Article

Quorum Sensing in Some Representative Species of Halomonadaceae

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Abstract: Cell-to-cell communication, or quorum-sensing (QS), systems are employed by bacteria for promoting collective behaviour within a population. An analysis to detect QS signal molecules in 43 species of the Halomonadaceae family revealed that they produced N-acyl homoserine lactones (AHLs), which suggests that the QS system is widespread throughout this group of bacteria. Thin-layer chromatography (TLC) analysis of crude AHL extracts, using Agrobacterium tumefaciens NTL4 (pZLR4) as biosensor strain, resulted in different profiles, which were not related to the various habitats of the species in question. To confirm AHL production in the Halomonadaceae species, PCR and DNA sequencing approaches were used to study the distribution of the luxI-type synthase gene. Phylogenetic analysis using sequence data revealed that 29 of the species studied contained a LuxI homolog. Phylogenetic analysis showed that sequences from Halomonadaceae species grouped together and were distinct from other members of the Gammaproteobacteria and also from species belonging to the Alphaproteobacteria and Betaproteobacteria.

Keywords: hypersaline environments; halophiles; quorum sensing; N-acyl homoserine lactones; Halomonadaceae
1. Introduction

The definition of extreme environments has been the subject of considerable controversy and as yet, due to its complexity, no consensus of opinion has been reached. In 1979 Brock defined extreme environments as those habitats with low species diversity [1]. It has been suggested, for example, that in hypersaline environments, a typical extreme habitat, environmental factors such as a high salt concentration along with low oxygen concentrations, high or low temperatures, basic pH and solar radiation may contribute to limiting their biodiversity [2]. Nevertheless, the results of a large number of ecological studies conducted recently in extreme environments indicate that in fact they contain a fairly high diversity of species [3–6].

According to 16S rDNA gene-sequence analysis, the family Halomonadaceae [7], within the order Oceanospirillales, forms a separate phylogenetic lineage within the class Gammaproteobacteria. Halomonadaceae contains the largest number of halophilic species described to date [8], including 10 genera of halophilic and halotolerant bacteria [9]: Aidingimonas (one species), Carnimonas (one species), Chromohalobacter (nine species), Cobetia (two species), Halomonas (80 species), Kushneria (five species), Modiscosalibacter (one species) and Salinicola (three species); as well as two genera of non-halophilic bacteria: Halotalea (one species) and Zymobacter (one species). Many members of the Halomonadaceae family are moderately halophilic since they grow best in media containing from 0.5 M to 2.5 M NaCl [10], although some can grow over a very broad range of salt concentrations due to their ability to accumulate organic compounds to adapt themselves to changes in environmental osmolarity. Compatible solutes such as betaine can be taken up from the external medium or others, such as ectoine, synthesized by the cells themselves [8,11,12]. Halomonadaceae species have been isolated from very different habitats, including the sea, salterns, saline soils and endorheic lakes, and have also been found in some seafoods, marine invertebrates and even in a mural painting [9,13].

Extensive studies carried out over the last 30 years into this group of halophiles have led us to a better understanding of their biodiversity, phylogenetic relationships, physiological and haloadaptive mechanisms and, more recently, their biotechnological applications. Some moderate halophiles are recognised for their potential use in biotechnology because of their capacity to produce exopolysaccharides, enzymes and compatible solutes such as ectoine—used as a stabilizer for enzymes—as well as for their active role in the process of denitrification and the degradation of aromatic compounds [6,13,14].

Within the Halomonadaceae, Halomonas is one of the genera most frequently isolated from hypersaline waters and soils by conventional-culture techniques [11,15]. Molecular-ecology techniques based on 16S rDNA sequence analysis suggest that Halomonas ventosae is the predominant species in these habitats [16]. Nevertheless, the ecological role that Halomonas species play in these habitats and their relationships with other halophilic and non-halophilic microorganisms are still unknown.

Bacteria have evolved sophisticated mechanisms to co-ordinate gene expression, such as quorum sensing (QS) [17–19], which involves the production of signal molecules known as autoinducers. Autoinducers include, among others, N-acyl homoserine lactones (AHLs), produced by the Proteobacteria, oligopeptides, produced by the Firmicutes, and furanosylborate diester (AI-2), which is produced by both Proteobacteria and Firmicutes and used for interspecies communication [20,21]. The classical AHL-based systems contain a luxI homologue gene, which is responsible for the
synthesis of AHLs, and a luxR homologue gene, which is an AHL-dependent transcriptional regulator. AHL-based systems involve the accumulation of AHL molecules in the extracellular medium until a critical concentration is reached, at which point the AHLs bind a transcriptional activator, which triggers the expression of target genes, including the luxI gene, leading in turn to the production of more AHLs [22–24] and the expression of virulence factors and exoenzymes, conjugal DNA transfer, control of plasmid-copy number, production of and susceptibility to antibiotics, biofilm formation and exopolysaccharide production [20,21].

AHL-dependent systems have been reported in many genera belonging to the phylum Proteobacteria. In addition, studies based on genome sequencing have revealed that many bacteria contain possible luxI/luxR homologues and, in some cases, multiple coexisting QS systems [25]. Nevertheless, little is known about the QS systems in halophilic microorganisms. In our experiments we have found that four exopolysaccharide-producing species of the genus Halomonas produce AHL-autoinducer molecules [26]. We have also described the structure of AHL molecules produced by Halomonas anticariensis (C₄-HSL, C₆-HSL, C₈-HSL and C₁₂-HSL) [26] and identified and characterized the QS genes hanR/hanI involved in their production [27]. In addition, we have proved that the QS system is regulated by a GacS/GacA two-component system, suggesting its integral involvement in the intercellular communication strategies of this bacterium [28].

In this present study we have detected and identified quorum-sensing systems that rely upon the production of AHLs in 43 species belonging to the Halomonadaceae family. Using PCR and DNA sequencing approaches, we have studied the distribution of the LuxI-type synthase in 29 of these species and constructed a phylogenetic tree, that was based on a partial sequence of this protein.

2. Results and Discussion

2.1. Detection of AHLs in the Halomonadaceae Family

We have investigated the existence of AHL-dependent QS systems in 43 species of the Halomonadaceae family. These 43 bacteria, the type strains of their respective species, were isolated from very different saline habitats, including salterns, saline soils, marshes and seawater, among others (Table 1). They include 12 species discovered by our research group during the course of ecological and taxonomic studies conducted in hypersaline environments in Spain, Chile and Morocco [29–41]. The strains used in this study include representatives of the following genera: Chromohalobacter (one species), Cobetia (one species), Halomonas (33 species), Halotalea (one species), Kushneria (three species), Modicisalibacter (one species) and Salinicola (two species). So far AHL signal molecules have only been detected in four exopolysaccharide-producing species of Halomonas described in our laboratory: H. eurihalina, H. maura, H. ventosae and H. anticariensis [26].

To overcome the limitation of the “cross-streak” method [42], in which each couple of sensor test strains must be cultured under optimum conditions without interfering with each other, we extracted AHLs from all the strains assayed and added them to agar plates upon which the biosensor strain had already been spread (see Experimental Section). Our choice of biosensor strains was ultimately based on previous experience in our laboratory [26]. Thus we chose the Chromobacterium violaceum strain CV026, a mutant which cannot synthesize its own quorum-sensing signal molecules and responds to
exogenously added short-chain AHLs (C₄-C₆-HSLs), producing a pigment called violacein [43], and also Agrobacterium tumefaciens NTL4 (pZLR4), a sensitive, broad-spectrum AHL-responsive reporter that is unable to produce its own AHLs and contains a lacZ fusion to the quorum-sensing regulated gene traG. This latter strain is sensitive to AHLs with medium-to-long acyl chains that, when added exogenously, activate lacZ fusion, which is detectable by the appearance of a blue stain in the presence of X-Gal [44]. All the strains tested synthesized signal molecules to activate the biosensor A. tumefaciens NTL4 (pZLR4) (see Figure 1 for some examples). No signal was detected when a sample from an uninoculated cultured medium of MY 7.5% (w/v) was tested as negative control (data not shown). These results initially suggested that most strains were able to produce AHLs and therefore probably possess at least one AHL-QS system. Nevertheless, only Halomonas rifensis HK31ᵀ and H. anticariensis FP35ᵀ, used as control, produced AHLs in sufficient quantities to activate C. violaceum CV026 under our assay conditions. As is demonstrated below, these two strains produce about five times more AHL than the rest of the species tested (Figure 2). We have in fact already described how some species of Halomonas, such as H. anticariensis FP35ᵀ, synthesize much greater quantities of AHLs than others, such as H. eurihalina, H. maura and H. ventosae [26].

Figure 1. N-acyl homoserine lactone (AHL) production by Halomonas salina F8-11ᵀ, H. eurihalina F9-6ᵀ, H. pacifica DSM 4742ᵀ and H. variabilis DSM 3051ᵀ. A volume of 5µL of AHLs previously extracted from the bacterial cultures were visualized on agar plate diffusion assay by means of the indicator strain A. tumefaciens NTL4 (pZLR4).

2.2. Characterization of the AHLs

The use of the indicator organisms in combination with thin-layer chromatography (TLC) provides a simple, rapid way of determining the number and nature of the AHLs produced by a particular strain [44]. We analysed the culture extracts of the 43 Halomonadaceae strains (Table 1) using TLC in combination with the biosensor A. tumefaciens NTL4 (pZLR4). This analysis showed the production of different AHL profiles amongst the various genera e.g. Chromohalobacter salexigens (Figure 2, lane 2), Cobetia marina (Figure 2, lane 3), Halomonas anticariensis (Figure 2, lane 6), Halotalea alkalilenta (Figure 2, lane 37), Kushneria marisflavi (Figure 2, lane 40) and Salinicola halophilus (Figure 2, lane 42) and also among certain species such as Halomonas alimentaria YKJ-16ᵀ (Figure 2, lane 4), H. anticariensis FP35ᵀ (Figure 2, lane 6), H. desiderata FB2ᵀ (Figure 2, lane 11) and...
H. eurihalina F9-6T (Figure 2, lane 14). Similarly, in a previous study [26] we found that strains belonging to the same species showed the same AHL profiles whilst different species showed different profiles. In just the same way, significant differences have been identified in the AHL profiles of the marine species Vibrio salmonicida [45] and V. anguillarum [46].

**Figure 2.** Thin-layer chromatography (TLC) analysis of the AHLs produced by the 43 species of Halomonadaceae: lane 1, Carnimonas nigificans CTCBS1T; lane 2, Chromohalobacter salexigens DSM3043T; lane 3, Cobetia marina 219T; lane 4, Halomonas alimentaria YKJ-16T; lane 5, H. almeriensis M8T; lane 6, H. anticariensis FP35T; lane 7, H. aquamarina 558T; lane 8, H. campaniensis 5AGT; lane 9, H. cerina SP4T; lane 10, H. denitrificans M29T; lane 11, H. desiderata FB2T; lane 12, H. elongata 1H9T; lane 13, H. eurihalina F9-6T, lane 14, H. fontilapidosi 5CRT; lane 15, H. gudaonensis SL014B-69T; lane 16, H. halmophilae ACAM 71T; lane 17, H. halodenitrificans ATCC 13511T; lane 18, H. halodurans DSM 5160T; lane 19, H. koreensis SS20T; lane 20, H. magadiensis 21 MIT; lane 21, H. maura S-31T; lane 22, H. meridiana ACAM 246T; lane 23, H. mongoliensis Z-7009T; lane 24, H. nitroreducens 11-ST; lane 25, H. organivorans G-16.1T; lane 26, H. pacifica DSM 4742T; lane 27, H. pantelleriensis AAPT; lane 28, H. rambllicola RS-16T; lane 29, H. rifensis HK31T; lane 30, H. saccharevitans AJ275T; lane 31, H. salina F8-11T; lane 32, H. shengliensis SL014B-85T; lane 33, H. stenophila N12T; lane 34, H. subglaciescola ACAM 12T; lane 35, H. variabilis DSM 3051T; lane 36, H. ventosae Al-12T; lane 37, Halotalea alkalilenta AW-7T; lane 38, Kushneria avicenniae MW2aT; lane 39, K. indalinina CG2.1T; lane 40, K. marisflavi SW32T; lane 41, Modicisalibacter tunisiensis LIT2T; lane 42, Salinicola halophilus CG4.1T; lane 43, S. salarius M27T. Each lane contains 10 µL of AHL crude extract except for lanes 6 and 29, which contain 5 µL. Lane S; synthetic AHL standards: o xo-C6-HSL (4.7 pmol), C6-HSL (804 pmol), C8-HSL (31.6 pmol), C10-HSL (2 nmol), C12-HSL (4.8 nmol).
The AHL patterns from Halomonadaceae species contain from one to three spots with mobilities similar to those of the C₈-HSL, C₆-HSL and 3-oxo-C₆-HSL standards. Differences were also observed in the quantities of AHLs synthesized, Halomonas anticariensis FP35ᵀ and Halomonas rifensis HK31ᵀ synthesizing about five times more AHLs than any of the other 41 species examined (Figure 2, lanes 6 and 29). Halomonas variabilis DSM 3051ᵀ (Figure 2, lane 35) and Salinicola salarius M27ᵀ (Figure 2, lane 43) produce as low an amount of signal as that from the uninoculated cultured medium MY 7.5% (w/v) [26], although, their AHL-extracts did activate the A. tumefaciens NTL4 (pZLR4) indicator strain when the diffusion plate assay was carried out (data not shown). The most predominant AHL molecule was C₆-HSL. In H. anticariensis FP35ᵀ this AHL had been previously identified by gas chromatography/mass spectrometry (GM/MS) and electrospray ionization tandem mass spectrometry (ESI MS/MS) [26], suggesting that this signal molecule may well be biologically active in intercellular communication strategies within the Halomonadaceae family. The synthesis of short-chain-acyl AHLs, such as C₆-HSL and C₈-HSL, is also very common among the species belonging to the Vibrionaceae family, which are ubiquitous in marine environments [45,46].

2.3. Distribution of the Autoinducer Synthase Gene

The luxI autoinducer synthase gene has been reported to be responsible for AHL production [18,47]. Therefore we tested its presence in the genome of 43 Halomonadaceae species, using luxI primers to amplify by PCR a fragment (300–400 bp) which forms part of the active site of the enzyme [48]. In this way we identified them in 29 species. On sequencing they turned out to be homologous to a luxI gene fragment. We did not, however, detect PCR fragments in the other 14 AHL-producing strains, possibly due to primer mismatching. In the cases of Chromohalobacter salexigens and Halomonas elongata, both of which do produce AHLs and the sequenced genomes of which are available, no luxI gene could be identified, due probably to their having different AHL synthases, either belonging to the LuxM protein family found in the genus Vibrio [49,50] or to the HdtS protein family identified in Pseudomonas fluorescens [51].

To carry out a phylogenetic analysis of the LuxI synthase in the 29 positive species of the Halomonadaceae family we conducted a multiple sequence alignment, including amino-acid sequences of LuxI synthase that had been experimentally determined in other members of the phyla Alphaproteobacteria, Betaproteobacteria and Gammaproteobacteria. The phylogenetic tree constructed according to the neighbour-joining method showed that all the amino-acid sequences from the Halomonadaceae family grouped together and were distinct from the rest of the Gammaproteobacteria analysed, and also from the species belonging to the Alphaproteobacteria and Betaproteobacteria (Figure 3a). The clustering of the 29 Halomonadaceae in which the luxI fragment was detected was not related to the habitat from which they were isolated (Table 1). The distribution of the Halomonadaceae family with respect to the rest of species analysed in the phylogenetic tree based on the 16S rDNA sequences (Figure 3b) was similar to that obtained from the LuxI sequence (Figure 3a). This result indicates that the LuxI amino-acid partial region used in this study is conserved among the family Halomonadaceae members.
Figure 3. Phylogenetic trees based on LuxI sequences (a) and 16S rDNA sequences (b) found in some members of Alphaproteobacteria, Betaproteobacteria and Gammaproteobacteria, including the species studied here belonging to the Halomonadaceae family. Abbreviations for bacterial genus names: A, Aliivibrio; Ac, Acidithiobacillus; Ae, Aeromonas; Ag, Agrobacterium; Az, Azospirillum; B, Burkholderia; Car, Carnimonas; C, Chromobacterium; Er, Erwinia; En, Enterobacter; H, Halomonas; Halot, Halotalea; K, Kushneria; M, M. Medicinalibacter; Me, Mesorhizobium; Ps, Pseudomonas; Rh, Rhizobium; Ra, Ralstonia; Rho, Rhodobacter; Si, Sinorhizobium; S, Salinicola; Se, Serratia; V, Vibrio; Y, Yersinia. The scale bar indicates the mean number of substitutions per site. Bootstrap values were obtained from 1,000 replicates via neighbour-joining algorithms using the MEGA program. Only branches with values >50% are shown. The branches highlighted in red are sequences from Alphaproteobacteria, in green from Betaproteobacteria and in blue from Gammaproteobacteria. The sequence accession numbers are given in brackets.
Figure 3. Cont.
3. Experimental Section

**Bacterial strains.** We used the type strains of 43 species belonging to the *Halomonadaceae* family (Table 1). Strains were cultured at 32 °C in MY medium (3 g malt extract, 3 g yeast extract, 10 g glucose and 5 g peptone per litre) \[52,53\] modified with a balanced mixture of sea salts \[54\].

**Table 1.** Species of the *Halomonadaceae* family included in this study \[9\].

| Species                        | Strain        | Ecological Niches                                                                 |
|--------------------------------|---------------|-----------------------------------------------------------------------------------|
| 1. *Carnimonas nigrificans*    | CTCBS1 \(^T\) | Cured meat, Spain                                                                  |
| 2. *Chromohalobacter salexigens*| DSM 3043 \(^T\)| Solar saltern, Netherlands                                                         |
| 3. *Cobetia marina*            | 219 \(^T\)    | Sea water, USA                                                                     |
| 4. *Halomonas alimentaria*     | YKJ-16 \(^T\) | Jeotgal, a traditional Korean fermented seafood, Korea                             |
| 5. *H. almeriensis*            | M8 \(^T\)     | Solar saltern, south-east Spain                                                    |
| 6. *H. anticariensis*          | FP35 \(^T\)   | Saline wetland, southern Spain                                                     |
| 7. *H. aquamarina*             | 558 \(^T\)    | Pacific ocean                                                                      |
| 8. *H. campaniensis*          | 5AG \(^T\)    | Mineral pool, Italy                                                                |
| 9. *H. cerina*                | SP4 \(^T\)    | Saline soil, Spain                                                                 |
| 10. *H. denitrificans*         | M29 \(^T\)    | Saline water, Korea                                                                |
| 11. *H. desiderata*            | FB2 \(^T\)    | Municipal sewage works, Germany                                                    |
| 12. *H. elongata*              | 1H9 \(^T\)    | Solar saltern, Netherlands                                                         |
| 13. *H. eurihalina*            | F9-6 \(^T\)   | Saline soil, Spain                                                                 |
| 14. *H. fontilapidosi*         | CR-5 \(^T\)   | Saline soil, southern Spain                                                        |
| 15. *H. gudaonensis*           | SL014B-69 \(^T\)| Saline soil contaminated by crude oil, China                                       |
| 16. *H. halophila*             | ACAM 71 \(^T\)| Dead Sea, Israel                                                                  |
| 17. *H. halodenitrificans*     | ATCC 13511 \(^T\)| Meat in brine                                                                    |
| 18. *H. halodurans*            | DSM 5160 \(^T\)| Great Bay estuary, USA                                                             |
| 19. *H. koreensis*             | SS20 \(^T\)   | Solar saltern, Korea                                                               |
| 20. *H. magadiensis*           | 21 MI \(^T\)  | Soda lake, East-African Rift Valley                                               |
| 21. *H. maura*                 | S-31 \(^T\)   | Saltern, Morocco                                                                   |
| 22. *H. meridiana*             | ACAM 246 \(^T\)| Saline lake, Antarctic                                                             |
| 23. *H. mongoliensis*          | Z-7009 \(^T\) | Soda lake, Mongolia                                                                |
| 24. *H. nitroreducens*         | 11-S \(^T\)   | Solar saltern, Chile                                                               |
| 25. *H. organivorans*          | G-16.1 \(^T\) | Hypersaline habitats contaminated by aromatic organic compounds, southern Spain   |
| 26. *H. pacifica*              | DSM 4742 \(^T\)| Pacific ocean                                                                   |
| 27. *H. pantelleriensis*       | AAP \(^T\)    | Hard lake sand, Pantelleria island, Italy                                           |
| 28. *H. ramblicola*            | RS-16 \(^T\)  | Saline soil, south-east Spain                                                     |
| 29. *H. rifensis*              | HK-31 \(^T\)  | Solar saltern, Morocco                                                             |
| 30. *H. saccharevitans*        | AJ275 \(^T\)  | Salt lake and a subterranean saline well, China                                   |
| 31. *H. salina*                | F8-11 \(^T\)  | Saline soil, Spain                                                                 |
| 32. *H. shengliensis*          | SLO14B-85 \(^T\)| Saline soil contaminated with crude oil, China                                    |
| 33. *H. stenophila*            | N12 \(^T\)    | Saline soil, Spain                                                                 |
| 34. *H. subglaciescra*         | ACAM 12 \(^T\)| Antarctic hypersaline, meromictic lake                                             |
| 35. *H. variabilis*            | DSM 3051 \(^T\)| Great Salt Lake, USA                                                              |
Table 1. Cont.

| Species                        | Strain | Ecological Niches                                                                 |
|--------------------------------|--------|----------------------------------------------------------------------------------|
| 36. *H. ventosae*              | Al-12\textsuperscript{T} | Saline soil, south-eastern Spain                                                  |
| 37. *Halotalea alkalilenta*    | AW-7\textsuperscript{T}  | Alkaline olive-mill waste (alpechin), Greece                                     |
| 38. *Kushneria avicenniae*     | MW2a\textsuperscript{T}  | Salty leaves of *Avicennia germnans* trees growing near solar salterns, Puerto Rico |
| 39. *K. indalinina*            | CG2.1\textsuperscript{T} | Solar saltern, south-east Spain                                                   |
| 40. *K. marisflavi*            | SW32\textsuperscript{T}  | Water from the Yellow Sea, Korea                                                   |
| 41. *Modicisalibacter tunisiensis* | LIT2\textsuperscript{T} | Oilfield-water injection sample, southern Tunisia                                |
| 42. *Salinicola halophilus*    | CG4.1\textsuperscript{T} | Solar saltern, south-east Spain                                                   |
| 43. *S. salarius*              | M27\textsuperscript{T}  | Saline water, Korea                                                              |

Note: Species shaded in grey indicates that the LuxI homolog has been detected by PCR.

*Chromobacterium violaceum* CV026 was cultured at 30 °C in LB medium supplemented with 2.5 mM CaCl\textsubscript{2} and 2.5 mM MgSO\textsubscript{4} (LB/MC) and containing 50 µg kanamycin per mL [43]. *Agrobacterium tumefaciens* NTL4 (pZLR4) was cultured at 30 °C in LB medium supplemented with 2.5 mM CaCl\textsubscript{2} and 2.5 mM MgSO\textsubscript{4} (LB/MC), in MGM minimal medium (11 g Na\textsubscript{2}HPO\textsubscript{4}, 3 g KH\textsubscript{2}PO\textsubscript{4}, 0.5 g NaCl, 1 g glutamate, 10 g mannitol, 1 mg biotin, 27.8 mg CaCl\textsubscript{2} and 246 mg MgSO\textsubscript{4} per litre) containing 50 µg gentamycin per ml, and in AB medium [44,55].

Extraction and detection of AHLs. AHL molecules were extracted following the technique described in our previous studies [56,57]. Briefly, 20 mL cultures were grown until the early stationary phase (optical density of approximately 2.8 at 600 nm) and then extracted twice with equal volumes of dichloromethane. The extracts were dried and suspended in 40 µL of 70% v/v methanol.

To detect AHLs, an overnight culture of one of the AHL indicator strains [*Chromobacterium violaceum* CV026 or *Agrobacterium tumefaciens* NTL4 (pZLR4)] was diluted 1:100 in 5mL of the corresponding medium and poured onto LB/MC and AB supplemented with 80 µg of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) per ml agar plates. Once the plates were dry, paper disks 5 mm in diameter were placed onto them and the AHL samples applied. The assay plates were incubated overnight at 32 °C to allow the indicator organisms to grow and surround the paper disks with either purple or blue haloes.

Thin-layer chromatography analysis of AHLs. To characterize the AHLs, the samples were subjected to analytical and preparative thin-layer chromatography (TLC). AHL samples and standards were spotted onto a TLC plate and developed with 70% v/v methanol in water. The plate was air-dried and overlaid with top agar containing the *A. tumefaciens* NTL4 (pZLR4) indicator strain before being incubated at 32 °C. For the *A. tumefaciens* NTL4 (pZLR4) overlay, a 6–8 h culture in MGM medium was mixed with an equal volume of fresh medium, 1.5% w/v Bacto Agar and 80 µg of X-Gal per mL [26].

The standard AHLs used were: N-β-ketocaproyl-dl-homoserine lactone (3-oxo-C\textsubscript{6}-HSL), N-hexanoyl-dl-homoserine lactone (C\textsubscript{6}-HSL), N-octanoyl-dl-homoserine lactone (C\textsubscript{8}-HSL) and N-decanoyl-dl-homoserine lactone (C\textsubscript{10}-HSL) (Sigma\textsuperscript{®}).

Chromosomal DNA extraction, autoinducer synthase gene amplification and sequencing. Chromosomal DNA was isolated and purified according to Marmur's protocol [58], modified by Martín-Platero and co-workers [59]. The purified DNA was dissolved in 50 µL doubly distilled water.
and checked by agarose gel electrophoresis [60] An internal segment of the autoinducer synthase gene was amplified from approximately 100 ng of chromosomal DNA by using the primers \textit{luxI}-F: 5'-GGGAGATATATACTGTAA-3' and \textit{luxI}-R: 5'-TGAGGTATTATTTCTGCAA-3'. These primers were designed to target a highly conserved region of the \textit{hanI} autoinducer synthase gene of \textit{Halomonas anticariensis} FP35\textsuperscript{T}. The \textit{hanI} gene is about 645 bp and the primer pair amplified approximately 386 bp of the conserved active site of the enzyme which contains the three conserved amino acids Arg71 (R71), Glu101 (E101) and Arg104 (R104) [27]. PCR entailed 30 cycles of 30 s at 95 °C, 30 s at 50 °C and 30 s at 72 °C. The annealing temperature was determined by PCR with a temperature gradient from 40 °C to 60 °C. All of the PCRs were run in a T100\textsuperscript{TM} thermal cycler (Bio-Rad).

The PCR fragments were purified and sequenced with \textit{luxI}-F or \textit{luxI}-R primers using a BigDye Terminator Cycle Sequencing Kit in an ABI 3100 DNA sequencer (Applied Biosystems). The DNA sequences thus obtained were analysed using a BLAST search of the GenBank database [61] to align homologous regions of autoinducer synthase gene sequences from different isolates.

\textit{Phylogenetic analysis}. A phylogenetic tree was constructed using version 4 of the MEGA (Molecular Evolutionary Genetics Analysis) software [62] after multiple alignments of the data by CLUSTALW [63] and the alignments were checked manually. Distances and clustering were determined according to the neighbour-joining method and bootstrap values were measured on the basis of 1,000 replications.

\textit{Nucleotide sequence accession number}. The autoinducer synthase DNA sequences reported here have been deposited in the GenBank database under accession numbers from KC244227 to KC244255.

4. Conclusions

Screening for AHL signal molecules in 43 species belonging to the \textit{Halomonadaceae} family revealed that the AHL-QS system is widespread within this group of bacteria. We did however find diversity within the AHL-profile signalling molecules produced by the different genera, and even between the molecules produced by different species from the same genus. Such variety would seem to be consistent with the ecological, physiological, metabolic and taxonomic diversity among them. The role of QS signalling in these extremophilic microorganisms remains to be elucidated and further work needs to be done to explore this bacterial cell-cell communication process in the multispecies communities.

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