Solo acylhomoserine lactone synthase from predatory myxobacterium suggests beneficial participation in interspecies cross talk

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Abstract: The prototypical intraspecies quorum signaling systems mediated by acylhomoserine lactones are abundant in proteobacteria, and considerable efforts have provided insight into the regulated physiological features impacted by such systems. However, the high occurrence of orphaned AHL receptors present in bacterial species that do not produce cognate AHL signals suggests the involvement of AHL signals in interspecies interactions within polymicrobial communities. The specific benefits of these interactions are mostly unknown. Considered a key taxon in microbial communities, myxobacteria exist as coordinated swarms that utilize an excreted combination of lytic enzymes and specialized metabolites to facilitate predation of numerous microbial phyla. Of all the biosynthetic gene clusters associated with myxobacteria deposited in the antiSMASH database, only one putative acylhomoserine lactone synthase, *agpI*, was observed in genome data from the myxobacterium *Archangium gephyra*. Without a cognate AHL receptor, we consider AgpI an orphaned AHL synthase. Herein we report the bioinformatic assessment of AgpI and discovery of a second myxobacterial AHL synthase from *Vitiosangium* sp. strain GDMCC 1.1324. Heterologous expression of each synthase in *Escherichia coli* provided detectible quantities of 3 AHL signals including 2 known AHLs, C8-AHL and C9-AHL. The functional, orphaned AHL synthase, AgpI, from the predatory myxobacterium *A. gephyra* provides unique support for beneficial interspecies crosstalk within polymicrobial communities.

Importance: The presence of orphaned quorum signal receptors and associated recognition and response to acylhomoserine lactone quorum signals provides evidence for small molecule-mediated interspecies interactions about microbial communities. A solo signal synthase from a predatory myxobacterium provides an alternative perspective

on the evolution and benefits of quorum signaling systems within these communities. Ultimately our results support and supplement the hypothetical benefits of interspecies cross talk within diverse microbial communities.

Ubiquitous throughout soils and marine sediments, bacteriovorus myxobacteria utilize cooperative features to facilitate uniquely social lifestyles and exhibit organized predation of microbial prey (1-3). Often attributed to their predatory capabilities, an extraordinary number of biologically active specialized metabolites have been discovered from myxobacteria (4-8). Interest in this chemical space and the therapeutic potential associated with each elucidated natural product has motivated significant efforts towards continued discovery. A recent survey of the unexplored, observable biosynthetic space from myxobacteria included in the antiSMASH database determined that the potential for such discovery from cultivable myxobacteria remains high (9-12). An oddity reported by this survey was the presence of a solo acylhomoserine lactone (AHL) synthase within the genome of the myxobacterium *Archangium gephyra* (9, 13, 14). Considered the prototypical class of quorum signals, AHL quorum signaling (QS) systems are abundant throughout the proteobacteria at large (15). While a recent assessment of AHL-associated QS receptors included within or nearby specialized metabolite biosynthetic gene clusters (BGCs) reported the presence of a putative AHL receptor from the marine myxobacterium *Haliangium ochraceum* DSM 14365, myxobacteria are not known to participate in AHL-mediated quorum signaling, and no AHL quorum signals have been reported from myxobacteria (16). Intriguingly, the model myxobacterium *Myxococcus xanthus* demonstrates enhanced predatory features when exposed to a variety of exogenous AHLs despite having no obvious AHL receptor within its genome (17). This phenomenon, often referred to as “eavesdropping,” has become a generally accepted cornerstone in hypotheses surrounding interspecies cross talk within polymicrobial communities, and the presence of solo or orphan AHL receptors from species that do not

produce AHL signals supports such communication (17-24). However, *A. gephyra* does not appear to harbor a cognate AHL receptor suggesting an input-driven participation in such cross talk unique from eavesdropping. Considering the abundance of AHL QS systems throughout proteobacteria other than myxobacteria, the uniqueness of this AHL synthase from *A. gephyra*, and the generalist diet of predatory myxobacteria that includes large swaths of AHL signaling proteobacteria, we hypothesize this AHL synthase was acquired horizontally (3, 25-27). Conversely, the benefit AHL production might provide a predatory myxobacterium remains non-obvious. Herein we report bioinformatic analysis, functional assessment, and heterologous expression of the myxobacterial AHL synthase AgpI.

Results

AgpI is highly homologous to functional AHL synthases.

Located in the 20.6kb BGC referenced as cluster 33 from *A. gephyra* (NZ_CP011509) deposited in the antiSMASH database (version 4.2.1) the 210aa gene product, AgpI (WP_047862734.1), is annotated as a putative autoinducer synthesis protein homologous to the GNAT family *N*-acetyltransferase, LuxI class of AHL synthases (Figure 1) (10, 11, 18). None of the other annotated features proximal to *agpI* are obviously associated with AHL biosynthesis or chemical modifications. Assessment of highly homologous AHL synthases provided a second putative AHL synthase within the genome of the myxobacterium *Vitiosangium* sp. GDMCC 1.1324, deemed VitI (WP_108069305.1), with 98% coverage and 68.12% identity when comparing amino acid sequence data with AgpI (28). The absence of genome data for *V.* sp. in version 4.2.1 of the antiSMASH database explains the omission of this putative AHL synthase from our

previous survey of myxobacterial biosynthetic space. The next highest scoring sequence from this analysis a GNAT family *N*-acetyltransferase (WP_055459978.1) from *Chelatococcus sambhunathii* has 96% coverage and 56.44% identity with AgpI (29, 30). Alignment and phylogenetic analysis of AgpI and VitI against an assortment of 17 AHL synthases experimentally validated to produce AHL QS molecules, suggests common ancestry with the LuxI, LasI, and TraI AHL synthases from *Aliivibrio fischeri*, *Pseudomonas aeruginosa*, and *Rhizobium radiobacter* thus supporting our hypothesis that AgpI was horizontally acquired (Figure 2) (31-42). Utilizing the genomic enzymology web tool EFI-EST developed by the Enzyme Function Initiative (EFI) to construct a sequence similarity network (SSN) that included 1,001 homologous entities as nodes and 124,346 edges, both AgpI and VitI are included in the central cluster family that contains the vast majority of homologous AHL synthases from proteobacteria (Figure 3) (43). From these data we conclude that both AgpI and VitI are likely AHL synthases as originally predicted by antiSMASH analysis. We also suggest that the shared ancestry observed from phylogenetic analysis and general absence of such synthases from other myxobacterial phyla supports our hypothesis that these synthases were horizontally acquired.

Absence of a cognate AHL receptor in the genome of *A. gephyra*.

While no obvious AHL-binding LuxR homolog was identified in the chromosome of *A. gephyra*, we sought to determine the presence of any potential AHL-binding domain using the conserved sequence for autoinducer binding domains (PF03472). Utilizing the blastp suite at NCBI, we assessed all 3,014 domains within the pfam database classified as autoinducer binding domains for homology against the deposited genome of *A. gephyra*

(14, 44). No features within the proteome of *A. gephyra* were sufficiently homologous to be considered an autoinducer binding. We next queried the associated Hidden Markov Model (HMM) associated with autoinducer binding domains deposited in Pfam against the proteome of *A. gephyra* using HMMSEARCH (supplemental data)(45, 46). The most significant hit (E-value 0.0015) a PAS domain S-box-containing protein also annotated as a GAF-domain-containing protein (WP_053066299.1) does not include significant sequence homology with LuxR-type, AHL receptors. Interestingly, similar analysis of *V.* sp. GDMCC 1.1324 provided a highly homologous LuxR-type receptor (WP_108076247.1). While the AHL receptor identified in the genome of *V.* sp. is not clustered near *vitI* as is typical of LuxI-LuxR type synthase-receptor pairs, we cannot assume both are unpaired orphans and instead consider VitI might not be a truly solo AHL synthase. From these data we determined AgpI to be an orphaned AHL synthase without any cognate AHL receptor present in the genome of *A. gephyra.*

A. gephyra does not produce AHLs during axenic cultivation.

Cultivation of *A. gephyra* on VY/2 agar plates at 30°C for 21 days provided fully developed, wispy myxobacterial swarms encompassing the entirety of the plate surface. Homogenized agar and cellular contents were extracted using traditional organic phase techniques to provide extracts for LC-MS/MS analysis. The resulting datasets from LC-MS/MS analysis of *A. gephyra* extracts were analyzed against datasets generated from analytical standards for a variety of AHLs including C6-AHL, 3-oxo-C6-AHL, C8-AHL, and C11-AHL to determine the presence of any produced AHL-like metabolites. Data from resulting mass spectra were scrutinized using the Global Natural Products Social Molecular Networking (GNPS) platform to generate molecular networks depicting

similarities in detected metabolite scaffolds inferred from ionized fragment commonalities (47). No metabolites that included the diagnostic AHL-associated fragments at 102.0547 m/z and 74.0599 m/z associated with the core homoserine lactone moiety were detected in extracts from *A. gephyra* (48, 49). This data supports any one of the following conclusions *A. gephyra* does not produce AHL-like metabolites when grown axenically but may be active under other growth conditions; metabolites produced by AgpI do not possess structural similarity with typical AHL metabolites; or AgpI is simply nonfunctional.

Heterologous expression of AgpI confirms functional production of AHLs.

To explore the functionality of both AgpI and VitI and assumed biosynthesis of AHL-like metabolites, inducible codon-optimized constructs of *agpI* and *vitI* included in replicating plasmids suitable for expression in *Escherichia coli* were purchased. Heterologous expression of AgpI and VitI and subsequent extraction, LC-MS/MS analysis, and evaluation of molecular networks rendered by GNPS as previously described, provided a cluster family including 2 of 3 total nodes identified as C8-AHL (228.159 m/z) and C9-AHL (242.174 m/z) from internal GNPS public datasets as well as a third AHL metabolite detected at 226.144 m/z (Figure 4) (47). This cluster family was identical in both heterologous expression experiments suggesting that AgpI and VitI produce the same 3 AHL metabolites with similar detected intensities for each AHL. Both C8-AHL and C9-AHL were confirmed to be present in AgpI and VitI extracts using analytical standards. Based on associated intensities, C8-AHL was the most abundant and the metabolite detected at 226.144 m/z was the least abundant AHL. No AHL-like entities were detected in control extracts from *E. coli* containing an empty pET28b expression plasmid. From the mass difference between C8-AHL and the unknown AHL detected at 226.144 m/z (2.015

Da measured vs. 2.01565 theoretical), as well as shared fragmentation patterns, we determined the metabolite detected at 226.144 m/z was likely an unsaturated analog of C8-AHL (Figure 5). From these experiments we determined that both AgpI and VitI are functional AHL synthases capable of producing the previously characterized AHLs C8-AHL and C9-AHL. These results also suggest *A. gephyra* produces these AHLs and likely requires environmental cues or specific nutrients not present during our axenic cultivation conditions.

Discussion

Ultimately we conclude that the myxobacteria *A. gephyra* and *V.* sp. possess functional AHL synthases that produce the AHL signals C8-AHL and C9-AHL when heterologously expressed in *E. coli*. Considering the strong precedent for heterologous expression of AHL synthases in *E. coli* to determine produced AHL metabolites, we suggest that both *A. gephyra* and *V.* sp. capably produce one or all of the observed AHL signals and that AgpI is merely silent or cryptic during axenic cultivation of *A. gephyra* (50-55). However, we should also consider that these synthases could instead utilize an acyl-ACP precursor not available to the heterologous *E. coli* host, and we are actively exploring cultivation conditions that might induce native AHL production from *A. gephyra* (54, 55). While numerous bacteria have been observed to possess orphaned LuxR-type AHL receptors, production of AHL metabolites from a solo AHL synthase without any cognate AHL receptor with homology to LuxR also present in the genome of *A. gephyra* is the first to be reported (19, 21, 23, 56). Although a functional orphaned LuxI-type synthase capable of producing AHLs has been reported from the sponge symbiont *Ruegeria* sp. KLH11, the strain also harbors 2 pairs of clustered LuxI/LuxR homologues (23, 57). We suggest

that production of quorum signals by myxobacteria supports the theoretical benefits of interspecies cross talk similar to functional, solo AHL receptors (21, 58-60). We also propose that the more typical abundance of orphan AHL receptors reported from a variety of bacterial species compared to the seemingly exceptional solo AHL synthase reported here might correlate with the rarity of bacteriovorus micropredators (19, 27). The absence of any AHL metabolites during axenic cultivation of *A. gephyra* suggests an unknown regulatory mechanism independent of the typical LuxR receptor to be involved. However, previously reported eavesdropping by *M. xanthus* and response to exogenous AHLs despite the absence of any AHL receptor with homology to LuxR suggests myxobacteria may possess an undiscovered, alternative means of AHL detection (17). While the benefit afforded predatory myxobacteria remains unclear, production of AHL signals known to regulate QS-associated physiological functions such as biofilm formation, specialized metabolism, and motility offers some insight (15). Predatory disruption of any one of these functions would likely improve predation of quorum signaling prey. We consider these observations provide a unique perspective and support the continued investigation of small molecule interactions that contribute to microbial community structures and trophic levels.

Materials and Methods

Cultivation of *A. gephyra*. *Archangium gephyra* (DSM 2261) initially obtained from German Collection of Microorganisms in Braunschweig was grown on VY/2 agar (5 g/L baker's yeast, 1.36 g/L $CaCl_2$, 0.5 mg/L vitamin B_{12}, 15 g/L agar, pH 7.2).

Bioinformatic assessment of AgpI. The amino acid sequence for AgpI (WP_047862734.1) was submitted for blastp analysis and EFI-EST analysis

(https://efi.igb.illinois.edu/efi-est/) using the default settings. Results from EFI-EST analysis were visualized using Cytoscape and are provided as supplemental data. Alignments from ClustalW and minimum evolution phylogenetic trees were rendered using MEGA7 (61, 62).

Autoinducer binding site search. All 3,014 domains annotated as autoinducer binding domains (PF03472) deposited in Pfam were subjected to blastp analysis against the *A. gephyra* genome (NZ_CP011509.1). For HMMSEARCH analysis, the raw HHM for autoinducer binding domains was downloaded from Pfam (PF03472) and utilized as input for profile-HMM vs protein sequence database via HMMSEARCH with the taxonomy restrictions set to limit analysis to *A. gephyra* or *V.* sp. Results from this analysis are provided as supplemental data.

Heterologous expression of AgpI and VitI in *E. coli*. Constructs of AgpI and VitI codon optimized for expression in *E. coli* situated in pET28b were purchased from Genscript (Piscataway, NJ). Sequence data for these constructs are provided as supplemental data. Heterologous host *E. coli* K207-3 was grown at 37°C in LB broth supplemented with 50μg/mL kanamycin, induced with 1μM IPTG at OD_{600}=0.6, and grown overnight at 14°C to facilitate heterologous protein expression.

Metabolite extraction and analysis. After 21 days of cultivation, *A. gephyra* plates were manually diced and extracted with excess EtOAc. Pooled EtOAc was filtered and dried *in vacuo* to provide crude extracts for LC-MS/MS analysis. Extracts from heterologous strains of *E. coli* were generated by Amberlite XAD-16 absorber resin facilitated extraction of clarified culture broths following cell lysis. LC-MS/MS analysis of the extracted samples was performed on an Orbitrap Fusion instrument (Thermo Scientific, San Jose, CA)

controlled with Xcalibur version 2.0.7 and coupled to a Dionex Ultimate 3000 nanoUHPLC system. Samples were loaded onto a PepMap 100 C18 column (0.3 mm × 150 mm, 2 µm, Thermo Fisher Scientific). Separation of the samples was performed using mobile phase A (0.1% formic acid in water) and mobile phase B (0.1% formic acid in acetonitrile) at a rate of 6 µL/min. The samples were eluted with a gradient consisting of 5 to 60% solvent B over 15 min, ramped to 95 % B over 2 min, held for 3 min, and then returned to 5% B over 3 min and held for 8 min. All data were acquired in positive ion mode. Collision-induced dissociation (CID) was used to fragment molecules, with an isolation width of 3 m/z units. The spray voltage was set to 3600 volts, and the temperature of the heated capillary was set to 300 °C. In CID mode, full MS scans were acquired from m/z 150 to 1200 followed by eight subsequent MS2 scans on the top eight most abundant peaks. The orbitrap resolution for both the MS1 and MS2 scans was 120000. The expected mass accuracy was <3 ppm.

GNPS dataset. Generated data were converted to .mzXML files using MS-Convert and mass spectrometry molecular networks were generated using the GNPS platform (http://gnps.ucsd.edu) (47). The corresponding Cytoscape file is provided as supplemental information. LC-MS/MS data for this analysis were also deposited in the MassIVE Public GNPS data set (MSV000084574).

Acknowledgements.

The authors appreciate funding and support from the National Institute of Allergy and Infectious Diseases (R15AI137996), Fulbright (H.A), and the University of Mississippi.

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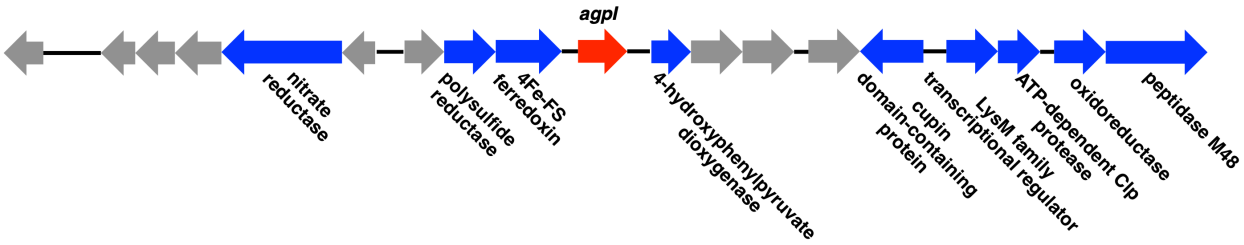
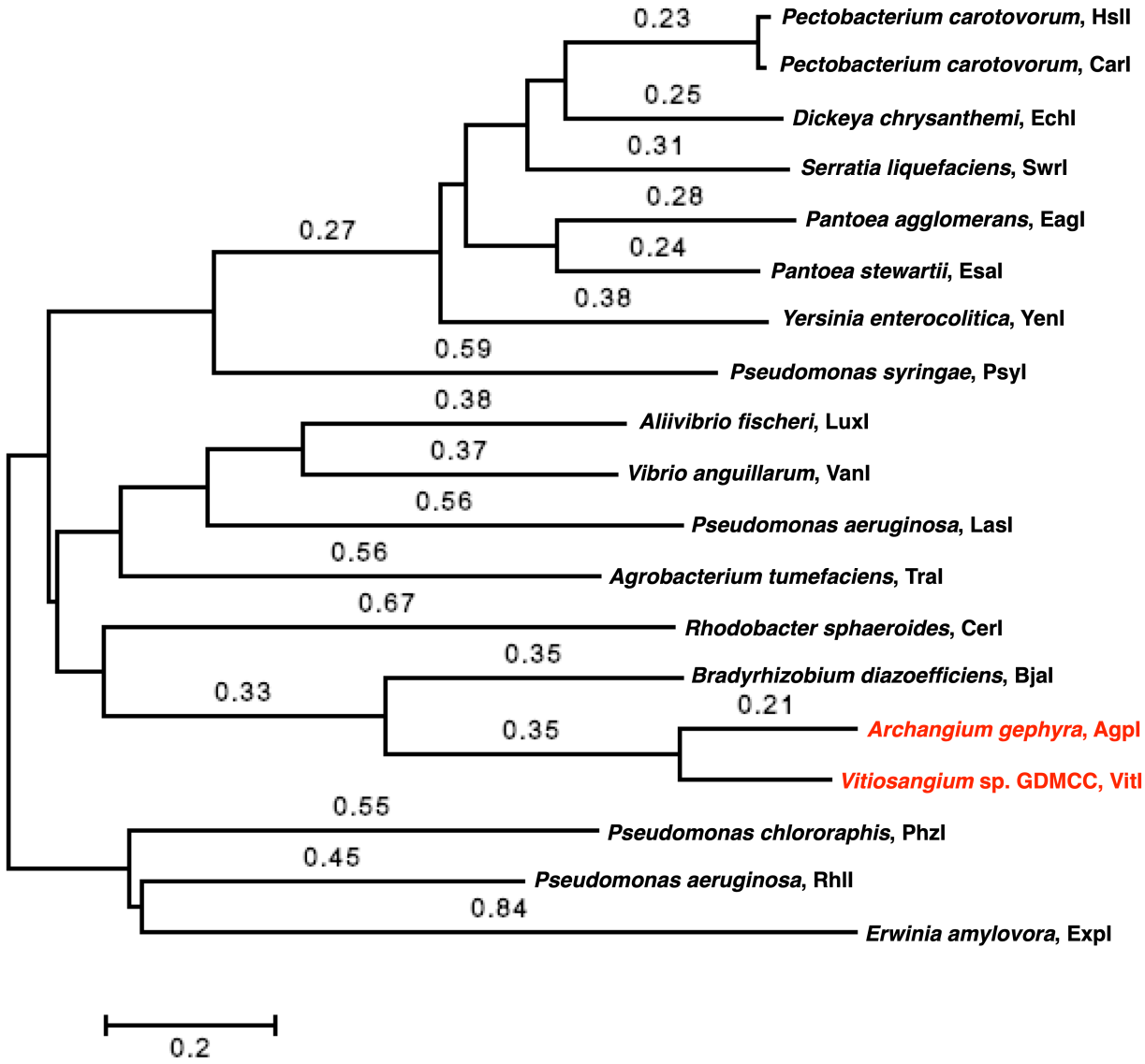


Figure 1: Cluster 33 from *A. gephyra* deposited in the antiSMASH database which includes the putative AHL synthase, *agpI*. All annotations included in the antiSMASH database provided and all hypothetical features are in grey (10, 11).

1



2 **Figure 2:** Minimum Evolution tree including AgpI and VitI rendered in MEGA7 using

3 ClustalW aligned with AHL synthases experimentally confirmed to produce AHLs (62).

4 Branch lengths ≤ 0.2 not depicted.

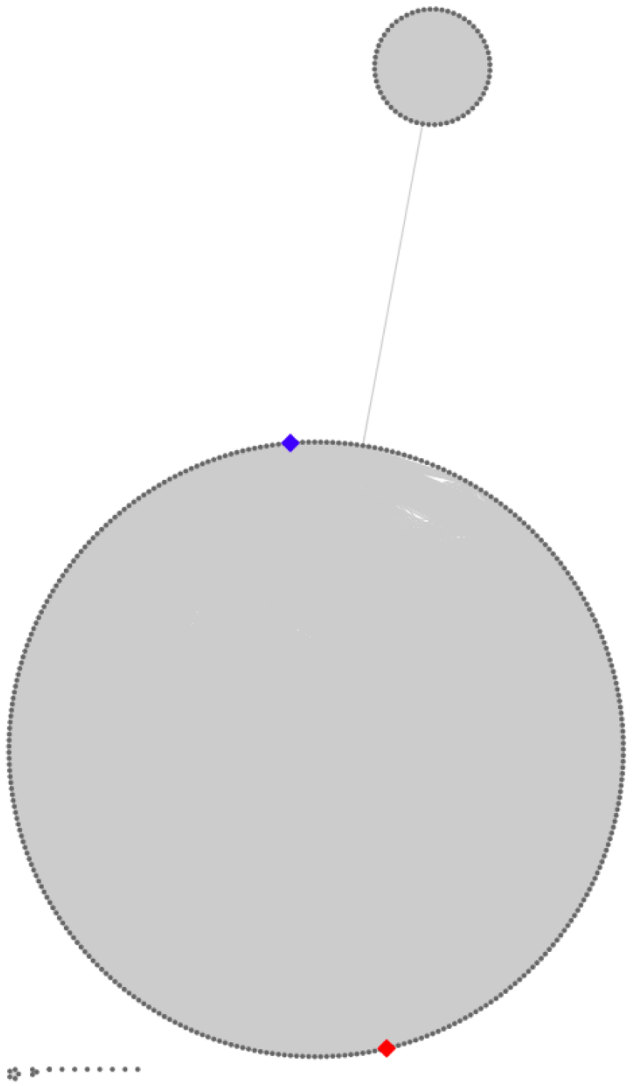
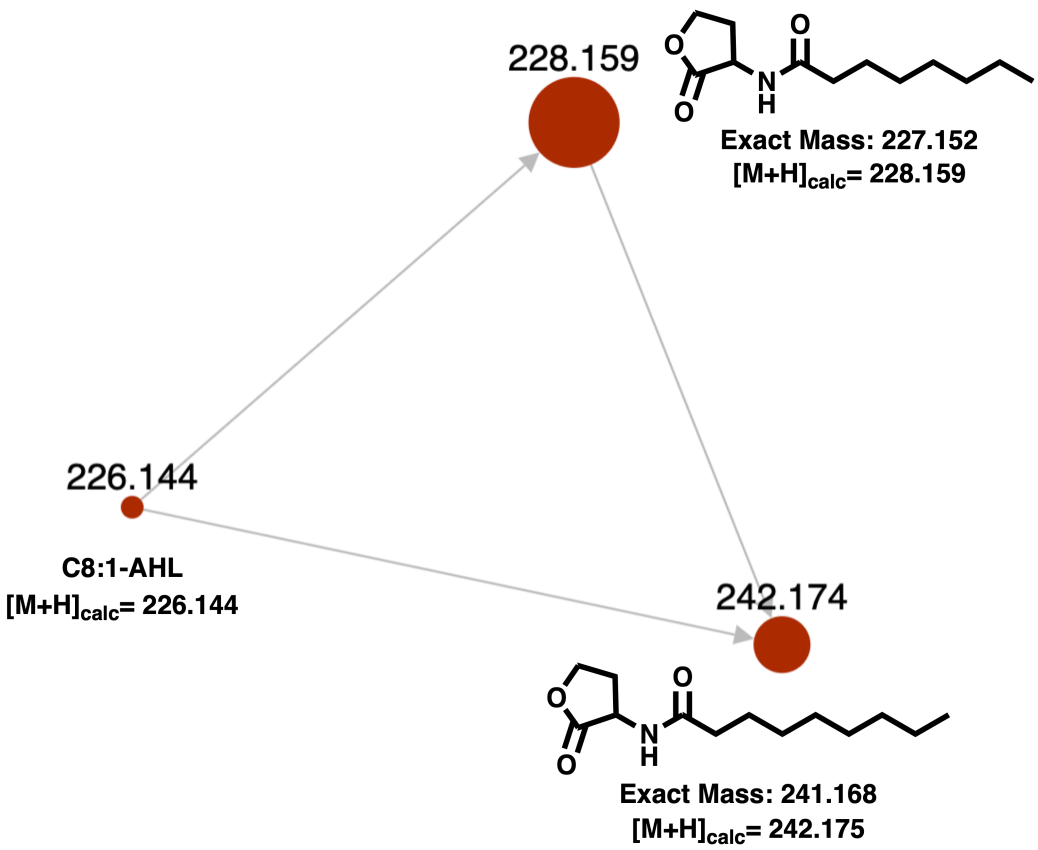


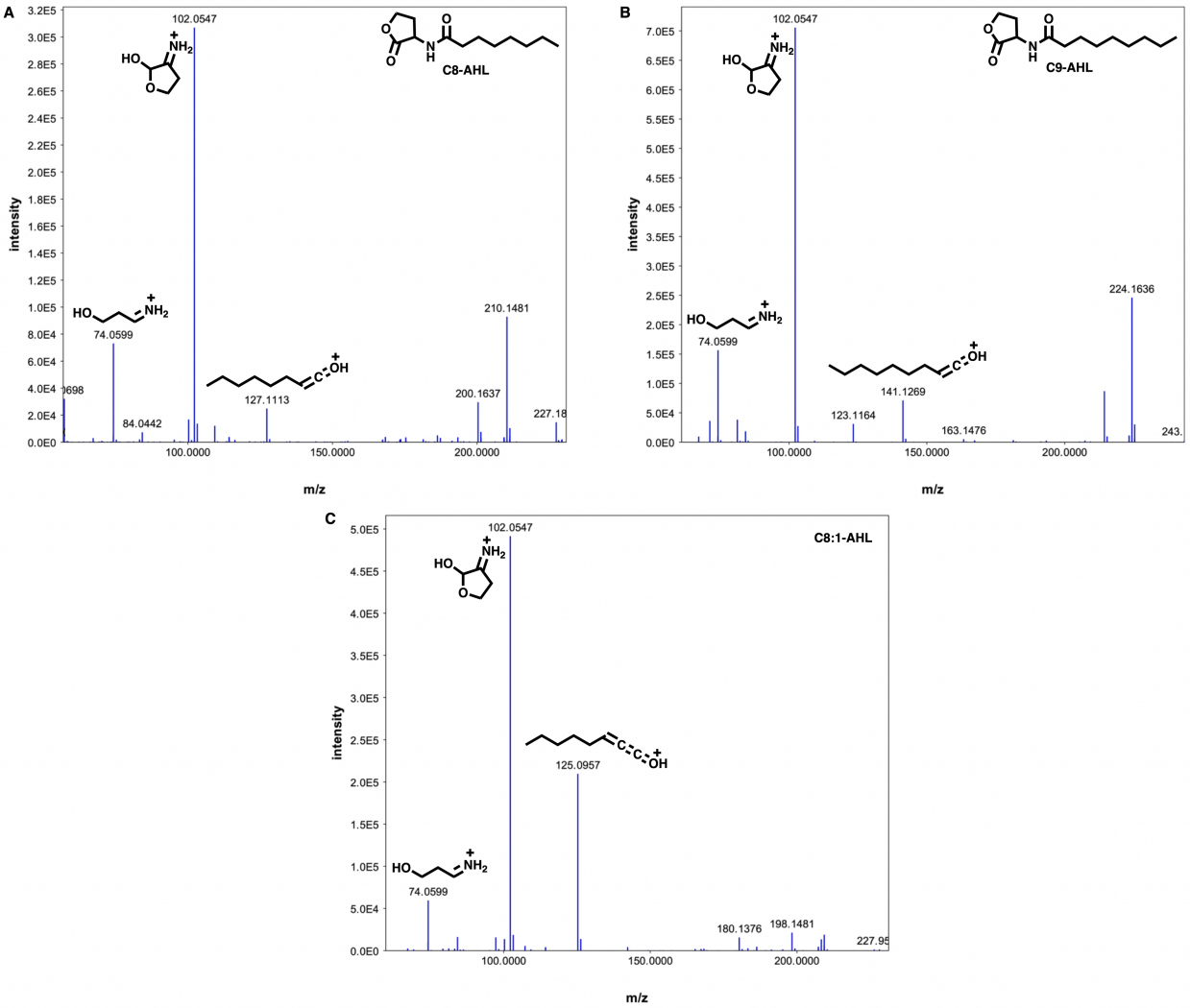
Figure 3: Sequence similarity network rendered by EFI-EST analysis of AgpI amino acid sequence data with AgpI (red diamond) and VitI (blue diamond) indicated (43). To reduce complexity all nodes with ≥90% sequence similarity are represented as an individual aggregate node.

1



14 **Figure 4:** Molecular family from the molecular network of LC-MS/MS datasets from
15 extracts of heterologous *E. coli* expressing AgpI rendered by GNPS (47). Detected m/z
16 values from raw data positioned over each node with node diameter depicting associated
17 intensities for each AHL.

1



2 **Figure 5:** MS/MS fragmentation spectra with diagnostic fragments indicated for each AHL

3 detected in extracts from heterologous *E. coli* expressing AgpI.

4