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ORIGINAL ARTICLE

Quorum sensing in Cyanobacteria: *N*-octanoyl-homoserine lactone release and response, by the epilithic colonial cyanobacterium *Gloeothece* PCC6909

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Quorum sensing involving acyl homoserine lactones (AHLs) is a density-dependent form of intercellular communication that occurs in many different members of the group Proteobacteria. However, to date, there have been few investigations of its occurrence in cyanobacteria. Here, using both a bioreporter *Agrobacterium tumefaciens* NTL4 (PZLR4) and mass spectrometry, we provide evidence of *N*-octanoyl homoserine lactone (C8-AHL) production in axenic cultures of the cyanobacterium *Gloeothece* PCC6909 and its sheathless mutant PCC6909/1. Accumulation of C8-AHL in the culture medium of laboratory cultures of *Gloeothece* followed a pattern characteristic of the phenomenon of autoinduction, a common feature of functional AHL-based quorum-sensing systems. Analysis by two-dimensional gel electrophoresis showed that, in response to treatment with C8-AHL, early growth-stage cells of PCC6909/1 showed changes in expression of 43 proteins compared with untreated cells. Among the 15 proteins that showed more than a twofold change in expression were RuBisCo, glutamate synthase, chorismate synthase, a member of the LysR family of transcriptional regulators (all upregulated), and enolase and aldolase, both of which were downregulated. The significance of such changes in response to C8-AHL is discussed in relation to carbohydrate and amino-acid metabolism and involvement of *Gloeothece* in biofilms.
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Introduction

Environmental stress and adaptation to it are of major importance to living organisms. Cyanobacteria, a group of photosynthetic prokaryotes, can adapt to such stress (Michel and Pistorius, 2004; Schwarz and Forchhammer, 2005) utilizing various sensing mechanisms and intracellular signalling systems (Forchhammer *et al.*, 2004; Murata and Suzuki, 2006; Montgomery, 2007). Many bacteria also have an intercellular signalling system, in which excretion of chemical signal(s) leads to communal responses that facilitate adaptation through cooperative changes in physiological status (Shapiro, 1998). Studies of intra- and intercellular signal systems of epilithic colonial cyanobacteria, such as *Gloeothece*, may prove to be important in understanding various adaptive capabilities of cyanobacteria to stressful conditions, such as nutrient deficiency (Schwarz and Forchhammer, 2005). Furthermore, such information may be significant to an understanding of biofilm formation involving *Gloeothece* and its interaction with other microorganisms that leads to the deterioration of buildings and monuments, a current issue concerned in the preservation of important archaeological sites (Ortega-Morales *et al.*, 2000; Crispim *et al.*, 2003). 'Quorum sensing', a cell density-dependent mechanism of intercellular communication generally associated with physiological changes that occur at high cell densities (Fuqua *et al.*, 2001), occurs in a number of organisms (Kleerebezem *et al.*, 1997; Whitehead *et al.*, 2001; Nickerson *et al.*, 2006; Sprague and Winans, 2006). However, it has not yet been detected in cyanobacteria. The quorum-sensing signal system most extensively studied features acyl homoserine lactones (AHLs), which have only been reported in Gram-negative bacteria within the phylum Proteobacteria (Whitehead *et al.*, 2001; d'Angelo-Picard *et al.*, 2005). These diffusible signals are released from cells constitutively and, when a critical threshold concentration has been

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reached, may induce expression of genes (Fuqua *et al.*, 2001; Whitehead *et al.*, 2001), resulting in the cells concerned acting coordinately and functioning like a multicellular organism (Miller and Bassler, 2001; Waters and Bassler, 2005). They can act in a manner termed as 'autoinduction', and consequently the signals are also referred to as 'autoinducers' (Miller and Bassler, 2001). AHLs are components of quorum-sensing systems involved in biofilm formation (Rice *et al.*, 2005), pathogenicity (Camara *et al.*, 2002), plant-microbial interactions (Cha *et al.*, 1998), motility and swarming (Daniels *et al.*, 2004), conjugation (Whitehead *et al.*, 2001) and growth inhibition (Gonzalez and Marketon, 2003). They are characterized by having a common lactone ring with a 4-18 carbon acyl chain attached, although the nature of substitution of the acyl chain varies with the individual type (Cha *et al.*, 1998). The concept of quorum sensing cannot be defined simply in terms of cell density, as the system is influenced by environmental conditions and nutrients (Horswill *et al.*, 2007; Schuster and Greenberg, 2007). Furthermore, AHLs can be involved in 'efficiency-sensing' in which the spatial distribution of microorganisms can determine how efficiently they can respond to the signalling molecule (Hense *et al.*, 2007).

There are examples of cooperation among colonial cyanobacteria, for example in reducing self-shading by alteration of pigment packing inside the colony (Agusti and Philips, 1992) and adaptability to environmental stress (Kellar and Paerl, 1980; Paerl, 1984). The involvement of cooperative behaviour may suggest a role for autoinducer-like compounds in such responses. The epilithic cyanobacterium *Gloeothece* also forms microcolonies, with the cells being surrounded by a layer of multilaminated sheath that is reported to act as a diffusion barrier against toxic inhibitors (Meeks *et al.*, 1978). Such organization of cells may also facilitate autoinducer accumulation in an efficiency-sensing manner. As quorum sensing has been linked to biofilm formation (Davies *et al.*, 1998), it is possible that *Gloeothece* (which also form biofilms) has a potential to produce AHLs as quorum-sensing molecules. These molecules have been found in cyanobacterial assemblages (Bachofen and Schenk, 1998; Braun and Bachofen, 2004) and also in phototropic biofilms that involve other algae species (McLean *et al.*, 1997). However, in both reports, mixed cultures were analysed and so, to date, there are no reports of AHL production that may be specifically attributed to cyanobacteria. In this study, we examined axenic cultures of *Gloeothece* for evidence of AHL production and for evidence that such a signal behaved in an autoinducer-like manner. Cultures of *Gloeothece* were also examined to determine whether gene expression was altered in response to AHL. This represents the first study of quorum sensing in cyanobacteria via homoserine lactones and will improve our understanding of how communication between this organism and other microorganisms may occur.

Materials and methods

Chemicals and reagents

All materials were Analar grade and were purchased from Sigma Aldrich (Dorset, UK), with the exception of methanol (Fisher Scientific, Loughborough, UK), sequencing-grade trypsin (Promega, Southampton, UK) and Kasil (PQ Corporation, Amersfoort, The Netherlands). Water was prepared in-house (Mill-Q and Elix Water purification system, Millipore, Watford, UK). All homoserine lactone standards were purchased from Fluka Bio-chemical (Dorset, UK).

Growth and culture conditions of Cyanobacteria

Gloeothece strains PCC 6909 and PCC 6909/1, a sheathless mutant of PCC 6909 (referred to in this manuscript as PCC 6909 and PCC 6909/1 and obtained from the Pasteur culture collection, Institut Pasteur, Paris), were grown and maintained in BG11^0 medium (Stanier *et al.*, 1971), at 26 °C under alternating 12 h light (30 μmol m^{-2} s^{-1}, over the waveband 400–700 nm, measured at the surface of the culture) and 12 h darkness on an orbital shaker (70 r.p.m.). Stock cultures were maintained by subculturing every 50 days. For the growth curve experiment, 750 ml of a 1-month-old culture of PCC 6909/1 was harvested under sterile conditions and centrifuged at 7000 *g* for 10 min. The pellet was resuspended in 200 ml of sterile BG11^0 (nitrate-omitted) medium and 20 ml of the resulting inoculum was used in 10 flasks, each containing 100 ml of fresh sterile BG11^0 medium. The inoculated flasks were transferred to the conditions described (above) and were maintained until the appropriate sampling time. A single flask was used for each growth point measurement and also utilized for the measurement of AHL concentration. Growth was monitored through measurement of turbidity at 436 nm (UV 1601, SHIMADZU) and also by determination of protein concentration using the Lowry method (Lowry *et al.*, 1951). Difficulties were experienced in monitoring growth of, and extracting proteins from, the wild type, caused by aggregation of cells and interference associated with the sheath. Consequently, cultures of PCC6909/1 (sheathless mutant) were used for both the growth experiments and analysis of protein expression.

Extraction of homoserine lactones from culture supernatants

Acyl homoserine lactones were extracted from culture supernatant as described in previous reports (Yates *et al.*, 2002), with some modifications. A sample (100 ml) of culture was centrifuged (for 10 min at 8000 *g*) to remove cells, and the pH of

the resulting supernatant was adjusted to 2.0. The sample was stored for 16 h at 4 °C, after which it was partitioned (three times) against dichloromethane (2:1 (v/v) culture medium: dichloromethane). The combined dichloromethane extracts were filtered and dried with anhydrous magnesium sulphate using a rotary film evaporator at 35 °C. The resulting residue was redissolved in 500 µl of acetonitrile (high-pressure liquid chromatography (HPLC) grade), transferred to a glass vial and the solvent was removed under a stream of filtered nitrogen. Samples were stored at −20 °C until required. Before use, for HPLC-MS, the residue was redissolved in 20 µl methanol/water (1:1 v/v) containing 0.1% (v/v) formic acid, whereas the bioreporter assays used 20–50 µl of acetonitrile.

Bioreporter assay and thin-layer chromatography of extracted samples using Agrobacterium tumefaciens *NTL4 (PZLR4)*

Stocks of *Agrobacterium tumefaciens* bioreporter NTL4 (PZLR4) (Cha *et al*., 1998, a gift from Stephen K. Farrand, Department of Crop Sciences and Microbiology, Univeristy of Illinois) were stored at −80 °C. When required, a sample of stock culture was transferred into 5 ml Agro-medium (Q-Biogene, UK) supplemented with gentamicin (5 µg ml^{-1}) and yeast extract (0.1% (w/v)) and incubated for 16 h at 28 °C, on an orbital shaker (200 r.p.m.). 2 ml of the culture was then used to inoculate 20 ml fresh medium, which was incubated (as previously) until late exponential phase (*ca.* five hours). Bioassay dishes were prepared by adding 10 ml of this 5 h culture to 130 ml of melted Agro-medium, supplemented with 1.2% agar and 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) (40 µg ml^{-1} Melford laboratories, UK) and transferring 30 ml into a sterile Petri dish. Aliquots of controls (acetonitrile), samples and standards (5-, 10- or 20 picomoles, DL-Octanoyl homoserine lactone) prepared in acetonitrile were pipetted onto individual antibiotic assay discs (Whatman Ref No. 2017 006), which were air-dried and placed on the agar surface of the bioassay plates. The dishes were placed at 28 °C for 16 h at the end of which time plates were inspected visually for the presence of blue zones representing the presence of AHL in the sample. Thin-layer chromatography (TLC) of extracts prepared from cell-free culture supernatants and AHL detection using the *A. tumefaciens* bioreporter (NLT4) was carried out as described by Shaw *et al.* (1997). TLC utilized C18 reverse phase plates 2 m (2 mm layer, 20 × 20 cm, Baker, Warrington, UK). A mixture of AHL standards was applied to the plate, alongside test samples, and the chromatogram was developed in methanol–water (60:40, v/v). After development, the solvent was evaporated and the chromatogram was overlaid with the indicator culture of *A. tumefaciens*. The overlay was prepared by inoculating 5 ml of NLT4 (PZLR4) 16 h culture in 50 ml of fresh agro medium, which was subsequently incubated under the conditions described above. The culture was then transferred into 100 ml of freshly melted Agro agar medium (0.7% agar), maintained at 45 °C, containing 60 mg ml^{-1} of X-gal. The culture was mixed and poured immediately over the TLC plate, which was allowed to cool and then it was incubated at 28 °C for 12–18 h.

Mass spectrometric identification and quantitation of homoserine lactones

Acyl homoserine lactones were studied by HPLC-electrospray mass spectrometry on a C18 stationary phase column using a Dionex 'Ultimate' pump and FAMOS autosampler (Camberley, UK). A column (15 cm × 300 µm i.d.) was prepared from dried stationary phase (Polaris C18, a gift from Varian, UK) as described in the Supplementary Material. Samples were injected onto the column, which was then eluted at a flow rate of 4 µl min^{-1} with a solvent consisting of 50:50 mixture of methanol, containing 0.1% (v/v) formic acid (A):water, containing 0.1% (v/v) formic acid (B), for 5 min, followed by a linear increase over 5 min to a final solvent mixture of A:B 95:5. This final solvent mixture was maintained for

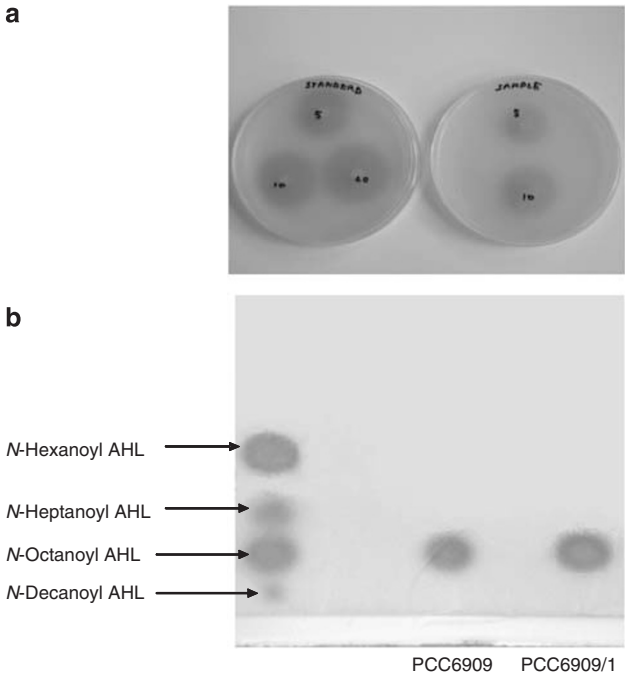
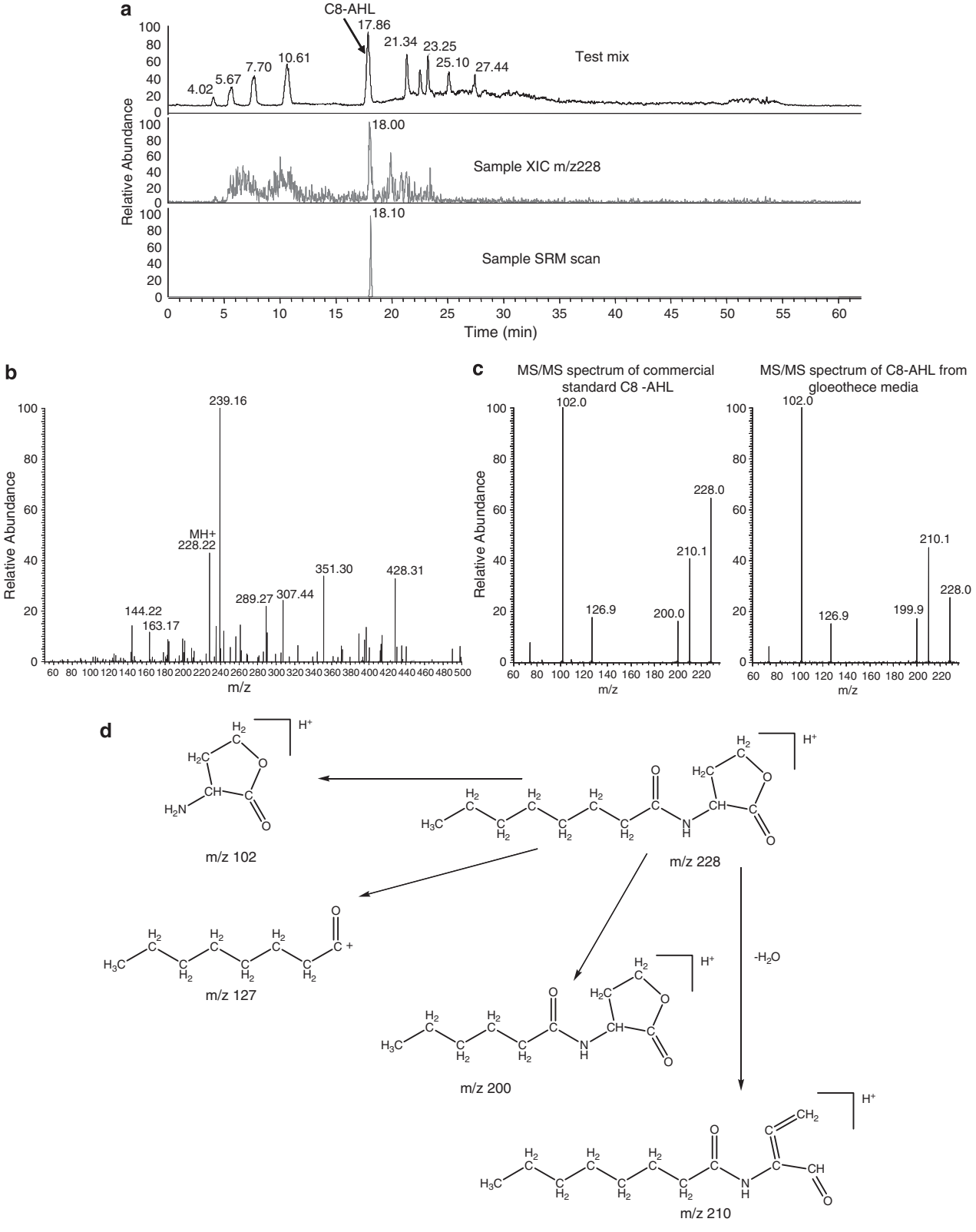


Figure 1 Identification of AHL activity in culture medium of *Gloeothece*. (**a**) A sample of an extract prepared from the culture medium of *Gloeothece* PCC6909 (Materials and methods) was assayed with the *A. tumefaciens* reporter (Materials and Methods), using 5 µl (top sample, right plate) or 10 µl (bottom) extract. For comparison, the results of an assay carried out on samples of a standard solution of C8-AHL containing 5, 10 or 20 pmol (left plate) are included. (**b**) Separation by TLC followed by visualization with the *A. tumefaciens* reporter of standard AHLs and a sample of extracts from the cultures of 'wild type' (PCC6909) and sheathless mutant (PCC6909/1) *Gloeothece* medium.

30 min before a return to the initial eluent composition, which was maintained for 20 min. The eluent from the HPLC was linked directly to an LCQ Deca XP Ion Trap mass spectrometer (Thermo Electron, Hemel Hempstead, UK). The mass spectrometer was operated in positive ion mode with a spray voltage of 3.5 kV, a capillary voltage of 23 V, a capillary temperature of 200 °C and a sheath gas flow of 30



(arbitrary units). For full-scan analysis, the mass spectrometer was scanned from 50 Da to 500 Da, with a maximum injection time of 50 ms. For tandem mass spectrometric analysis scans, an isolation window of ± 0.5 Da was used when selecting the desired parent ion. A collision energy of 35 arbitrary units and an activation time of 30 ms were applied. For identification of AHLs, the mass spectrometer was operated once in full-scan mode to determine if any signals indicative of the compounds could be detected. If such signals were detected, MS/MS was also performed. Only if the HPLC retention time, the full-scan MS and subsequent fragmentation analysis were in agreement with that of the commercial standards was the AHL reported as being present. For quantitation of *N*-octanoyl homoserine lactone (C8-AHL), an HPLC-single reaction monitoring-MS/MS method was used. The transition from protonated C8-AHL (*m/z* 228) to the product ion at *m/z* 102 (± 0.5 Da) was monitored. The sensitivity of the method was determined by analysing various concentrations of C8-AHL (between 2.5 pmol and 50 fmol per injection), demonstrating the linearity of the mass spectrometric response versus analyte concentration over the defined range and the limits of detection of the HPLC-single reaction monitoring-MS/MS assay. Quantitation of C8-AHLs took advantage of the proven linearity and reproducibility of the mass spectrometric response of C8-AHL. The experimental sample was divided into two equal fractions. To one of these, 5 µl of mobile phase was added, whereas 5 µl mobile phase containing 1 pmol of C8-AHL was added to the other. The amount of C8-AHL naturally occurring in the sample was calculated, as the increase in signal produced by the addition of C8-AHL to the experimental sample is equivalent to the response produced by 1 pmol of C8-AHL. The response of the sample to which no AHL had been added was then compared to this increased signal and expressed as a fraction. This allowed potential difficulties relating to altered sensitivity of the mass spectrometric system over time to be overcome.

Analysis of protein expression

Preparation of cultures and addition of synthetic AHL to the culture. To eliminate any effects caused by endogenous AHLs, cells were washed and resuspended in fresh BG11^0 medium, as follows: 1 l of *Gloeothece* PCC6909/1 (60 days old) was harvested under sterile conditions by centrifugation at 7000 *g* for 10 min at 20 °C. The resulting pellet was resuspended in 100 ml of sterile BG11^0 medium and 50 ml was used to inoculate each of two flasks containing 750 ml of sterile BG11^0 medium. The resulting two separate cultures were grown for a further 10 days under the conditions described previously, after which one flask was treated with C8-AHL (final concentration: 10 µM; see the Supplementary Material for the rationale for this choice of concentration) and the other flask was used as a control (ethanol). After a further 24 h, the flasks were retreated with AHL or ethanol as appropriate, grown for another 24 h and then proteins were extracted for analysis.

Protein expression analysis and protein identification. Proteins were extracted from the AHL-treated and control cultures and three samples of each of the extracts were subjected to two dimensional (2D)-polyacrylamide gel electrophoresis (see Supplementary Material). Following silver staining, each individual gel was image-matched and analysed according to the 'Progenesis Same Spot' 2D software (Non linear dynamics Ltd., Newcastle upon Tyne, UK). Those proteins in the AHL treatment that were identified by the software as showing statistically significant differential expression compared with the control were excised and digested for MS identification. Identification was carried out using nano-HPLC electrospray MS/MS analysis (see Supplementary Material).

Results

Identification of C8-AHL in Gloeothece *culture medium*

The bioreporter assay of *Gloeothece* culture (PCC 6909) medium indicated the presence of an AHL (Figure 1a). A control assay dish containing medium and X-gal but without *A. tumefaciens* reporter revealed no activity in the *Gloeothece* extract capable of hydrolysing X-gal to produce a false-positive result (data not shown). In this format, the *A. tumefaciens* reporter system gives no information on the types(s) of AHL present. Consequently, to establish which AHLs may be present in the *Gloeothece* extract, a sample of the extract was analysed by thin-layer chromatography and the AHL(s) were detected with an overlay of the

Figure 2 HPLC-electrospray mass spectrometry analysis of AHLs. (**a**) Upper chromatogram—total ion chromatogram from HPLC-MS analysis of a mixture of AHLs, indicating the retention times of C2-AHL (5.67), C4-AHL (7.70), C6-AHL (10.61), C8-AHL (17.86), C10-AHL (21.34), C12-AHL (23.25) and C14-AHL (25.10). Middle chromatogram—extracted ion chromatogram for *m/z* 228 (protonated molecular ion of C8-AHL) from an analysis of an extract of PCC6909/1 culture medium carried out under the same conditions used for separation of the standards (upper chromatogram). Lower chromatogram—single reaction monitoring (SRM) (*m/z* 228–*m/z* 102) of an extract of PCC6909/1 culture medium carried out under the same conditions. (**b**) Full-scan mass spectrum from the extracted ion chromatogram (**a**, middle chromatogram), taken at 18 min, derived from the separation of an extract from culture medium of PCC6909/1 showing the presence of the protonated C8-AHL (*m/z* 228). (**c**) MS/MS analysis of authentic C8-AHL and the extracted putative C8-AHL. (**d**) Mass spectrometric fragmentation pathway of C8-AHL indicating the origins of the fragment ions detected in MS/MS analysis (Figure 2c).

A. tumefaciens reporter. Extracts from cultures of both PCC6909 and PCC6909/1 contained AHLs, the values for R_f suggesting the presence of only C8-AHL (Figure 1b—the absence of tailing for the samples indicates that the AHLs concerned do not possess an oxo-group). However, the *A. tumefaciens* reporter exhibits differing sensitivities to different homoserine lactones (Shaw *et al.*, 1997) and co-extracted substances other than AHL may also induce bioreporters (Holden *et al.*, 1999; Degrassi *et al.*, 2002). Consequently, the presence and identity of the homoserine lactone was confirmed using an HPLC-electrospray mass spectrometry method. On the basis of the retention time of the protonated molecular ion (*m/z* 228, Figure 2a), a full-scan mass spectrum (Figure 2b) recorded at 18 min (the time at which authentic C8-AHL is eluted, Figure 2a), and MS/MS analysis of the molecular ion (*m/z* 228, Figure 2c), analysis of extracts from both the wild-type and mutant *Gloeothece*, confirmed the presence of C8-AHL as the sole AHL. In each analysis, the data generated from the sample agrees well with the data obtained from a sample of authentic C8-AHL (Figures 2a–c). Furthermore, comparison of the fragmentation-derived product ions with the fragmentation pathway derived from examination of the MS/MS analysis of authentic C8-AHL (Figure 2d) indicated no unexpected product ions generated by the HPLC-MS/MS analysis of the extract (Figure 2c).

Change in concentration of C8-AHL in Gloeothece *culture during growth*

To establish the characteristics of accumulation of C8-AHL in *Gloeothece* culture, its concentration was monitored using HPLC-single reaction monitoring-MS/MS over a period of growth in cultures of PCC6909/1. Measurements with authentic C8-AHL demonstrated that the mass spectrometric response was linear over the range of 50 fmol to 2.5 pmol (see Supplementary Data), with an R2 value of 0.9913 and low standard error values. Samples removed at the time points indicated (Figure 3) were used to measure both growth and C-8 AHL production, to determine the pattern of C8-AHL accumulation throughout growth. C8-AHL accumulated slowly during the initial phase of growth when normalized to protein content, whereas culture density increased in a linear fashion (Figure 3). Optical density was utilized as a measure of growth and was measured as the absorbance of light at 436 nm (Figure 3). The amount of C8-AHL in the media relative to the population of the organism increased more markedly at the 55-day point and continued to accumulate more rapidly from this point forward (indicated by an increase in its concentration relative to the protein content). The cell density at the point at which C8-AHL production is seen to increase (55 days) was determined to be 1.7×10^7 cells ml^{-1}. This pattern suggests an increased production of C8-AHL in response to a defined threshold concentration of the molecule. The concentration of C8-AHL that accumulated reached a maximum value of 70 nM during the course of the experiment, which is consistent with levels produced by other AHL quorum-sensing bacteria (Kaplan and Greenberg, 1985).

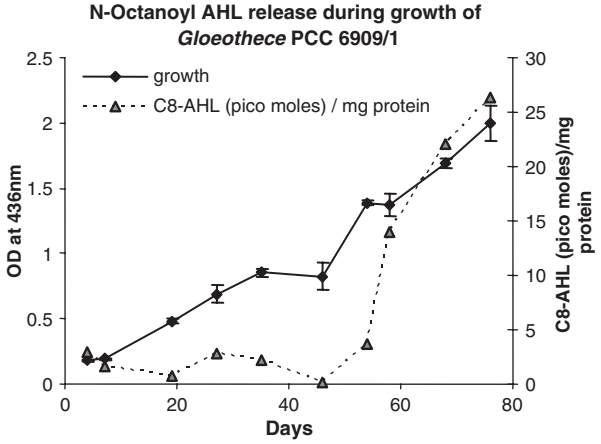


Figure 3 Accumulation of C8-AHL during growth of a culture of *Gloeothece* PCC6909/1. The C8-AHL content of the medium from a culture of PCC6909/1 was determined over the course of its growth as described in Materials and methods. Protein and o.d. at 436 nm were determined as an indicator of culture growth.

Proteomic analysis

Acyl homoserine lactones function in quorum sensing and are known to have a number of effects, many of these involving changes in gene expression. Therefore, the effect of C8-AHL on gene expression in *Gloeothece* was assessed in the early stages of growth via analysis of the effect of C8-AHL addition on protein expression. A silver-stained IEF 4–7 range, 2D-polyacrylamide gel electrophoresis gel showing the separation achieved for a sample of the proteins extracted from the C8-AHL-treated culture is presented in Figure 4. Of those proteins that showed differential expression compared with the control, those that could be identified are indicated with arrows. A representative example of one of the protein images derived from the 2D software (protein 5 in Figure 4) showing differential expression of that protein, on the basis of comparison of the triplicate analyses for C8-AHL-treated and control culture, is presented in Figure 5. Following determination of expression levels, the expression of 43 proteins was shown to be statistically different ($P<0.05$, Student's *t*-test) in the C8-AHL-treated culture compared with the control culture. For 15 of these proteins, the difference in expression level was twofold or more. Each of these proteins was subjected to tryptic digestion, and the sequences of the resultant peptides were identified by HPLC-MS/MS combined with database searches for protein identification (see Supplementary

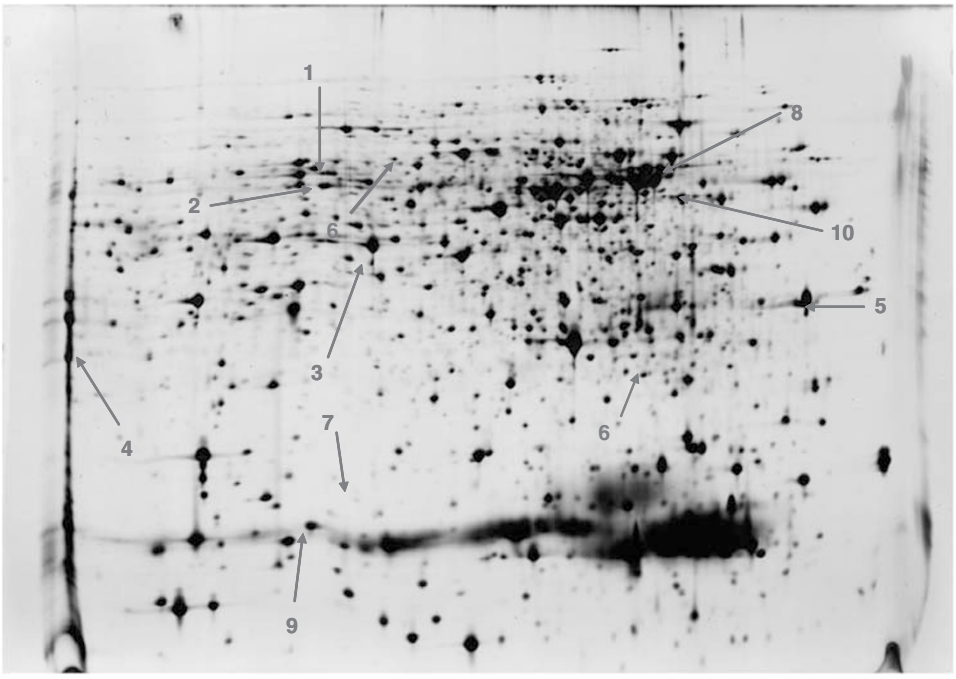


Figure 4 2D-polyacrylamide gel electrophoresis separation of extracted proteins, with proteins exhibiting expression changes highlighted.

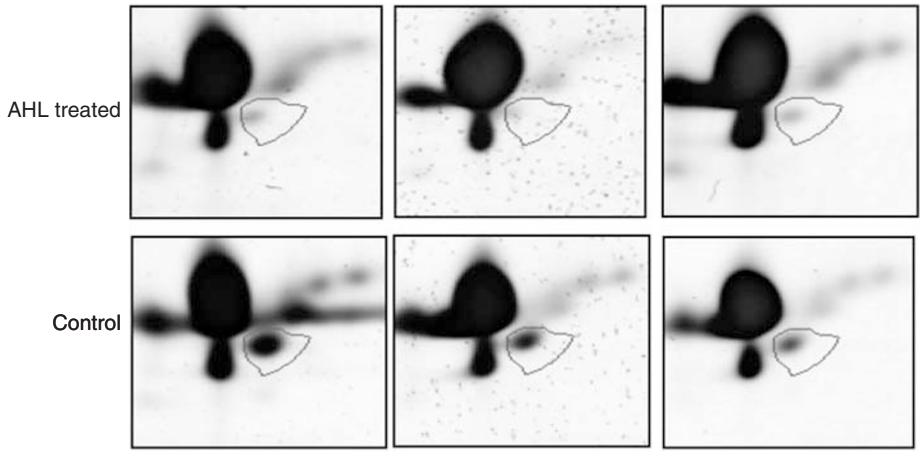


Figure 5 Differential protein expression in C8-AHL-treated *Gloeothece* PCC6909/1. The images shown are of proteins 5 (circled) from Figure 5 (AHL-treated) and the corresponding protein in the control sample. The protein was subsequently identified as aldolase, which exhibited a fourfold decrease in expression in response to C8-AHL treatment.

Material). Identification of proteins from *Gloeothece*, for which the genome has not been sequenced, relies on homology between the same proteins from different species and the occurrence of conserved sequences in those proteins. Of the 15 proteins that showed a significant change in expression in response to treatment with C8-AHL, 7 were identified in the database search, on the basis of matching the identity of more than one peptide (Table 1). All the peptides reported in Table 1 passed the acceptance criteria set for determination of peptide sequences from MS/MS data (judging the XCorr of individual peptides, personal communication with software manufacturer). However, identification of three proteins (proteins 4, 8 and 10; Table 1) is more tentative, depending on a single peptide match. Consequently, in the case of these proteins, the results of the database search were combined with other experimentally determined characteristics. For example, protein 4 produced only one peptide that led to a tentative identification of the protein as membrane-bound *O*-acyl transferase. The theoretical pI value of this protein is very basic, (approximately 10), and such a value is consistent with the position of the *Gloeothece* protein (at the basic end of the 2D gel—Figure 4).

Table 1 Proteins exhibiting differential expression upon C8-AHL addition identified by HPLC-MS/MS

No.	*Fold change*	P-*value*	*Protein*	*Species*	*Peptides*	*Z*	*Xcorr*	*Ions matched*
1	2.474	0.058	Glutamate synthase	*Plectonema boryanum*	R.ADRPGGLLMYGIPNMK.L	3	3.06	23/60
					R.GQSLVVWAFNEGR.G	2	3.74	19/24
				Bacillus halodurans	K.TNNFPEFTGR.V	2	2.47	15/18
					K.AGHWVTVFER.E	2	2.36	14/18
				Synechocystis sp. PCC 6803	R.AANNPWPEWPK.V	2	2.29	12/20
2	3.001	0.044	Ribulose-1,5-bisphosphate Carboxylase/oxygenase	*Synechoco*	K.TPIIMHDYLTGGFTANTTLAK.F	3	4.52	30/80
				Prochloron sp.	R.FLFVQEAIEK.A	2	3.25	16/18
					K.TFQGPPHGIQAER.D	2	2.80	15/24
				Euonymus	K.TFQGPPHGIQVEK.N	2	2.47	14/24
					R.NKGLLLHIHR.A	2	2.45	13/18
3	2.439	0.028	Chorismate synthase	*Nostoc punctiforme* PCC 73102	K.GLGEPVFDK.L	2	2.27	13/16
					K.YRPSHADATYDAK.Y	3	2.94	23/48
				Thermosynechococcus elongatus	R.SGGVQGGISNGENIILR.V	2	3.24	19/32
						3	3.28	25/64
				Brucella suis 1330	R.CGAVIDTASLIR.K	2	2.57	16/22
4	1.73	0.043	Membrane-bound *O*-acyl transferase		R.RANEASLFIDFVK.R	2	2.27	13/24
5	−4.029	0.0026	Aldolase		K.GILAADQSTGSIAK.R	2	2.96	20/26
					K.LQSIGZTBTZZBR.R	3	2.99	16/24
6	2.059	0.043	ABC transporter	*Nostoc sp.*	K.QMPAGYETQIGEGGGMLSGGQR.Q	3	3.10	26/84
			ABC-type amino-acid transporter		K.SYSAILGPGLSSQASAISPIAEK.G	3	2.66	28/88
7	−2.452	0.027	ATP synthase β-subunit/ ATPase β-subunit	*Penthorum sedoides*	R.FLSQPFFVAZVFTGSPGK.Y	2	4.02	21/34
				Lactobacillus gasseri	R.GMEVEDTGAPISVPVGK.D	2	3.52	18/32
				Synechococcus sp. PCC 7002	R.IFNVLGEPVDEK.G	2	2.95	17/22
				Amphidinium operculatum	R.VANAALTMAEYFR.D	2	2.93	16/24
				1711264A CF1	R.FVQAGSEVSALLGR.M	2	2.90	21/26
8	2.402	0.0245	Transcriptional regulator LYSR-type	*Escherichia coli* O157:H7	R.VDATFSSGCQLMINAIR.K	3	2.85	23/64
9	−1.949	0.0187	Enolase	*Paramecium multimicronucleatum*	K.VQIVGDDLLVTNPTR.V	2	4.09	19/28
				Azotobacter vinelandii	R.SGETEDTTIADLAVATR.A	2	3.32	22/32
						3	3.95	30/64
				Clostridium acetobutylicum	K.GIQLGVANSILIK.L	2	3.00	18/24
10	3.02	0.0084	PS II Mn-stabilizing polypeptide precursor	*Cyanothece*	K.RQEAEYVPGK.L	2	2.41	14/18

Encouragingly, although searches were carried out using a proteomic database on the basis of all the available proteomes, many of the protein identifications shown in Table 1 are from bacterial species. This suggests that, in these cases, reliance on conservation of amino-acid sequences for identification of proteins from the *Gloeothece* was sufficient.

Discussion

Intercellular communication between single-cell organisms may allow them to act coordinately like multicellular organisms and bring about a significant change in the phenotype (Shapiro, 1998), leading to expression of a communal response, for example, virulence (Camara *et al.*, 2002). Many such cooperative changes lead to a response that facilitates adaptation to changes in the environment and that increase survival of the organism (Shapiro, 1998). Quorum-sensing is a form of intercellular signalling that is dependent on the density of the population, and so changes in gene expression that arise via such signalling coordinate with the growth stage of a culture (Miller and Bassler, 2001).

Although colonial cyanobacteria such as *Gloeothece* do not have such distinct specialized cells, for example, the heterocysts that occur in *Anabaena*, coordination of their physiological and biochemical activities may allow cells to survive during conditions of stress, especially those encountered at high-cell densities such as limitations of nutrients or light (Schwarz and Forchhammer, 2005; Montgomery, 2007). As some activities of Gram-negative bacteria are coordinated in a density-dependent manner via quorum-sensing, we examined the cyanobacterium *Gloeothece* for evidence of such a system. Evidence that AHL(s) accumulate in the culture medium of both the wild-type (PCC6909) and the 'sheathless' mutant (PCC6909/1) was derived initially from bioassays using an *A. tumefaciens* reporter system (Figures 1a and b). Further mass spectrometric analysis identified C8-AHL as the sole AHL accumulated by both strains (Figures 2a–c). Before this study, there have been only two reports of AHL activity in cyanobacteria (Bachofen and Schenk, 1998; Braun and Bachofen, 2004). However, the cultures involved were taken from open lake water and were certain to contain numerous other microorganisms, including AHL-producing Gram-negative species. In such conditions, the origin of the signalling molecule is unclear. The *Gloeothece* cultures used in this study were grown axenically and were periodically tested for the presence of other microorganisms (by a combination of confocal microscopy and application of the cultures to a suitable bacterial medium on agar plates for identification of contaminating bacteria) and were always found to be free of other microorganisms (data not shown). This, then, is the first report of AHLs produced by an axenic strain of a cyanobacterium.

By itself, accumulation of AHL activity in a culture is not sufficient to indicate that an AHL-based signal system operates in that organism; it is possible that the C8-AHL identified has no physiological/biochemical role. However, further evidence presented in this report demonstrates that this is not so. The concentration of C8-AHL detected in the medium of PCC6909/1 (Figure 3) reached the low nM range (70 nM). Such concentrations are within the range over which quorum-sensing systems in other organisms appear to operate (Kaplan and Greenberg, 1985). Furthermore, the pattern of accumulation of C8-AHL in *Gloeothece* cultures appears to be a density-dependent one (Figure 3). The significant increase in the amount of C8-AHL, apparent when expressed per mg of protein, only occurs some time after 55 days, whereas the culture density up to this point, as measured by OD_{436}, has shown a linear increase to nearly fourfold its original value. Thus C8-AHL accumulation does not simply parallel growth; it appears that significant accumulation of C8-AHL occurs only when a critical cell density has been achieved, after which it exhibits a greatly increased rate and continues linearly until measurements were stopped. Such a pattern is characteristic of the phenomenon of positive feedback, a feature of many quorum-sensing systems. In response to treatment with authentic C8-AHL, changes in expression of particular proteins were observed in cultures of *Gloeothece,* the response predicted of a quorum-sensing molecule (Figures 4 and 5, Table 1). The level of change (43 proteins in total, ca. 11% of the proteome) is consistent with reports of changes in protein expression observed in other organisms during quorum sensing (Christensen *et al.*, 2003). The largest increase in expression in response to C8-AHL was observed for ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCo), which catalyses carbon dioxide fixation. Generally, AHLs activate expression of genes that are advantageous to a species when they are at a population density perceived as a quorum. An increase in the expression of RuBisCo in response to a quorum-sensing signal may offer such an advantage. The efficiency of RuBisCo is relatively low and its capacity to combine CO_2 with its co-substrate, ribulose-1, 5-bisphosphate (RuBP), can be a major limitation on the rate of net CO_2 assimilation. A rise in cell density would eventually lead to limitations in availability of nutrients (including CO_2), for example, from limited diffusion in biofilms. In such circumstances, an increase in the amount of RuBisCo may be expected to lead to restoration in the efficiency of CO_2 fixation. An AHL-based quorum-sensing system may, therefore, modify the population and allow it to cope with lower CO_2 concentrations. Furthermore, RuBisCo is increased in expression following treatment of *Medicago truncatula* with nM

concentrations of AHL (Mathesius *et al.*, 2003). A LysR-type transcriptional regulator is also upregulated 2.4-fold in *Gloeothece*, and these are transcriptional regulators of RuBisCo in most microorganisms (Shively *et al.*, 1998) Furthermore, the threefold upregulation of a polypeptide involved in photosystem II may also assist in overcoming CO_2 limitation. In contrast to RuBisCo, expression of enolase (2-phosphoglyceratelyase) and aldolase, both of which are associated with carbohydrate metabolism, are downregulated by treatment with AHL (Table 1). A decrease in the activity of enolase, which catalyses interconversion of 2-phosphoglycerate and phosphoenolpyruvate, may be expected to reduce flux through the glycolytic/gluconeogenic sequences. Similarly, a decrease in the activity of aldolase, which catalyses the interconversion of fructose 1,6-bisphosphate with glyceraldehyde 3-phosphate and dihydroxyacetone phosphate, may also reduce flux through the same pathways. Such a reduction coupled with increased RuBisCo could lead to increases in the concentrations of the intermediates in these pathways. As a consequence, glyceraldehyde 3-phosphate, dihydroxyacetone phosphate, intermediates such as 2- and 3-phosphoglycerate and members of the pentose phosphate pathway would become elevated. Increases in the latter group would maintain the concentration of RuBP and, because at times availability of RuBP limits CO_2 assimilation, may increase photosynthetic carbon fixation.

3-Phosphoglycerate is also a precursor in the synthesis of the α-keto acids corresponding to the amino acids, serine, glycine and cysteine, and so an increase in its concentration may lead to increase in the synthesis of these amino acids. In this regard, the increase in glutamate synthase in response to AHL treatment (Table 1) would be significant. Glutamate, together with glutamine, plays a central role in the assimilation of NH_4^+ into amino acids and then into other nitrogen-containing biomolecules. Glutamate is the source of amino groups for most other amino acids, and an increase in its availability may lead to a general increase in amino-acid synthesis. There is some evidence that this may be so. Chorismic acid is a key intermediate in the synthesis of the aromatic amino acids, and chorismate synthase exhibits a 2.5-fold increase in response to AHL treatment. Chorismate is synthesized from phosphoenolpyruvate and erythrose 4-phosphate, the latter arising from the activities of the oxidative and reductive pentose phosphate pathways. The combined changes in aldolase, enolase and RuBisCo activities discussed above would be expected to lead to an increase in E4P that would then be available for chorismate synthesis. In cyanobacteria, ammonium assimilation is under strict regulatory control to maintain C:N balance and homeostasis of the amino acid pools (Muro-Pastor *et al.*, 2005). It is possible that in *Gloeothece*, there is an AHL-based system that regulates the carbon and nitrogen pools as a response to increasing cell density.

The increased expression of an ABC-type transporter observed (Table 1) would be expected to increase the capacity for transmembrane transport of metabolites such as amino acids (Higgins, 1992), and so be consistent with an increase in synthesis of amino acids that may result from the changes in expression of the enzymes already discussed. There is evidence that cells of *Gloeothece* excrete amino acids during times when nitrogen fixation exceeds the capacity for growth and that these are reimported at times of limited nitrogen fixation (Flynn and Gallon, 1990). The increase in the transporter protein may reflect this situation. An acyl transferase is also upregulated in response to AHL and potentially may be involved in the demonstrated autoinduction of C8-AHL biosynthesis. Although no LuxI and LuxR homologues have been detected in cyanobacteria, alternative pathways for AHL biosynthesis are known, for example, the HtdS-utlizing pathway in *Pseudomonas fluorescens*. The HtdS enzyme involved belongs to the lysophosphatidic acid acyltransferase family and has been reported to direct the synthesis of some AHLs (Laue *et al.*, 2000). It is possible that C8-AHL production in *Gloeothece* occurs through such a route, although the genome of *Gloeothece* has not yet been characterized. The upregulation of an acyl transferase may reflect the requirement of a C8 carbon chain being available for AHL biosynthesis and may arise as an identified protein through homology to other similar enzymes such as the HtdS acyltransferase enzyme. Although this data can be used to suggest functions for these differentially expressed proteins and how such functions may interact, confirmation of the role(s) of the individual proteins requires further focused study of each protein. Despite these reservations, the tentative roles provide some insight into the effect of C8-AHL on the cyanobacterium.

Cells within densely clustered colonies are likely to encounter nutrient or light limitation and may tend towards decreased metabolic activity. It is possible that these stressed cells can undergo coordinated activities through quorum sensing, for example, the changes in carbohydrate and amino-acid metabolism discussed, which can allow growth and biofilm development under such conditions. It should not be overlooked that biofilms frequently represent mixed communities of organisms. It is possible that some of these other organisms also respond to C8-AHL, although the quality of the information such cross talk would convey would depend on many factors. However, in most environmental situations, it is unlikely that *Gloeothece* exists in isolation. So, release of C8-AHL from *Gloeothece* cells may initiate a response in members of the surrounding microbial community that can receive and interpret the signal. Depending on the nature of the response, such cross talk may facilitate interactions between *Gloeothece* and the other species in its environment. It is also

recognized that some species can degrade AHLs and can use them as a carbon or nitrogen source (Leadbetter and Greenberg, 2000; Flagan *et al.*, 2003). Even if the available AHLs do not supply sufficient carbon for growth, their degradation by some species would likely influence signal gradients to the extent that some *Gloeothece* cells in any microbial community would not experience sufficient AHL concentration to trigger a response.

The current results indicate that elements of a quorum-sensing system operate in *Gloeothece* but its function, especially in the context of the biofilm, is unclear. It remains to be established what factors influence the accumulation of AHLs and the consequences of such accumulation in terms of cell and community function. The organism's relation to other species that are found in association with *Gloeothece* has still to be investigated.

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