Production of N-acyl homoserine lactones by the sponge-associated marine actinobacteria Salinispora arenicola and Salinispora pacifica ☐

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Abstract

The structures of acyl homoserine lactone (AHL) compounds and their quantification were accomplished using an integrated liquid chromatography-mass spectrometry approach. The precursor and product ions, along with retention times of peaks, were searched against an in-house database of AHLs and structures confirmed by accurate mass and by comparison with authentic AHL standards. The two compounds, N-(3-oxodecanoyl)-L-homoserine lactone and N-(3-oxododecanoyl)-L-homoserine lactone, were characterised and quantified in Salinispora sp. cultures.

Keywords: marine bacteria, homoserine lactone, liquid chromatography, mass spectrometry

INTRODUCTION

Bacteria are highly interactive organisms, frequently using an array of signal molecules known as acyl homoserine lactones (AHLs) (Fuqua and Greenberg 2002), to facilitate communication. Such chemical signalling in bacteria demands the production and release of these compounds, and the detection and response to these relatively low molecular weight molecules. This phenomenon, termed ‘quorum sensing’ (QS), permits bacteria to respond to environmental influences such as the presence of other bacteria (whether in number or species) and then to consequently respond by changing behaviour on a population-wide scale. This process occurs following the interaction of AHLs with transcriptional activators and subsequent induction of target gene expression. Conditions triggering such an event include the achievement of a particular bacterial density threshold and the resultant concentration of AHL signalling molecule(s). The production of AHLs in terrestrial Gram-negative bacteria has been reported extensively (Williams et al.2007); however, much less information is known concerning the production of AHLs in the marine environment—especially from Gram-positive bacteria.

Marine sponges act as hosts for a range of significantly diverse microbial communities (Taylor et al.2007). Bacteria filtered from seawater primarily act as a food source for the sponge, and during this process some are retained in the sponge as endosymbionts (Hentschel et al.2012). Such bacterial symbionts can comprise more than half of the total sponge biomass. The diversity of bacterial communities coexisting in sponges varies between host sponge species but some 28 bacterial phyla, including the Actinobacteria, have been described to comprise the sponge microbiota (Hentschel et al.2012). Associations between sponges and microbes are widely known, and are, in some cases, unique relationships. It is believed that a number of such associations can be

mutually beneficial rather than existing as brief relationships resulting from the filter feeding activity of sponges. Studies of bacteria isolated from marine sponges suggest that the production of AHLs can play a key role in controlling the number of cells in the microbial community and also in the coupled relationships between the microbial communities and their host sponges (Taylor et al.2004). AHL signal molecules isolated from the sponge-associated Gram-negative Mesorhizobium sp. have been shown, in other studies, to be potentially involved in intergenus signalling in the sponge (Krick et al.2007).

Two distinct types of signalling molecules have been reported for Gram-negative and Gram-positive bacteria. The most frequently studied QS molecules in Gram-negative bacteria are the N-AHLs (Nealson and Hastings 1979). These homoserine lactones, as noted previously, have specific binding interactions with transcriptional regulators; the acyl side chain characteristics such as length and substitution being responsible for determining the specificity of interactions (Bassler 2002). The range of homoserine lactone molecules produced by organisms is generally stable; however, the patterns of AHL produced across species are, of themselves, variable. Gram-positive bacteria, on the other hand, communicate with each other using short peptide signals (Kleerebezem et al.1997), although Gram-negative bacteria are also known to produce similar chemistry (Dirix et al.2004). To date, only one study, to our knowledge, has reported the production of an AHL compound, C3-oxo-octanoyl homoserine lactone, from a Gram-positive marine bacterium in the Exiguobacterium genus (Biswa and Doble 2013). While there are no reports of AHL production in obligate marine Salinispora spp., this genus has been proven capable of synthesising a wide range of secondary metabolites including compounds with both anticancer and antibacterial potential (Bose et al.2014, 2015; Jensen, Moore and Fenical 2015 and references therein) and is likely to rely on QS.

The identification of AHLs in culture media has previously focused on the use of chemical methodologies such as thin-layer chromatography or biological assay techniques using a luminescence bacterial biosensor (Shaw et al.1997; Steindler and Venturi 2007). Such methods may, however, be problematic since they may be insufficiently sensitive or specific to detect and identify low concentrations of AHLs in biological systems or extracts thereof. Additionally, the chemical characterisation of any detected AHL is not possible using the above methodologies. Recent methods of AHL characterisation and quantitation have used liquid chromatography coupled to electrospray tandem mass spectrometry (LC-MS/MS) (Ortori et al.2007, 2011, 2014). Such a technique is capable of specifically quantifying a wide range of AHLs in biofluids and biological extracts and, when LC is coupled to a high-resolution mass spectrometer, this approach provides mass accuracy and confirmation of molecular formula (Watson 2013).

In this study, we describe a qualitative and quantitative investigation of AHLs in the mycelia cell mass extracts of a number of Salinispora arenicola and S. pacifica strains, harvested from Great Barrier Reef sponges, using LC-MS/MS.

MATERIALS AND METHODS

AHL standards and derivatives (Fig. 1A and B) were sourced as previously described (Ortori et al.2011, 2014). Standards of the above compounds were prepared in acetonitrile (0.220 µM) and stored at–80°C prior to use. Methanol and acetonitrile were purchased from Fisher Scientific (Loughborough, UK). Formic acid (MS grade) was obtained from Sigma-Aldrich (Poole, UK) and purified water (>18MΩ/cm) was from an Elga Maxima system (Elga Ltd, High Wycombe, UK).

Figure 1.

(A) Structures of homoserine lactones. (B) Predicted mass spectral fragmentation of N-(3-oxododecanoyl)-L-homoserine lactone.

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Cultivation and metabolite extraction

Taxonomic identification of Salinispora pure culture isolates was accomplished according to Vidgen, Hooper and Fuerst (2011). Isolates were grown on starch yeast extract peptone agar medium containing 3% w/w artificial seawater at 28°C until each strain established a black colony colouration. The mycelial cell mass from these strains was collected from the growth medium after ~56 days by careful removal with a scalpel blade, the cell mass was pooled (approximately 0.1 g) and placed in a 1.5 mL centrifuge tube. A similar procedure used a blank agar sample (0.1 g) to provide the blank extract. Secondary metabolites were extracted from the cell masses using ethyl acetate (1 mL) (Sigma Aldrich, Castle Hill, Sydney, Australia) and the mixture shaken for 90 min at room temperature. The ethyl acetate and aqueous, Salinispora extract, layers were allowed to separate and the ethyl acetate phase removed by pipette and placed in a clean centrifuge tube of volume capacity 1.5 mL. The ethyl acetate solvent was evaporated using a vacuum centrifuge

(Savant Instruments, Hicksville, NY, USA) and the dried residue resuspended by adding methanol (20 µL) and then ultra-pure water (80 µL; MilliQ, Millipore, Bedford, MA, USA). Before LC-MS/MS analysis, the samples were centrifuged for 5 min at 125 rpm after which a 30 µL aliquot of sample was transferred into a vial and maintained at 4°C–10°C. Where necessary, extracts were stored at –80°C until analysis following resuspension as above.

LC-MS data acquisition

AHL compounds from the Salinispora species (30 strains in total, 15 each from the two species Salinispora arenicola and S. pacifica, with biological replicates, where experimentally feasible) were identified and quantified by LC-MS/MS with slight modification to the method reported by Ortori et al. (2014).

Chromatographic separations were performed using 10AD binary pumps, an autosampler and column oven (Shimadzu, Columbia, MD, USA). Mass spectrometric detection was performed on a 4000 QTRAP QQQ linear ion trap mass spectrometer (AB SCIEX, Foster City, CA, USA) operated in positive electrospray mode with data acquisition and processing using Analyst (version 1.4.1). Separations were achieved by injecting 5 µL of sample extract onto a Phenomenex Gemini C18 column (150×2.1 mm, 3.0 µm particle size; Phenomenex, USA) operated at 50° C.

The mobile phase comprised solvents A (0.1% formic acid in Milli Q water) and B (0.1% formic acid in methanol) delivered at a flow rate of 0.45 mL min−1. The gradient used for multiple reaction monitoring (MRM) was as follows: 0% to 99% B for the first 5.5 min, held for 2 min at 99% B, returned over 0.2 min to 0% B and held at 0% for 0.8 min. The acquisition run time was 8.5 min with a further 1.2 min for the injection cycle. During a further analysis, an individual MRM signal above 5000 cps initiated a secondary experiment—enhanced product ion spectral generation for identity confirmation. Here, the following slower LC gradient was used: 0% to 99% B for first 16 min, held for 1 min at 99% B, returned over 1 min to 0% B and re-equilibrated for 2 min. Total acquisition run time was 20 min. During both types of analysis, the source was held at 400°C and the curtain, nebuliser and desorption gases were 20, 30 and 20, respectively. The ion spray voltage was set at 5000 V.

As noted above, AHL compounds were quantified using the method of Ortori et al. (2014) where the lower limit of detection for HSL was 0.5 nM and the lower limit of quantitation was 1.6 nM.

For further confirmation purposes, samples and authentic standards were also examined to acquire the accurate monoisotopic masses of the peaks of interest. All such experiments were conducted using an Accela U-HPLC system equipped with quaternary pumps coupled to an Exactive Orbitrap mass spectrometer (Thermo Scientific, Hemel Hempstead, UK), controlled by Xcalibur 2.1 software. The instrument was equipped with an ESI probe and operated in switching mode with the spray voltage set to 4 kV or –4 kV for positive or negative ion mode, respectively, with a capillary temperature of 300°C. The sheath gas, auxiliary and sweep gas flows were set to 30 (at 250°C), 15

and 5, respectively (arbitrary units). The instrument was operated at a resolution setting of 25 000 over a range of m/z 100–800 in full scan mode using an automatic gain control target of 10e6. The mass spectrometer was calibrated according to the manufacturer's guidelines prior to each use. LC gradient and column conditions were as for the second reported method above. The interpretation of nominal mass product ion spectra was assisted using Mass Frontier 7 SR3 (Thermo Scientific).

RESULTS AND DISCUSSION

Extracts of pure cultures, totalling 100 in number, arising from 15 strains each of Salinispora arenicola and S. pacifica (with their respective true biological replicates) were subjected to LC-MS/MS. Two AHL compounds, N-(3-oxodecanoyl)-L-homoserine lactone (3-oxo-C10-HSL) and N-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C12-HSL), were detected in 29 extracts (Fig. 1A and B). These compounds were identified by comparison of chromatographic retention times with authentic standards, by examination and assessment of the compound fragmentation patterns for samples and authentic standards (Table 1) and by comparison of their accurate monoisotopic masses. The extracted ion chromatograms and fragmentation patterns for authentic 3-oxo-C12-HSL (upper panel) and for a sample extracted from S. pacifica mycelial cell mass are shown in Fig. 2. The retention time of the sample peak (10.7 min) agreed with the authentic standard; the fragmentation patterns revealed shared fragments characteristic of AHLs, including the common product ion at m/z 102 that corresponds to the lactone ‘head’ group, formula ($C_4H_8NO_2$) (Iida, Ohnishi and Horinouchi 2008; Sharif et al.2008). Other ions present are those at m/z 298, representing $[M+H]^+$ for 3-oxo-C12-HSL ($C_{16}H_{27}NO_4$) and the spectrum base peak at m/z 197 representing the ionised difference product ($C_{12}H_{21}O_2$) remaining from the fragmentation of 3-oxo-C12-HSL and m/z 102 (Fig. 1C). The calculated accurate monoisotopic mass for 3-oxo-C12-HSL ($C_{16}H_{27}NO_4$) is 297.1940 Da and thus for $[M+H]^+$ is 298.2013 Da. This corresponds precisely with the data obtained for both sample and standard 3-oxo-C12-HSL accurate mass chromatograms, as shown in Fig. 3.

Figure 2.

Extracted ion (LC-MS/MS) chromatograms of 3-oxo-C12-HSL standard (upper panel) and S. pacifica mycelial cell mass extract sample (lower panel) with, as insets, the fragmentation patterns of the extracted ions.

Extracted ion (LC-MS/MS) chromatograms of 3-oxo-C12-HSL standard (upper panel) and S. pacifica mycelial cell mass extract sample (lower panel) with, as insets, the fragmentation patterns of the extracted ions.

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Figure 3.

Accurate mass spectra of 3-oxo-C12-HSL from S. pacifica mycelial cell mass sample (upper panel) and 3-oxo-C12-HSL standard (middle panel) obtained using accurate mass LC-MS. The predicted mass spectrum based on the formula C16H28O4N; m/z 298.201 (lower panel) is also included.

Accurate mass spectra of 3-oxo-C12-HSL from S. pacifica mycelial cell mass sample (upper panel) and 3-oxo-C12-HSL standard (middle panel) obtained using accurate mass LC-MS. The predicted mass spectrum based on the formula C16H28O4N; m/z 298.201 (lower panel) is also included.

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Table 1.

Predicted product ions from MS fragmentation of 3-oxo-C12-HSL.

The above two AHL compounds were quantified in 16 of the 29 extracts and in the remaining 13 extracts, the compounds were detectable but not quantifiable—i.e. the compounds were observed at concentrations greater than the assay limit of detection but were below the limit of quantitation. In those samples where AHLs could be quantified, the concentrations (expressed as pmol/g mycelial mass of S. pacifica) of 3-oxo-C10-HSL were in the following range: 0.105–0.574 pmol/g; in S. arenicola extracts, the concentrations of 3-oxo-C10-HSL were not detectable. The concentrations of 3-oxo-C12-HSL in S. pacifica ranged from 0.189 to 1676 pmol/g extract, whereas in S. arenicola these ranged from 0.044 to 1225 pmol/g (see Fig. 4). AHLs could not be detected in 71 of the 100 extracts. It is notable that substantial variability was observed in the concentration of AHLs both within and between species. Such variability in the occurrence of AHLs has been noted previously in bacteria associated with marine sponges but these reports have been confined to Gram-negative organisms (Taylor et al.2004). The use of pure cultures of the Salinospora spp. strains mitigates against potential contamination from other organisms, and analysis of the culture medium revealed no detectable levels of AHLs. We were also unable to discern any evidence of a positive correlation between AHL production and the species of host sponge (Dercitus xanthus, Cinachyrella australiensis

and Hyattella intestinalis) or between AHL production and the collection location in the Great Barrier Reef.

Figure 4.

Concentrations (pmol/g) of 3-oxo-C10-AHL and 3-oxo-C12-AHL in S. pacifica and S. arenicola mycelial cell mass extract samples. (Error bars denote standard deviations.)

Concentrations (pmol/g) of 3-oxo-C10-AHL and 3-oxo-C12-AHL in S. pacifica and S. arenicola mycelial cell mass extract samples. (Error bars denote standard deviations.)

Concentrations (pmol/g) of 3-oxo-C10-AHL and 3-oxo-C12-AHL in S. pacifica and S. arenicola mycelial cell mass extract samples. (Error bars denote standard deviations.)

Symbiotic relationships involving homoserine lactone QS are exemplified by relationship between the Gram-negative bacterium, Vibrio fischeri and squid (Boettcher and Ruby 1995) as well as by Gram-negative bacteria associated with marine sponges (Mohamed et al.2008). Another study has shown that QS involving AHLs occurs in marine situations where bacterial colony density in the sponge is a significant consideration in the sponge–bacterium association (Taylor et al.2004). In addition, a potential role for AHL signalling molecules has been recently proposed by Garderes et al. (2014), who have suggested the presence of a molecular communication system which may exist between a sponge host (Suberites domuncula) and symbiotic bacteria, such as system might play a role in sponge tolerance and population control of the bacterial species. Although most AHLs have thus far been found from Gram-negative bacteria, a recent study has reported the production of C3-oxo-octanoyl homoserine lactone in Gram-positive bacteria isolated from marine water (Biswa and Doble 2013). It is likely that a novel synthase enzyme is involved in the production of the 3-oxo-C10-HSL and 3-oxo-C12-HSL reported in these studies. A database search of S. arenicola and S. pacifica for the LuxI synthase and LuxR regulatory protein homologues has been unsuccessful.

We used LC-MS/MS to identify AHL compounds from marine-derived Gram-positive strains of Salinispora spp. This study thereby implies the presence of an AHL-based signalling system in these marine-derived bacterial species, which needs to be confirmed by further experimental work. Such studies could include developmental cycle analysis to identify when AHL synthesis is occurring and examining the role played by AHLs in antibiotic synthesis control, as occurs in Streptomyces species via γ-butyrolactone signal compounds (Takano 2006; Tan, Bai and Zhong 2013).

In conclusion, the production of AHLs by Salinispora species collected from marine sponges is reported herein for the first time. These results, in accord with the observed roles played by such signalling molecules in the bacterial colonisation of other higher organisms, support the proposal that AHLs may play a pivotal role in the development and maintenance of the association between bacteria and marine sponges.

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Conflict of interest.None declared.

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