Archaea join the conversation: detection of AHL-like activity across a range of archaeal isolates

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ABSTRACT

Quorum sensing is a mechanism of genetic control allowing single cell organisms to coordinate phenotypic response(s) across a local population and is often critical for ecosystem function. Although quorum sensing has been extensively studied in bacteria comparatively less is known about this mechanism in Archaea. Given the growing

significance of Archaea in both natural and anthropogenic settings, it is important to delineate how widespread this phenomenon of signaling is in this domain. Employing a plasmid-based AHL biosensor in conjunction with thin layer chromatography (TLC), the present study screened a broad range of euryarchaeota isolates for potential signaling activity. Data indicated the presence of 11 new Archaeal isolates with AHL-like activity against the LuxR-based AHL biosensor, including for the first time putative AHL activity in a thermophile. The presence of multiple signals and distinct changes between growth phases were also shown via TLC. Multiple signal molecules were detected using TLC in *Haloferax mucosum*, *Halorubrum kocurii, Natronococcus occultus* and *Halobacterium salinarium*. The finding of multiple novel signal producers suggests the potential for quorum sensing to play an important role not only in the regulation of complex phenotypes within Archaea but the potential for cross-talk with bacterial systems.

Introduction

Micro-organisms are capable of complex community level co-ordination of phenotypes through the process of communication termed quorum sensing. Early experiments with growth-mediated genetic control of bioluminescence were detected in *Vibrio fischeri* and *Vibrio harveyi* (Rosson and Nealson,1981), with AHL-mediated quorum sensing was first described in *Vibrio fischeri* which utilize N-acyl homoserine lactones (AHLs) as a messenger molecule (Dunlap, 1999). These signal molecules, commonly produced by an AHL synthase, such as LuxI, are released into the environment until the signal builds up to a critical threshold triggering a phenotypic change. This typically occurs in later growth phases due to the effects of diffusion (Redfield, 2002). AHL based quorum sensing is quite common among Gram-negative proteobacteria such as *Pseudomonas* and *Vibrio*. Initially AHL based quorum sensing was thought to be limited to Gram-negative bacteria, however this has been recently

challenged by discoveries of AHL molecules in cyanobacteria (Sharif *et al.* 2008) and members of *Bacteroidetes* (Romero *et al.* 2010). Additionally, bacteria belonging to *Actinobacteria* and *Firmicutes* phyla have been identified as producing AHL activity in a range of environments (Biswa and Doble, 2013; Ma *et al.* 2016; Bose *et al.* 2017) including Shark Bay microbial mats (Charlesworth *et al*. 2019). These discoveries together help to broaden the base of potential AHL communication within bacteria. There are a wide variety of non-AHL signal molecules including auto-inducing peptides, furansoyl borate diesters, quinolones, pyrones (Brachmann *et al.* 2013) and other molecules such as diketopiperazines (Holden *et al.* 1999) or rosmarinic acid (Corral-Lugo *et al.*, 2016), as well as synthetic ligands which can also interact with AHL signaling systems (Gerdt *et al.*, 2015). Eukaryotes are also capable of producing their own quorum sensing signals such as tyrosol detected in some yeasts (Chen *et al.* 2004), or production of AHL inhibitors such as furanones (Manefield *et al.* 1999; Joint *et al.* 2007).

The third domain of life, Archaea, are often considered to be an extreme branch of the tree of life, occupying environments other micro-organisms cannot tolerate, including conditions of extremes in temperature, salinity, and pH (Kanai *et al.* 1995; Rinker and Kelly, 1996; Goh *et al.* 2006). Extreme environments, while posing a significant challenge for cells, can also promote the production of secondary metabolites such as antibiotics or signaling molecules which could provide a competitive advantage to community members, including through biofilm formation (Charlesworth and Burns, 2015). These metabolites could be important in surviving extreme environmental conditions (Charlesworth and Burns 2015).

The first indication of quorum sensing signals in Archaea was shown in the Euryarchaeota phylum in the methanogen *Methanosaeta harundinacea*, which has been shown to produce

carboxylated AHLs (Zhang *et al.* 2012). Two other Archaea, also members of the Euryarchaoeta phylum have also shown AHL-like activity, *Haloterrigena hispanica* (Tommonaro *et al.* 2012) which is capable of producing diketopiperazines (DKPs), signal molecules that also interact with LuxR response elements (Holden *et al.* 1999), and *Natronoccous occultus* which is capable of inducing AHL biosensors in the late/stationary phase of growth (Paggi *et al.* 2003). This latter signal coincided with the induction of an extracellular protease (Paggi *et al.* 2003). Most recently, *Halorubrum lacusprofundi* also of the Euryarchaoeta phyla, has been shown to have AHL activity, and there were differences observed between planktonic and biofilm growth phenotypes (Liao *et al.* 2016). However, despite these few studies providing insights into the potential for quorum sensing in the Archaea, very little is still known (Charlesworth *et al.* 2017).

Archaea are both prevalent and important for ecosystem function in a wide range of environments (Jarrell *et al.* 2011; Wong *et al.* 2018), thus it is critical to obtain a better understanding of the scope of signalling in this domain. It is this gap in knowledge the present study is addressing: delineating the breadth of AHL or AHL-like signalling, AHL-like signalling being defined as metabolites capable of activating AHL response genes without being an AHL across a range of Euryarchaeota isolates.

Methods

Archaeal strains and growth conditions. The Archaea analysed in the present study are shown in Table 1. These were chosen to cover a range of environments and conditions. *Halorubrumlacusprofundi, Halobacteria* sp. DL1, *Haloarchaea* isolate DL31 were originally isolated from Antarctic lakes. *Halorubrum kocurii* was isolated from a saline lake in China

and *Natronococcus occultus* and *Natronococcus amylolyticus* were originally isolated from haloalkaliphillic lakes. Strains were grown in specified media and conditions outlined below: *Natronococcus occultus* and *Natronococcus amylolyticus* were grown in haloalkaliphilic (pH 10) growth medium (Tindall *et al.* 1984) at 37˚C shaking at 200 rpm. *Haloferax mucosum*, *Halococcus hamelinensis*, and *Halogeometricum borinquense* were grown in DSM-97 medium (Goh *et al.* 2011) at 37˚C shaking at 200 rpm. *Halorubrum kocurii* and *Halobacterium salinarium* strain NRC-1 were both grown in JCM 168 medium (Kamekura and Dyall-Smith, 1995) at 200 rpm. *Haloferax volcanii* was grown in Hv-YPC medium (Chimileski *et al.* 2014). *Halobacteria* sp. DL1, Haloarchaea isolate DL31, *Haloquadratum walsbyi* and *Halohasta litchfieldiae* were grown in DCBM2 at 30˚C shaking at 200 rpm. *H. lacusprofundi* was grown in DBCM2 media with 1% tryptone at 30˚C as previously described (Liao *et al.* 2016). *Thermococcus celericrescens* was grown in filtered marine medium per DSMZ at 80 ºC anaerobically. *Methanosarcina mazei* was grown anaerobically at 37˚C in DSMZ 318 medium.

To ensure any downstream findings on AHL activity could be attributed to the Archaea under investigation and not any bacterial contamination, a number of quality control checks were performed. Initial purity checks were undertaken by extracting genomic DNA from isolate cultures using the xanthogenate protocol (Tillett and Neilan, 2000) that has been previously shown to extract bacterial and archaeal DNA successfully (Leuko *et al*. 2008, Goh *et al*. 2009, Leuko *et al*. 2009), and these were tested for the absence of bacteria using universal bacterial PCR primers described below. The purity of axenic cultures were continually confirmed on an ongoing basis via a 16S rRNA colony PCR as described previously (Woodman 2008). Primers used were 27F/1492R for bacteria (Zimmer *et al.* 2014) and

21F/985R for Archaea (Goh *et al.* 2006). Additionally, the plate morphologies of the Archaea were routinely inspected prior to experimentation as well as light microscopy to ensure no other cell types present. Only cultures without bacterial contamination and confirmed sequence identity were used for further analysis.

Screen for AHL activity using *E. coli* strain MT102. The AHL biosensor selected for this study is a plasmid-based biosensor, pJBA132 borne in *E. coli* strain MT102. This particular biosensor is based around the LuxR receptor protein and produces GFP in response to AHL stimulus (Andersen *et al.* 2001). This biosensor was chosen as it has a broad range of activity with a high level of sensitivity to a number of AHL variants, ranging from C4 to C12 (Charlesworth *et al.* 2015). To screen for AHL activity, 50 ml cultures were grown in the conditions described above. After ethyl acetate extraction and AHL biosensor screening described below, several haloarchaea were selected for more detailed analyses of potential growth-phase dependent signal molecule production, whereby AHL activity was determined and compared in early log phase vs stationary growth phase cultures, based on growth curve data (Supplementary Figure 4). Experimental set-up involved growing duplicate 200 mL cultures (except for *H. lacusprofundi* where 1 L cultures were used) of *H. hamelinensis*, *H. mucosum*, *H. borinquense*, *N. occultus*, *H. volcanii*, *H. salinarium* strain NRC-1, *T. celericrescens*, *Halobacteria* sp. DL1 and *Haloarchaea* isolate DL31, to early log and stationary growth phase stages according to published growth data (Rinker and Kelly, 1996; Paggi *et al.* 2003; Goh *et al.* 2006; Allen *et al.* 2008).

After growth of strains to the selected phase (early log or stationary), cultures were centrifuged at 5500 rpm for 20 min and supernatant was extracted with acidified ethyl acetate

(0.1% acetic acid) (Shaw *et al.* 1997). For extractions of haloalkaliphillic medium supernatant, an additional acidification step was used to prevent any degradation of signal molecules as per previously published studies (Paggi *et al.* 2003). Following the overnight evaporation of ethyl acetate, extracts were re-dissolved in 200 µL of HPLC grade methanol with 0.1% formic acid, and stored at -20 °C until AHL screening with the *E. coli* strain MT102.

The LuxR-based biosensor *E. coli* strain MT102 (Andersen *et al.* 2001) was employed in a 96 well assay using a Clariostar plate reader to determine relative fluorescence produced in response to exogenous signaling molecules, as described previously (Charlesworth *et al*. 2015). AHL activity of culture extracts was determined against growth medium alone extracts, to prevent any background fluorescence producing false positives. The relative fluorescence units were determined using the media-alone subtracted results and normalized against protein concentrations of the cell pellet as described previously (Liao *et al.* 2016). The one exception was *T. celericrescens*, where there was insufficient biomass to facilitate protein determination in these cultures.

Thin layer chromatography. TLC analysis using *E. coli* MT102 utilized a GE Typhoon phosphorimager for fluorescence analysis of an overlaid TLC sheet, according to methods previously established (Charlesworth *et al.* 2015). The culture extracts with strong activity against the 96 well assay were loaded onto a non-fluorescent reverse phase C18 Silica sheet, in addition to the following AHL standards (Sigma): *N*-3-oxohexanoyl homoserine lactone (OHHL), *N*-octanoyl homoserine lactone (OHL), *N*-hexanoyl homoserine lactone (HHL), *N*-3-oxododecanoyl-l-homoserine lactone (OdDHL), *N*-decanoyl-l-homoserine lactone (DHL)

and *N*-butyryl homoserine lactone (BHL). TLC sheets were then developed in 60:40 methanol to MilliQ water solution prior to overlay with the *E. coli* biosensor. Following 24 h incubation at 30 °C plates were then examined using a GE typhoon phosphorimager, employing the EGFP protocol with excitation source of 489 nm and an emission spectrum of 520 nm. Images were examined using the Image quant software package made available by the manufacturer.

Potential regulation of biofilm production by signaling in *Halorubrum lacusprofundi*. As a recent study demonstrated an increase in AHL biosensor activation in biofilm compared to planktonic cells in *H. lacusprofundi* (Liu *et al.* 2016), this archaeon was examined further to assess whether biofilm production could be influenced by quorum sensing. To test this, conditioned media - media in which cells are grown to a certain point then the cells removed by centrifugation - was prepared as described (Paggi *et al.* 2010), by growing *H. lacusprofundi* to stationary phase under culturing conditions described above. This conditioned media should contain any putative signal molecules produced and excreted and/or passively released into culture supernatants. To assess whether Archaeal biofilm formation may be influenced by any signal molecules produced, cultures of *H. lacusprofundi* were incubated with either 1.6 ml of fresh media (as a control) or conditioned media in tissue culture plates at 30˚C (static) for 3 or 7 days. Plates were contained in zip-lock bags with a moist tissue to reduce evaporation. Biofilm formation was assessed using methods described previously (Stepanovic *et al.* 2007) - a microtitre-based assay that measures adherence of cells in a well after growth to stationary phase - with the modification that the concentration of Hucker Crystal Violet was increased from 0.2% to 2% (w/v). Independent t-tests were conducted to determine any significant difference in biofilm formation.

Results

Identification of Archaea with potentially novel AHL-like signaling activity. A broad screen of multiple Archaea encompassing differing physiologies from the *Euryarcheota* phylum was carried out to determine which strains could activate an AHL biosensor. All strains were confirmed to be free of bacterial contamination by microscopy and none were positive for bacterial 16S rRNA gene using PCR. Activity was determined as inducing fluorescence in the AHL biosensor *E. coli* MT102. Negative controls were extracts of the isolate growth medium without Archaea. Initial screens of these Archaea with a LuxR-based biosensor revealed 11 new isolates possessing AHL-like activity, including the first putative AHL activity from a thermophilic archaeon, *T. celericrescens*. The results of this screen delineating these novel findings are shown in Table 1. There was also confirmation here of *N. occultus* having AHL-like activity, shown previously using the TraR-based biosensor assay (Paggi et al., 2003). A methanogen previously noted as potentially having carboxylated AHLs, *M. mazei* (Zhang *et al.* 2012), was also shown to induce the *E. coli* MT102 biosensor in the present study. Strains that did not induce the biosensor here included *H. walsbyi*, *N. amylolyticus*, *H. litchfieldiae*, *M. taylorii*, and *M. burtonii*. It is important to note that while this particular biosensor is both sensitive and has a broad range of detection, it is possible that some of the Archaea with negative results may be found to possess activity against other biosensors carrying AHL receptor proteins with differing specificities, such as those with longer or shorter chain response proteins. It is also unclear whether the active molecules are actively transported out of the cell or are a by-product of cell lysis.

Growth phase analyses of AHL activity. In order to determine if putative signal strength increased with growth in a manner similar to quorum sensing systems in bacteria, early and stationary phase growth cultures from several Archaea were screened for any potential signal difference between the two phases. Early log phase cultures were extracted on day 3 and stationary phase cultures extracted on day 14 similar to previous experiments conducted with haloarchaea (Liao et al., 2016), and for *T. celericrescens* cultures were extracted at 12 h and 48 h due to the rapid growth rate (Rinker and Kelly, 1996). The results of the fluorescence-based LuxR biosensor are shown in Figure 1. Putative signal is stronger in stationary phase cultures, which is reminiscent of quorum sensing systems in bacteria where the signal is often, though not always, much stronger in later phase cultures. The majority of the Archaea tested had up to 3-fold higher levels of signal activation in stationary phase compared to early phase cultures (Figure 1). Any culture supernatant AHL-like activity was compared to respective media-alone extract controls, and this resulted in some early phase extracts producing no significant fluorescence (Figure 1). Consistent with an earlier study using alternate biosensors (Paggi *et al.* 2003), the activity of stationary phase extracts of *N. occultus* in comparison to early phase extracts was also shown here to have increased.

Semi-qualitative analysis of putative AHL signals via thin-layer chromatography.

Following the fluorescence-based 96 well assays, TLC was performed to visualize the signal profile of selected Archaeal isolates and to observe any differences between the growth phases. Extracts were separated based on polarity and activity was determined by the position as well as size and intensity of spots. A representative TLC plate from these analyses is shown in Figure 2, illustrating signal molecule separation in *H. volcanii* and *N. occultus*. Retardation factor (Rf) values were calculated for each individual spot detected in addition to

known AHL standards (Table 2). Further examples of separation of putative signal molecules by TLC are provided in Supplementary Figures 1-3. The general trend of the TLC data indicates stronger signal presence in stationary growth phase extracts compared to early phase, reinforcing the well-based assay results in the present study. In some cases, there appears to be a shift in number of signal molecule between growth phases with *N. occultus*, *H. mucosum*, *H. kocurii*, *H. salinarium*, *H. lacusprofundi*, and *H. borinquense* all producing multiple signals in the latter phases of growth. The polarity of the signal molecules indicates Archaeal signals tend to exhibit more polarity, and it is clear that several different signal molecules are found in the Archaea screened in this study. Interestingly, *N. occultus*, *H. mucosum*, *H. kocurii*, *H. salinarium*, *H. lacusprofundi*, and *H. borinquense* are all capable of producing multiple signaling molecules with differing Rf values, indicative of differing polarities and likely chemical structures (Table 2).

It does appear some putative signal molecules may be common across organisms with a Rf value between 0.71 and 0.78 present in *H. salinarium*, *H. mucosum*, *H. volcanii*, *N. occultus*, *H. kocurii* and *H. borinquense*. Other potentially similar signals were identified in *H. kocurii*, *N. occultus*, *H. hamelinensis*, *H. mucosum*, *H. borinquense* and *H. salinarium* strain NRC-1 with Rf values between 0.50 and 0.56. Generally, TLC can be used to identify signal molecules based on polarity compared to known standards such as AHLs, however given the unknown nature of signal molecules in Archaea (e.g. possible modifications such as carboxylation), this TLC data cannot be used to conclusively identify these molecules. However, the polarity of the signals appears to be consistent with lower chain AHLs used as standards in this study such as *N*-hexanoyl homoserine lactone (HHL). *H. kocurii* and *H. hamelinesis* both presented signals with the lower Rf values of 0.48 and 0.56 respectively which tend to be more consistent with slightly longer AHLs such as *N*-decanoyl-l-

homoserine lactone (DHL) with a Rf value of 0.40 or *N*-octanoyl homoserine lactone (OHL) with a Rf value of 0.61.

Potential quorum sensing induced biofilm formation in *H. lacusprofundi*. To ascertain whether biofilm formation in Archaea may be regulated by quorum sensing, one of the strains, *H. lacusprofundi,* was chosen for further analyses. Given recent findings indicating increased activation of an AHL bisosensor by biofilm cells (Liao *et al.* 2016), it was hypothesized that incubating *H. lacusprofundi* with conditioned media would result in stronger biofilm formation compared to cultures incubated in fresh media. In this instance 'conditioned' media, also referred to as spent media, is any growth medium in which cultures are grown, and the cells centrifuged leaving the "spent media" behind, containing any putative signal molecules (Liao *et al.* 2016). Crude extract from stationary-phase culture supernatant of *H. lacusprofundi* (conditioned media), was added to starting cultures and biofilm formation in *H. lacusprofundi* incubated in fresh media was compared to those incubated in conditioned media. Cultures grown in conditioned media were shown to generate biofilms with significantly more biomass compared to controls (Figure 3), suggesting that quorum sensing could potentially regulate biofilm formation in this archaeon, though other mechanisms could also be responsible.

Discussion

Despite their critical importance in a number of environments and global biogeochemical cycles (Jarrell *et al.* 2011), our understanding of Archaeal communication has been limited (Paggi *et al.* 2003; Zhang *et al.* 2012; Liao *et al.* 2016). The idea of microbial communication now being present in all three domains of life is a breakthrough concept for microbial

ecology, in particular the ability to identify signals in even the most extreme environments. The present study represents the most comprehensive screen of AHL-like activity in archaea to date, identifying several new organisms with potential quorum sensing activity, including the first evidence of activity in a hyperthermophilic Archaeon.

While quorum sensing has not been investigated extensively in halophilic environments, a number of moderately halophilic *Halomonas* spp. have been identified as possessing AHL production capabilities (Llamas *et al.* 2005). These systems are thought to control the production of extracellular polymeric substances which are crucial to forming biofilms (Llamas *et al.* 2005; Amoozegar *et al.* 2012). Biofilm formation within haloarchaea has previously been indicated to be widespread (Fröls *et al.* 2012) however regulation of biofilm formation within Archaea is poorly understood (Fröls, 2013). A recent work has identified the biofilm producing archaeon *H. lacusprofundi* possessing increased AHL activity in biofilm compared to planktonic cells (Liao *et al.* 2016). The present study built on this finding, with TLC data here indicating multiple signal molecules produced by *H. lacusprofundi* at the latter stages of growth (Table 2). In addition, a strong link to quorum sensing controlled biofilm production in *H. lacusprofundi* was made, with a significant increase in biofilms observed when statonary phase supernatant (containing putative signal molecules) was added to early phase cultures (Figure 3). Whether these specific signal molecules identified by TLC are involved in regulating biofilm production in *H. lacusprofundi* remains to be determined and is an avenue for future research.

Signaling in halophilic Archaea may also be important in constructing and maintaining biofilm communities in microbialites (geobiological microbial communities also rich in

biofilms), which are often predicated by signal induced exopolymeric substance (EPS) production (Hammer and Bassler, 2003; Burns et al, 2009, Decho *et al.* 2009). Microbialites have been investigated for quorum sensing properties with several quorum sensing inhibitors found including those produced by Archaea (Abed *et al.* 2013), and there is the potential for quorum sensing-mediated interactions in these systems (Decho *et al.* 2009; Wong *et al.* 2018; Charlesworth *et al.* 2019). Of particular relevance to the present study, two of the halophilic Archaea analysed in the present study, *H. hamelinensis* and *H. mucosum*, were isolated from the microbialites in Shark Bay (Goh *et al.* 2006; Leuko *et al*. 2007; Allen *et al.* 2008). The role of quorum sensing in these hypersaline environments is still unclear, however during the course of the current study metagenome analyses of the Shark Bay microbialites revealed the presence of potential bacterial quorum sensing and quenching systems (Wong *et al.* 2018). It has been suggested quorum sensing may be responsible for microbialite formation as well as co-ordination of metabolisms within these complex ecosystems (Decho *et al.* 2009; Wong *et al.* 2017).

In addition to the halophilic Archaea, two anaerobic Archaea, *M. mazei*, a methanogen, and the hyperthermophile *T. celericrescens* were shown to activate the AHL biosensor (Table 1). Previous work has suggested *M. mazei* is capable of producing carboxylated AHLs (Zhang *et al.* 2012) which could be responsible for biosensor activation observed in the present study. The potential for carboxylated AHLs in *M. mazei* is further supported by the presence of a potential FilI homologue in the genome (Zhang *et al.* 2012). The AHL-like activity detected in *T. celericrescens* is the first direct evidence for potential quorum sensing in a thermophilic archaeon. There is little known about quorum sensing in thermophilic environments with most studies finding the presence of AHL degrading enzymes in thermophilic bacteria,

although it has been suggested quorum sensing may play a role in thermophilic environments such as regulating EPS production in thermophilic bacteria (Johnson *et al.* 2005).

Results from the present study suggest there is a range of potential signal molecules in the Archaea analysed (Table 2). This is not an uncommon feature as several bacteria are capable of producing multiple signals often at different time points in growth to control multiple phenotypes (Fekete *et al.* 2010; Hansen *et al.* 2015). It is possible a similar mechanism is occurring in Archaea. The archaeon *H. salinarium* strain NRC-1 has been identified here as appearing to possess three separate signal molecules (Table 2). Some of the Archaea analysed here also appear to change signal molecules produced between early log phase and stationary growth phases suggesting some phenotypes may be triggered after others. Further support for multiple signals in Archaea is seen in work on the methanogen *M. harundinacea* where at least three separate carboxlyated AHLs were detected in the supernatant of the organism (Zhang *et al.* 2012).

The results from TLC analysis in the present study also suggest the signal molecules present in the Euryarchaeota isolates investigated tend to possess higher polarities consistent with medium chain AHLs or some DKPs (Table 2, Figure 2), and this is consistent with recent work suggesting LuxR homologues in Archaea may bind medium chain length AHLs or DKPs (Rajput and Kumar 2017). It is unknown the effect modifications such as carboxylation or other novel substitutions can have on the polarity of the signal molecule, and future chemical separation and analysis through HPLC may help confirm these findings.

Conclusions. The broad range of Euryarchaeota isolates reported here to be able to activate an AHL biosensor suggests AHL or AHL-like communication may be more universal amongst Euryarchaeota and possibly Archaea than first thought. We hypothesise that Archaea-Bacteria communication may be prevalent in environments where the two domains are common. This intriguing possibility of cross talk between the two domains could lead to a greater understanding of mixed Archaeal-Bacterial population dynamics. Our data indicated the number and polarity of signal molecules within several Archaea that can be used to inform later purification and analysis techniques such as HPLC and NMR, to confirm the structure and type of signaling molecules detected in this study. It should also be noted that a diverse range of non-AHL signals have been shown in bacteria that include pheromones and fatty-acid derived signals, and such signals could potentially be prevalent in archaea. The prevalence of quorum sensing signals in extreme environments also raises questions of how tolerant these molecules are to environmental stresses and what role signaling plays in these environments. Finally, it is likely novel synthesis and receptor genes are present within archaea given traditional LuxI enzymes act on acyl groups carried by ACPs, a protein not present within archaea (Lombard *et al.* 2012).

Conflicts of interest. No competing financial interests exist. The authors declare that there is no conflict of interests regarding the publication of this paper.

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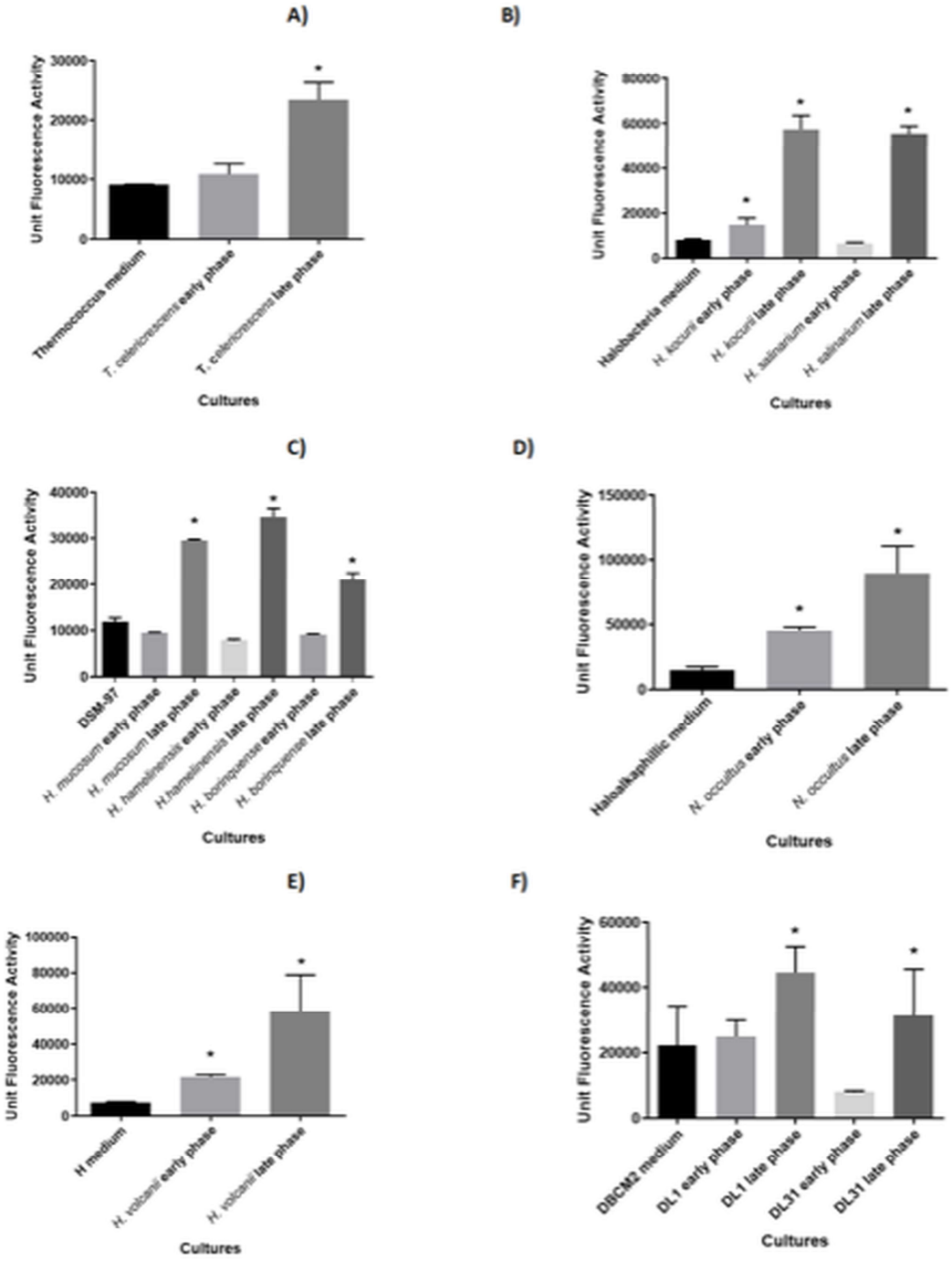

Figure 1. Fluorescence intensity of *E. coli* MT102 AHL biosensor in response to archaea and medium extract negative controls. A) Early and late phase extracts of *Thermococcus*

celericrescens B) Early and late phase extracts of *Halobacterium salinarium* strain NRC-1 and *Halorubrum kocurii* C) Early and late phase extracts of *Haloferax mucosum, Halococcus hamelinensis,* and *Halogeometricum borinquense* D) Early and late phase extractions of *Natronococcusoccultus* E) Early and late phase extractions of *Haloferax volcanii* F) Early and late phase extractions of *Halobacteria sp.* DL1 and *Haloarchaea* isolate DL31. All extracts were analysed in triplicate on 96 well plates.

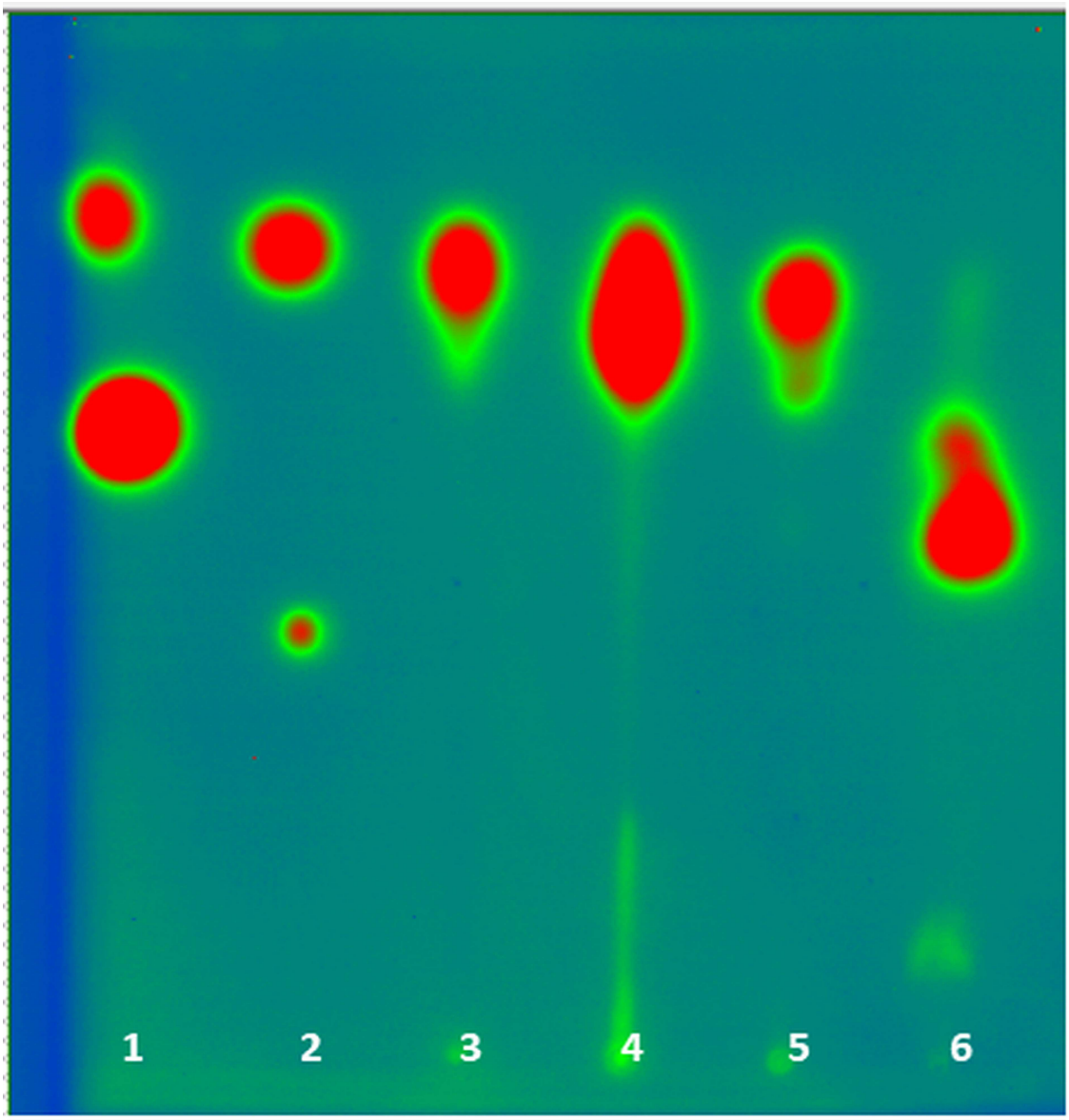

Figure 2. Representative results of signal molecule detection by thin-layer chromatography. Archaeal extracts from either early or late phase cultures of *Haloferax volcanii* and *Natronococcusoccultus* were overlaid with the *E. coli* MT102 biosensor. Lanes 1-2 are standards and Lanes 3-6 archaeal extracts. Lane 1: *N*-3-oxohexanoyl homoserine lactone (0.32 ng), *N*-octanoyl homoserine lactone (22.7 ng); Lane 2: *N*-hexanoyl homoserine lactone (0.6 ng); *N*-decanoyl-l-homoserine lactone (102.16 ng); Lane 3: *H.volcanii* (early

growth phase); Lane 4: *H. volcanii* (late growth phase); Lane 5: *N. occultus* (early growth phase); Lane 6: *N. occultus* (late growth phase).

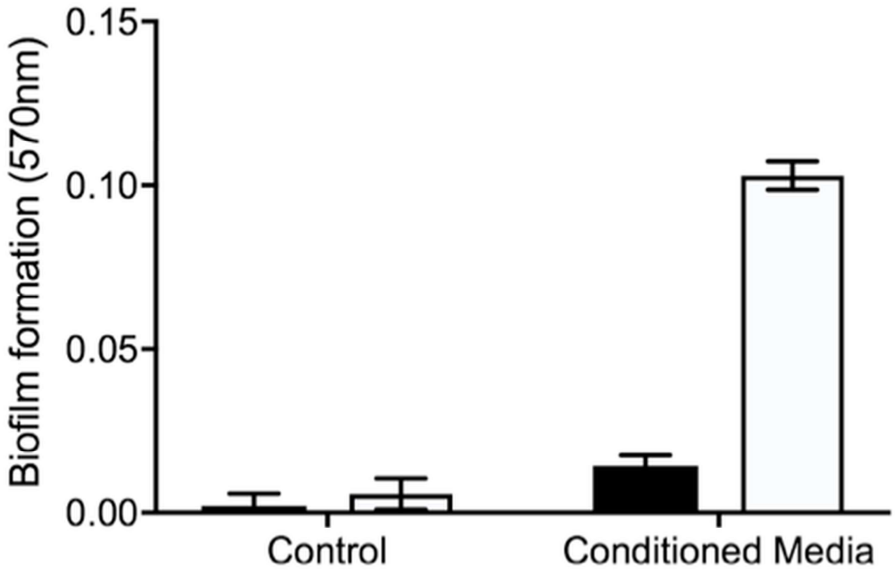

Figure 3. Putative quorum sensing-induced biofilm formation in *Halorubrum lacusprofundi*. Cultures were incubated in cell-free spent supernatant from late phase grown *H. lacusprofundi* containing any excreted signal molecules, and biofilm formation compared to cells incubated with fresh media. The absorbance of crystal violet was measured at 570 nm as a proxy for biofilm growth. Day 3 (black bars), day 7 (white bars). Error bars represent SE of triplicates.

Table 1. AHL activity of archaeal isolates using an *E. coli* MT102 LuxR-based biosensor.

Organism	AHL-like activity*	Isolated from	First identification of activity
Halococcus hamelinensis	Yes	Stromatolite	This study
Haloferax mucosum	Yes	Microbial mat	This study
Halogeometricum borinquense	Yes	Saltern crystallizer	This study
Natronococcus occultus	Yes	Haloalkaphilic lake	Paggi *et al*., 2003
Natronococcus amylolyticus	No	Haloalkaphilic lake	-
Halobacterium salinarum	Yes	Hypersaline lake	This study
Haloferax volcanii	Yes	Dead Sea	This study
Haloquadratum walsbyi	No	Saltern crystallizer	-
Halorubrum lacusprofundi	Yes	Antarctic lake	Liao *et al*., 2016
Halorubrum kocurii	Yes	Hypersaline lake	This study
Halohasta litchfieldiae	No	Antarctic lake	-
DL1	Yes	Antarctic lake	This study
DL31	Yes	Antarctic lake	This study
Thermococcus celericrescens	Yes	Thermophilic	This study
Methanosarcina mazei	Yes	Wastewater	This study
Methanococcoides burtonii	No	Antarctic lake	-
Methanolobus taylorii	No	Estuarine sediments	-

*Activity was observed in triplicate extractions

Table 2. Thin layer chromatography analysis of AHL signal production in early and late-phase cultures of select archaea.

Archaea	Growth phase	No. of AHL-like molecules identified	Rf values*
Haloferax volcanii	early	1	0.76
Haloferax volcanii	late	1	0.75
Natronococcus occultus	early	2	0.75, 0.67 0.54
Natronococcus occultus	late	3	0.75, 0.64, 0.61
Halorubrum kocurii	early	1	0.72
Halorubrum kocurii	late	2	0.71, 0.48
Halobacterium salinarum	early	none	ND
Halobacterium salinarum	late	3	0.78, 0.66, 0.50
Thermococcus celericrescens	early	none	ND
Thermococcus celericrescens	late	none	ND
Haloarchaea isolate DL31	early	none	ND
Haloarchaea isolate DL31	late	1	0.78
Halococcus hamelinensis	early	none	ND
Halococcus hamelinensis	late	1	0.56
Halogeometricum borinquense	early	none	ND
Halogeometricum borinquense	late	2	0.75, 0.52
Haloferax mucosum	early	none	ND
Haloferax mucosum	late	1	0.75, 0.54
Halorubrum lacusprofundi	early	1	0.74

Halorubrum lacusprofundi	late	2	0.74, 0.83

* RF values are given for archaeal extracts on silica sheets overlaid with *E. coli* MT102 biosensor. AHL standards Rf values were calculated as: OHHL – 0.81; OHL – 0.61; HHL – 0.78; DHL – 0.40; BHL – 0.86; OdDHL – 0.34. ND – Not determined.