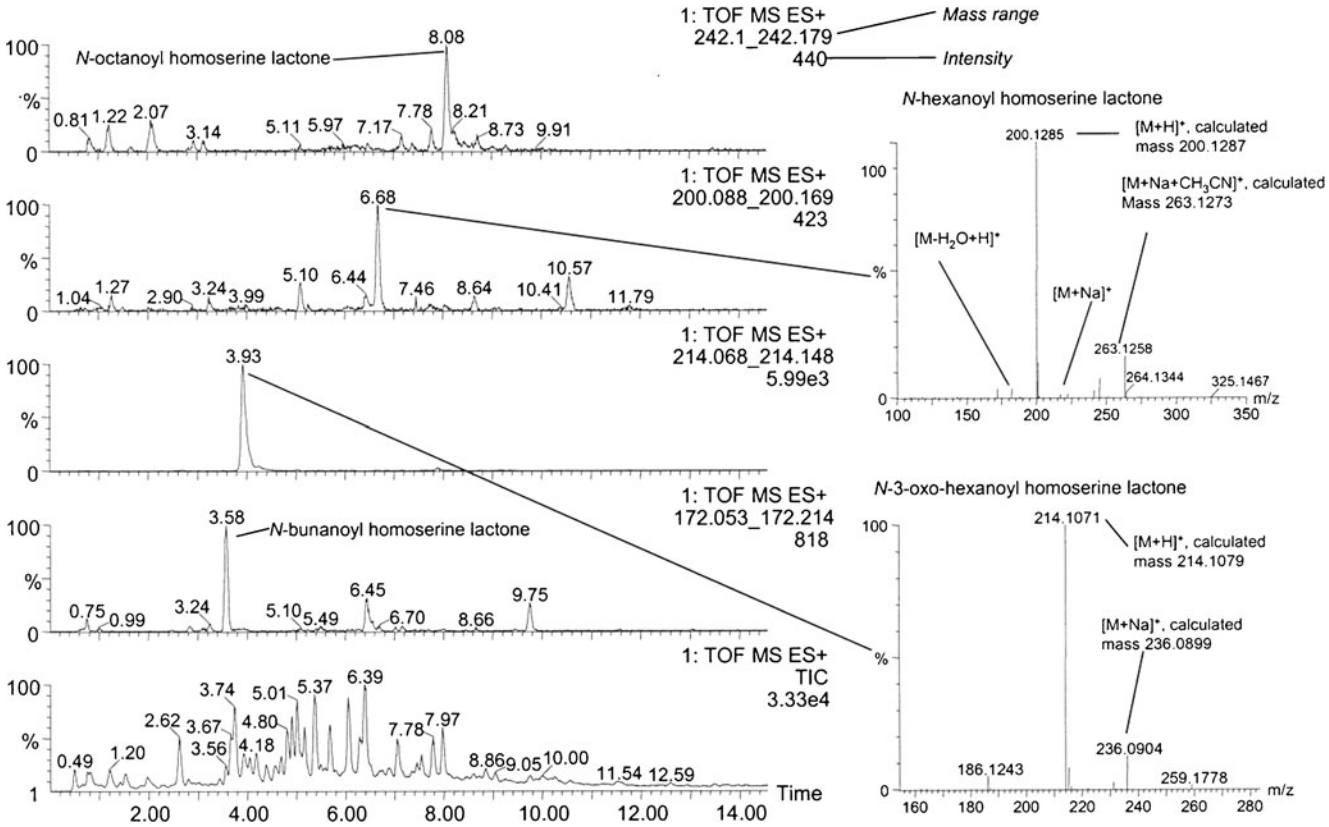

Fig. 8 LC–ESI–TOF MS with the *lower-left panel* showing the total ion current (TIC) chromatogram and the *upper four panels on the left* showing XICs for the $[M + H]^+$ adducts of four detected homoserine lactones. The mass spectra for *N*-hexanoyl homoserine lactone and *N*-3-oxo-hexanoyl homoserine lactone are shown with the masses calculated for the predominant adducts. (Reproduced from Ref. [68], with permission)

component of interest in accordance with its chromatographic retention time.

Ease of use is the essential driver for utilization of high-resolution mass spectrometry—using high resolution and accurate mass capability to filter away chemical noise and capture more information per scan. An interesting example of high-resolution time-of-flight (TOF) MS in LC–MS analysis applied to commercially produced vacuum-packed meat samples, was reported by Bruhn et al. [68]. Ninety-six AHL-producing bacteria were isolated, and 92 were identified as *Enterobacteriaceae*. *Hafnia alvei* was the most commonly identified AHL-producing bacterium. These Authors confirmed the presence of *N*-3-oxo-hexanoyl, *N*-hexanoyl, *N*-octanoyl, and *N*-butanoyl-homoserine lactones on the basis of the accurate masses of their protonated and sodiated adducts in pure cultures of *H. alvei* (Fig. 8).

Among recent advances in quadrupole time-of-flight (qTOF) mass spectrometry, high resolving power and accurate mass capability are combined with high-speed data acquisition, making those instruments compatible with UPLC separations [58]. This has become an important technique for complex mixture analysis, because of its sensitivity and mass accuracy, with typical mass errors below 2 mDa. One example of its application is our studies of enzymatic homoserine lactone hydrolysis in *Pseudomonas putida IsoF* cell culture, in which AHL can be degraded by lactonase enzymes producing homoserines [69]. The analytical approach showed peak-area linear response between 37 $nmol L^{-1}$ and 1.8 $\mu mol L^{-1}$ for oxo-C10 AHL and enabled the detection of 10 nmol L^{-1} (unpublished data). In this case, the substrate and its reaction product are detected by use of extracted ion chromatograms (XICs) set at $\Delta m/z$=0.0010 (Fig. 9). It is important to emphasize that sample clean-up and preconcentration strategies using solid-phase extraction techniques can improve the sensitivity of a method.

AHLs are also sensitive to pH, temperature, and abiotic stress. If the bacterial supernatant undergoes a pH increase, lactone ring opening of AHLs in the presence of water occurs. Such a chemical reaction leads to a mass increase of 18.01056 Da, corresponding to a water molecule as confirmed by accurate mass analysis [70]. In addition, during the ESI process the opened ring homoserine can be converted to a homoserine lactone and two peaks can be detected at the same

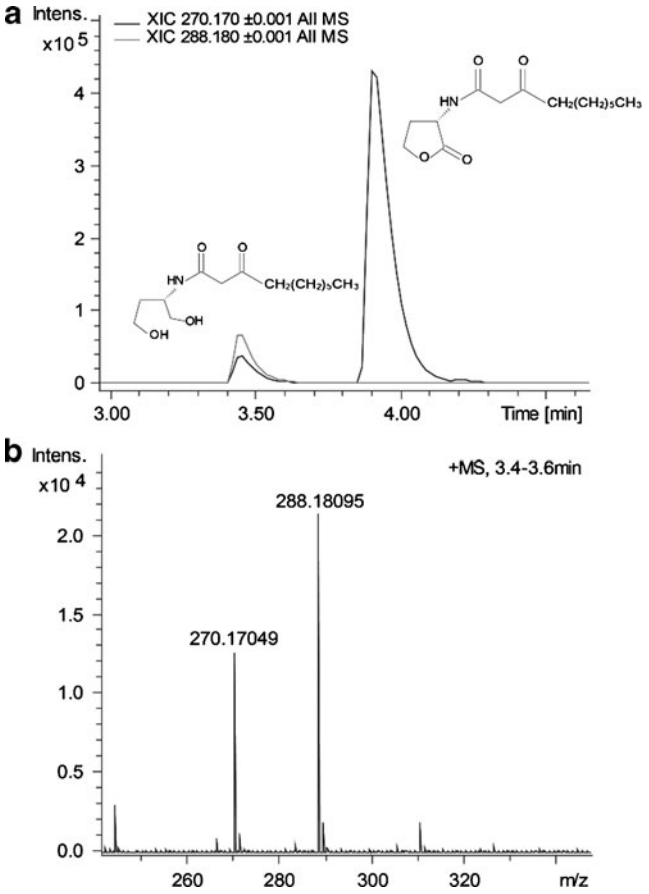

Fig. 9 UPLC–ESI–qTOF MS XICs in ESI positive mode of *Pseudomonas putida IsoF* cell culture supernatant. Chromatographic conditions: 150 mm×1.0 mm, 1.7 μm particle size BEH C_{18} column; the mobile phase was a linear gradient prepared from water containing 10 % acetonitrile and 0.1 % formic acid (component A) and acetonitrile (component B); the gradient program was from 0 to 90 % B in 5 min; the flow rate was 0.3 mL min^{-1}, the column temperature was 60 °C, and the injection volume was 10 μL. **a** Extracted-ion chromatogram shows the presence of $3OC_{10}$-HSL (*m*/*z* 270.170) at t_R 4.0 min and its lactonase product $3OC_{10}$-HS (*m*/*z* 288.180) at t_R 3.5 min. **b** The extracted mass spectra in the chromatographic time range 3.4–3.6 min show two *m*/*z* signals at the same retention time characteristic of homoserine chromatographic peaks. (see also Electronic Supplementary Material)

retention by using extracted ion chromatograms in LC–ESI MS analysis [25]. This characteristic conversion phenomenon of HS into HSL enables better confirmation of the presence of homoserine molecules originating after enzymatic reaction. This is in agreement with the chemical composition of some signaling molecules occurring as lactone-opened ring forms. The extent to which these related compounds are biologically and ecologically relevant still needs careful investigation. Another interesting example is production of the active signaling molecule $3OC_6$-HSL from C_6-HSL by abiotic oxidation (e.g., photolysis) processes; these have been investigated by ESI-qTOF MS. Other abundant oxidation products of C_6-HSL were acyl side-chain-oxidized C_6-HSLs, i.e. either positional isomers of $3OC_6$-HSL or the corresponding enol analogs [71]. Identification of unknown compounds is routinely achievable and work is in progress to apply the same analytical approach to other bacterial isolates and to achieve comprehensive profiling of the quorum sensing molecules. Conceivably, high-resolution mass spectrometry is a useful tool for accurate identification of unknown and untargeted molecules. FTICR and time-of-flight mass analyzers are widely used because of their high-speed in acquiring full mass spectra, thus facilitating discovery and identification of both signaling molecules and closely related compounds. Data acquisition can be performed without any prior knowledge of the investigated species; HRMS offers high mass accuracy which is advantageous for accurate determination of the elemental composition of molecules.

Concluding remarks

Analyzing bacterial signaling molecules is just the first step in understanding the communication of bacteria at a molecular level. Bioreporters, perhaps, exhibit a distinctive advantage on MS as they offer the possibility of measuring directly the bioavailability of any given chemical and the overall effect of the chemical on a living system. As a complementary tool, high-resolution measurements are becoming not only feasible but also necessary for confident, contemporary QS analysis. Despite seemingly higher complexity and cost, high-resolution MS can avoid errors that may otherwise lead to years of misdirected work. High-resolution MS provides highly selective chemical information, for example accurate mass, isotope distribution pattern for elemental formula determination, and characteristic fragmentation for structural elucidation or identification via spectral matching to authentic compound data, that is directly related to chemical structure. High-resolution MS, especially when used in conjunction with LC–ESI, furnishes highly informative data in targeted metabolite profiling and in non-targeted metabolomics (signalomics) as a complement to other profiling techniques. Although complex samples are regularly involved, either direct injection of supernatant isolates or extraction and consequent organic phase concentration can be used to check the occurrence of AHLs and related molecules. What is most interesting is that common and uncommon AHLs can easily be identified, thus facilitating the investigation of the function of QS signals and their effect in host responses to infection, biofilm formation, and the dynamics of these processes.

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