Genomic Analysis Reveals Versatile Organisms for Quorum Quenching Enzymes: Acyl-Homoserine Lactone-Acylase and -Lactonase

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Abstract: Microbial virulence and their resistance to multiple drugs have obliged researchers to look for novel drug targets. Virulence of pathogenic microbes is regulated by signal molecules such as acylated homoserine lactone (AHL) produced during a cell density dependent phenomenon of quorum sensing (QS). In contrast, certain microbes produce AHL-lactonases and -acylases to degrade QS signals, also termed as quorum quenching. Mining sequenced genome databases has revealed organisms possessing conserved domains for AHL-lactonases and –acylases: i) *Streptomyces* (Actinobacteria), ii) *Deinococcus* (Deinococcus-Thermus), iii) *Hyphomonas* (α-Proteobacteria), iv) *Ralstonia* (β-Proteobacteria), v) *Photorhabdus* (γ-Proteobacteria), and certain marine gamma proteobacterium. Presence of genes for both the enzymes within an organism was observed in the following: i) *Deinococcus radiodurans* R1, ii) *Hyphomonas neptunium* ATCC 15444 and iii) *Photorhabdus luminescens* subsp. *laumondii* TTO1. These observations are supported by the presence motifs for lactonase and acylase in these strains. Phylogenetic analysis and multiple sequence alignment of the gene sequences for AHL-lactonases and –acylases have revealed consensus sequences which can be used to design primers for amplifying these genes even among mixed cultures and metagenomes. Quorum quenching can be exploited to prevent food spoilage, bacterial infections and bioremediation.

Keywords: Acylhomoserine lactone; Acylase; *Bacillus*; Lactonase; Phylogeny; *Pseudomonas*; Quorum sensing; Quorum quenching.

INTRODUCTION

The advent of antibiotics in the beginning of twentieth century gave a new lease to human health and longevity to life span. Ironically, usage of antibiotics to counter the various diseases caused by pathogenic microbes has been proving quite ineffective during the last decade or so. The situation is turning alarmingly grave because microbes have become increasingly resistant to almost all the antibiotics including those which had been approved quite recently [1, 2]. Pharmaceutical companies find it uneconomical to be in this evolutionary race with multiple drug resistant microbes [3-6]. In fact, microbes have evolved defence mechanisms against antibiotics by exploiting their large reservoir of genetic variability [6]. The needs are to search new drug targets [7, 8] and /or attenuate bacterial pathogenicity [9] by preventing dissemination of genes conferring resistance and those responsible for their virulent characteristics [3] and design drugs accordingly.

Among the various microbial pathogens, those notorious for causing high morbidity and mortality among living beings initiate the infection process within specialized structures – biofilms, which enable microbes to withstand adverse environmental conditions, such as the host defence components and drugs including antibiotics used for treating the disease [10, 11]. Most pathogenic microbes develop it in a density dependent manner by a process termed as quorum sensing (QS) [12], which is regulated by the signal molecules released in their environment [13]. In Gram-negative bacteria, the most widely reported QS system is LuxR/I-type, where signals such as acylated homoserine lactones (AHLs) regulate the expression of virulence factors like elatase and pycocyanin production by *Pseudomonas aeruginosa* [14], antibiotic production by *Erwinia carotovora*, pigment and antibiotic production by *Serratia liquefaciens* [15]. The AHL-type pheromones differ in their specificity due to variations in acyl chain length, degree of saturation and substitutes at the third carbon of the lactone ring [16]. More than 70 bacterial species produce AHL type QS [15, 17].

In contrast to AHL mediated QS, which allows bacteria to compete and dominate the bacterial community, there are others which have developed mechanisms to degrade these signals. The process of interference with QS, known as quorum quenching (QQ), has been reported among prokaryotes and a few eukaryotic organisms [18, 19]. Bacteria are known to produce AHL-degrading enzymes such as AHL-lactonases and AHL-acylases. Lactonases hydrolyze the lactone ring of AHL and are found in numerous *Bacillus* spp. [20, 21]. Heterologous expression of *aiiA* (encoding for AHL-lactonase) from *Bacillus* spp. in *Pseudomonas*, *Burkholderia* and *Erwinia* resulted in marked decrease in their QS signal

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molecules [22-24]. AHL-acylases break the amide linkages of the AHLs and have been reported to be produced by bacteria such as *Ralstonia* spp. [25, 26] and *Pseudomonas* spp. [27-29]. *Rhodococcus erythropolis* W2 presents an interesting scenario, as it possess both types of AHL-degrading enzymes [30-32]. Recently proposals have been floated to use QS signal molecules as potential drug targets to attenuate the virulence of pathogenic bacteria and use QQ to design novel drugs [25]. The large reservoir of information available in sequenced genomes of many organisms encouraged us to search for potential organisms [4, 33-35], which may possess either or both the enzymes for usage as potential drugs or as supplement to antibiotic based treatments.

MATERIALS AND METHODOLOGY

Phylogeny of Acyl-Homoserine Lactone Degrading Enzymes

From the literature available in public domains, we collected protein sequences for AHL-lactonase from *Bacillus* sp. SB4 (Accession No. AAR85482.1) and AHL-acylase from *Ralstonia* sp. XJ12B (Accession No. AAO41113.1) from NCBI Protein database. The nucleotide sequences of corresponding protein sequences for the MEME signature analysis were downloaded from the NCBI Genbank. The conserved domains of these enzymes from reference organisms have been presented in Fig. (**1**). We used BLASTP for the similarity searches and NCBI Conserved Domains [36, 37], for conserved domain search. The selected sequences were analyzed to get similar sequences and Conserved Domains of each BLAST hit. We selected parameter for Max target sequences in BLASTP as ~250 hits (BLOSUM 62 Matrix), as it gives results with large number of sequences from same species.

SELECTION OF SEQUENCES BY HOMOLOGY SEARCH

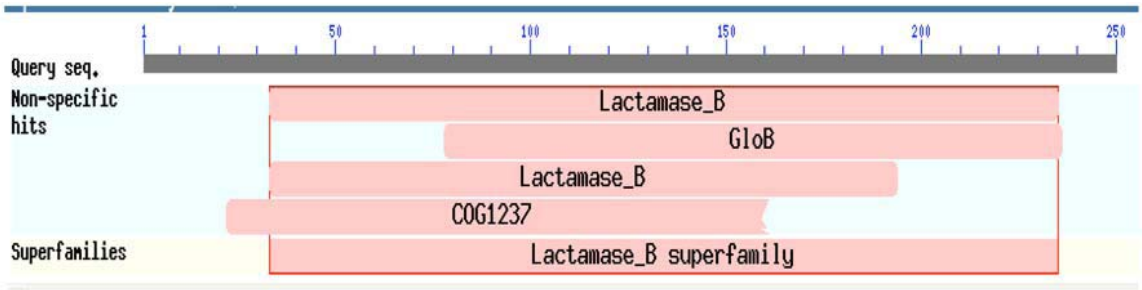
The number of sequences in the each Table was reduced with respect to the homology among the same species using multiple sequence alignment - Neighbor Joining algorithm. We selected the sequence if the value of divergence was more than 10% [38], others could be deleted. The homology search helped to reduce the number of sequences in the each Table to approximately 100. This was done primarily to bring down redundancy and enable preparation of phylogenetic trees of reasonable size.

MULTIPLE SEQUENCE ALIGNMENT (MSA) AND RECONSTRUCTION OF PHYLOGENETIC TREE

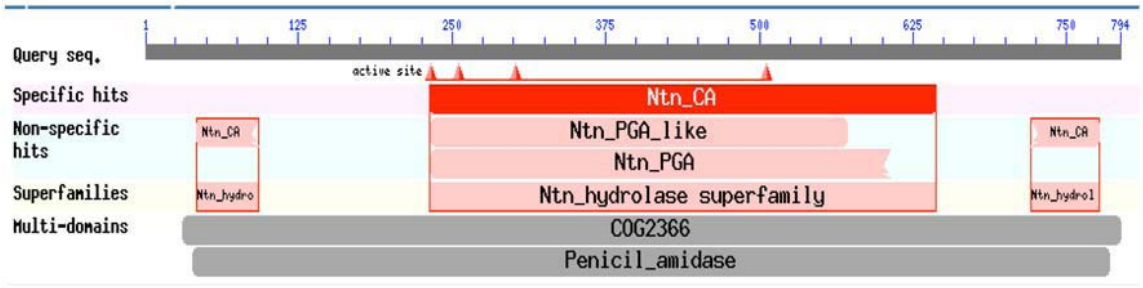
For the reconstruction of the tree we have collected all the protein sequences for AHL-lactonase and AHL-acylase from NCBI, for each Table nearly 100 sequences were selected. ClustalX 2.0.10 [39] and Tree view 1.40 [40] were used for the multiple sequence alignments and phylogenetic tree reconstruction. Neighbor Joining algorithm was used for the tree reconstruction and Boot Strap value used was 1000 (default value). And the tree constructed was visualized with the Tree view software. Multiple aligned sequences were viewed using Jalview to find out the conserved region among them [41, 24].

SIGNATURE ANALYSIS & FINDING REGULAR EXPRESSION, USING MEME

MEME (Multiple EM for Motif Elicitation) is used for searching for novel motifs or signatures in sets of biological sequences. MEME works by searching for repeated, ungapped sequence patterns that occur in the DNA or protein



A: Conserved domains on AHL-lactonase (*Bacillus* sp. SB4) [gi|40388447|gb|AAR85482.1]



B: Conserved domains on AHL-acylase (*Ralstonia* sp. XJ12B) [gi|28376389|gb|AAO41113.1

Fig. (1). A. Conserved domains on AHL-lactonase (*Bacillus* sp. SB4) [gi|40388447|gb|AAR85482.1], **B.** Conserved domains on AHL-acylase (*Ralstonia* sp. XJ12B) [gi|28376389|gb|AAO41113.1].

sequences [43-45]. MEME searches can be performed *via* the web server (http://meme.nbcr.net) and its mirror sites [44]. The same web server also allows access to motif alignment and search tool to search sequence databases for matches to motifs. To successfully discover motifs with MEME, it is necessary to choose and prepare the input sequences carefully. Ideally, the sequences should be <1000 base pairs long [46].

Data sets in FASTA format were submitted group wise in MEME program Version 4.0.0 (http://meme.nbcr.net/meme4/cgi-bin/meme.cgi). In order to obtain maximum number of motifs in our sequences, we modified default settings from 3 motifs to 10 motifs. MEME search stops when this number of motifs has been found, or when none can be found with E-value less than 10000 (http://meme.nbcr.net/meme4/meme-input.html#width). We used default setting zero or one motif per sequence to get the occurrence of single motif which is distributed among the sequences. The default value of motif widths, set between 6 (minimum) and 50 (maximum) were modified and re-set between 25 and 30, respectively. Each of the 10 signatures (25 to 30 nucleotides long) (Table **S2** to **S17**) was checked for its frequency of occurrence among all the sequences of a particular taxonomic group and the ones with highest frequency and did not appear in other taxonomic groups were considered unique to a particular reference taxonomic group.

In our analysis, sequences from each taxonomic group were analyzed for signature using MEME [43]. The MEME result gives a graphic representation and Motifs in regular expression format. MEME analysis were conducted for each taxa like Actinobacteria, Firmicutes, α-, β-, γ-, and δ-Proteobacteria, Ascomycota, Bacteroidetes, Euryarchaeota. The number of sequences of γ-Proteobacteria with reference to acylase from *Ralstonia* sp. Table were too large to be accommodated on line by the MEME Software (Word limit up to 60,000). In this case, we segregated the members of γ-bacterial group and made three case studies and searched for the signatures in i) *Pseudomonas* spp., ii) *Shewanella* spp. and iii) rest of the organisms (Designated as Others). The signatures obtained from the MEME program were cross-checked in other Tables and the other taxa in the same Table using online tool Sequence Manipulation Suite SMS [47].

VALIDATION OF SIGNATURES AND REGULAR EXPRESSIONS

The unique signatures - obtained after crosschecking with other taxonomic groups - were used as query sequence to BLAST against the sequenced microbial genomes available in NCBI database (http://www.ncbi.nlm.nih.gov/), to validate them [45]. Phylogenetic trees based on complete sequence of the gene and the strings based on the signatures were compared for AHL-lactonase among Firmicutes and AHL-acylase among γ-Proteobacteria – *Pseudomonas* spp. For developing strings, each of the 10 signatures was represented by a single alphabet - A to J.

RESULTS

Quorum sensing phenomenon has been reported from a wide range of organisms, which employ these primarily to sense environmental stress by releasing signal molecules: i) AHL by Gram-negative bacteria and ii) oligopeptides by Gram-positive bacteria. In contrast, quite a few organisms also have the abilities to degrade these signal molecules to ensure their survival. Here we are presenting the phylogenetic diversity of AHL inactivating enzymes: AHL-lactonases and AHL-acylases, which act by hydrolyzing the lactone ring and by cleaving the acyl chain, respectively.

PHYLOGENY OF AHL-LACTONASE AND AHL-ACYLASE

Using *Bacillus* sp. SB4 AHL-lactonase (Accession No. AAR854821) (Fig. **1**) amino acid sequences as reference, BLAST resulted in detecting 110 sequences in different organisms showing completely conserved domains (Table **S1**). Sequences for this enzyme were found to be distributed among organisms belonging to Actinobacteria, Acidobacteria, Bacteroidetes, Chloroflexi, Deinococcus-Thermus, Firmicutes, α-Proteobacteria, β-Proteobacteria, γ-Proteobacteria, δ-Proteobacteria, Euryarchaeota, Crenarchaeota, Sphingobacteria, Spirochaetales, Nitrospirales, and Planctomycetes. Among these, two major groups of organisms possessing AHL-lactonase were represented by Firmicutes – 12 genera and 22 spp. and α-Proteobacteria – 18 genera and 22 spp. (Table **S1**).

On the other hand, with *Ralstonia* sp. XJ12B AHL-acylase (Accession No: AAO41113.1) (Fig. **1**) as reference, BLAST resulted in detecting 94 sequences of different organisms showing completely conserved domains (Table **S1**). The distribution of sequences for AHL-acylase was rather limited in comparison to AHL-lactonase. It was found among Actinobacteria, Cyanobacteria, Bacteroidetes, Deinococcus-Thermus, Firmicutes, α-Proteobacteria, β-Proteobacteria, γ-Proteobacteria, δ-Proteobacteria, Euryarchaeota and Crenarchaeota. The distribution pattern of this enzyme was in clear contrast to that of AHL-lactonase. The largest group of organisms (59) possessing AHL-acylase belonged to γ-Proteobacteria - 20 genera and 31 spp. Here, Firmicutes and α-Proteobacteria were among the smaller groups constituted by 3 and 10 genera, respectively. The frequency of occurrence of AHL-acylase was relatively low among other Proteobacteria. Another contrasting scenario was observed among cyanobacterial members, which were found to possess only AHL-acylase. On the other hand, organisms belonging to Acidobacteria, Sphingobacteria, Spirochaetales, Nitrospirales and Planctomycetes were found to possess only AHL-lactonase (Table **S1**).

Multiple sequence alignment of all the sequences revealed that conserved regions for gene encoding for AHL-lactonase and AHL-acylase are located at the positions ranging i) from 467 to 484, 557 to 571 and 737 to 755 and ii) from 236 to 254 and 1892 to 1907 nts, respectively (Table **1**). These regions can be used for designing 'universal' primers for amplifying genes from diverse taxonomic classes. In addition, certain conserved regions were also observed within each taxonomic group, which can be exploited for amplifying genes specific to them. The following conserved sequences can be used for amplifying lactonase: i) Firmicutes - TA[CT]ATTA at positions 535 to 542 nts, and ATATCGT at 774 to 780 nts, ii) β-Proteobacteria - CGCA-GACCA at 681 to 689 nts, and CGG[AC]CG at 521 to 523

nts, etc. Similar regions were also observed among γ-Proteobacteria, δ-Proteobacteria and Ascomycota for lactonase gene (Table **1**) and acylase type gene among Actinobacteria, Cyanobacteria, α-Proteobacteria, β-Proteobacteria and Bacteroidetes (Table **1**).

PHYLOGENETIC DISCREPANCIES

The phylogenetic trees (Figs. **2**-**5**) based on organisms found to possess AHL-lactonase and AHL-acylase followed the taxonomic distribution, which is expected on the basis of

Table 1. Location of Conserved Sequences in AHL-Lactonase and AHL-Acylase Genes of Taxonomically Diverse Bacterial Groups

Taxonomic Group	Position (nts)[a]	Conserved Regions
AHL-lactonase gene		
All[b]	467-484	TGGTC[AG]AAATGCAGATGGGT
	557-571	ATTTCGACCATGCCG
	737-755	AGCGATCACGCGTGTCC
Actinobacteria	**560-570**	**TCGACCACG[CG]CG**
Firmicutes	535-542	TA[CT]ATTA
	559-566	**TTCGATCA**
	774-780	ATATCGT
Alphaproteobacteria	**562-571**	**GACCAT[ACG]TCGT**
Betaproteobacteria	521-523	CGG[AC]CG
	681-689	CGCAGACCA
Gammaproteobacteria	527-534	ATGTCGAG
	548-552	CCCAT
Deltaproteobacteria	518-522	A[GT]CCC
Euryarchaeota	**560-569**	**T[CT]GACCACGC**
Ascomycota	338-347	TTGAXCATCC
	520-539	ATCAAAGACGTCAAGGCAGT
	550-571	**CA[CT]TTACATCTCGA[CT]CACGCAG**
	628-633	CACGAG
AHL-acylase gene		
All[c]	236-254	TGATCGCCGAAATCCGGCG
	1892-1907	GTATGGGTCCAGGCCA
Actinobacteria	704-713	TACCGGGTCG
	1177-1181	AC[AC]C[CG]GTCA[CG]C
	1208-1217	CCA[CG]GAGCTG
Cyanobacteria	1522-1527	TTTGAAA
Alphaproteobacteria	486-495	TCGATGCG
Betaproteobacteria	328-340	GCGCAGGCAACC
Gammaproteobacteria	309-320	CGGCATCGGTTA
	586-595	GGCTTCAACC
Bacteroidetes	1490-1497	TC[CG]GCATA

[a]: Nucleotides in the gene from 5' to 3'.
[b]: Gene sequences from taxonomic groups listed under AHL-lactonase gene.
[c]: Gene sequences from taxonomic groups listed under AHL-acylase gene.

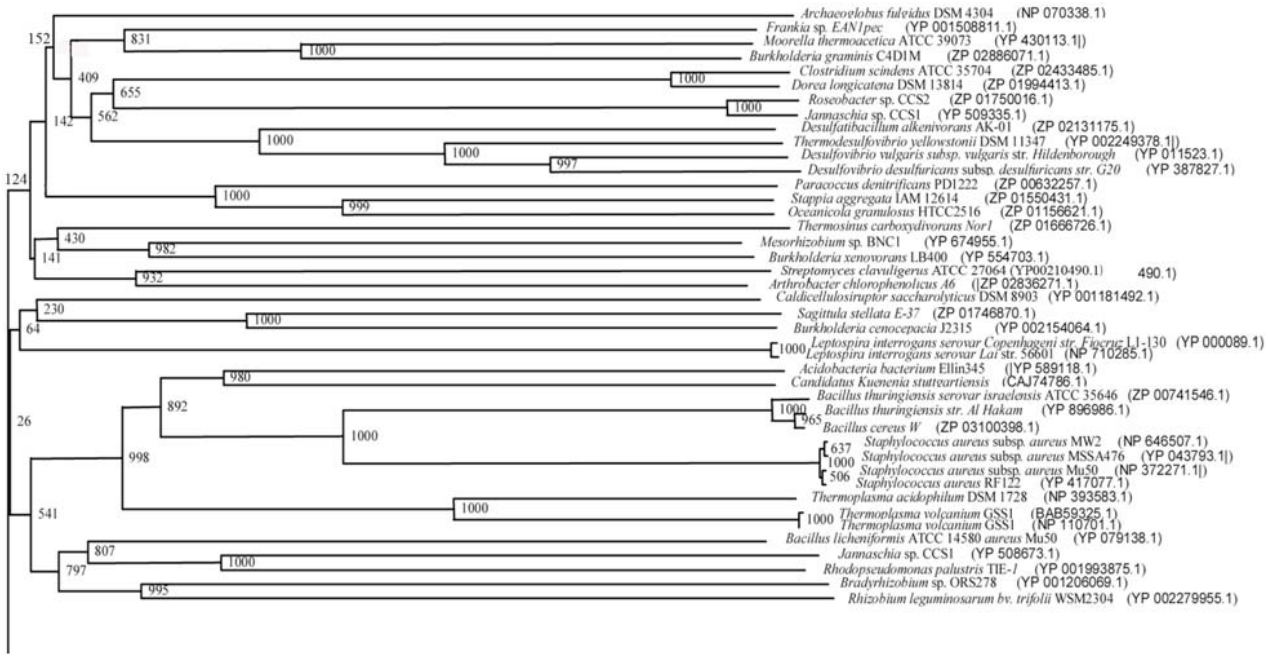


Fig. (2). Phylogenetic tree (Upper half) based on protein sequences of organisms containing conserved domains with respect to *Bacillus* sp. SB4 AHL lactonase as reference (For Taxonomic details refer to Additional file 3). A Neighbor-Joining analysis with Jukes-Cantor correction and bootstrap support was performed on the protein sequences. Bootstrap values are given at nodes, 1000 bootstrap replicates were run. The leaf contains Accession number and name of the organism.

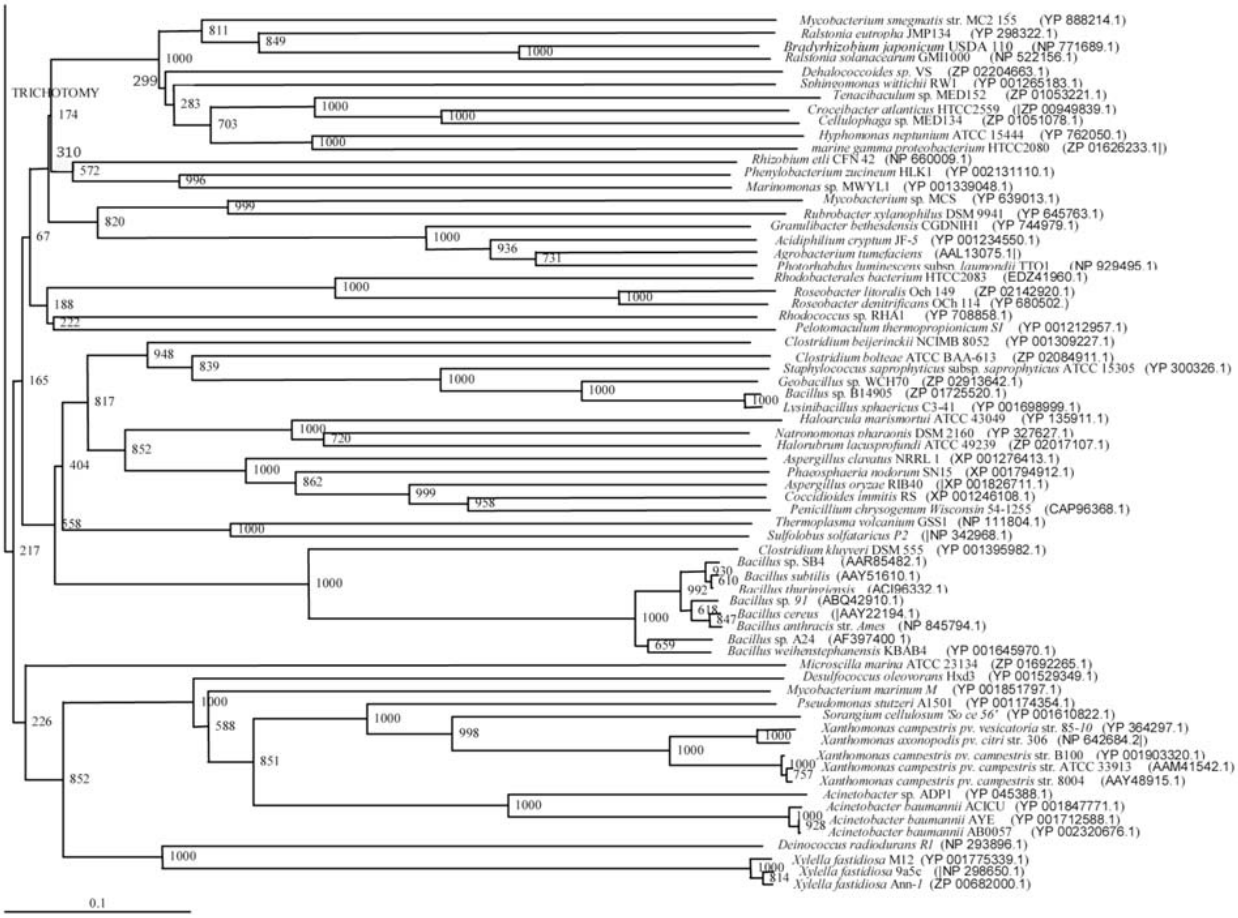


Fig. (3). Phylogenetic tree (Lower half) based on protein sequences of organisms containing conserved domains with respect to *Bacillus* sp. SB4 AHL lactonase as reference (For Taxonomic details refer to Additional file 3). A Neighbor-Joining analysis with Jukes-Cantor correction and bootstrap support was performed on the protein sequences. Bootstrap values are given at nodes, 1000 bootstrap replicates were run. The leaf contains Accession number and name of the organism.

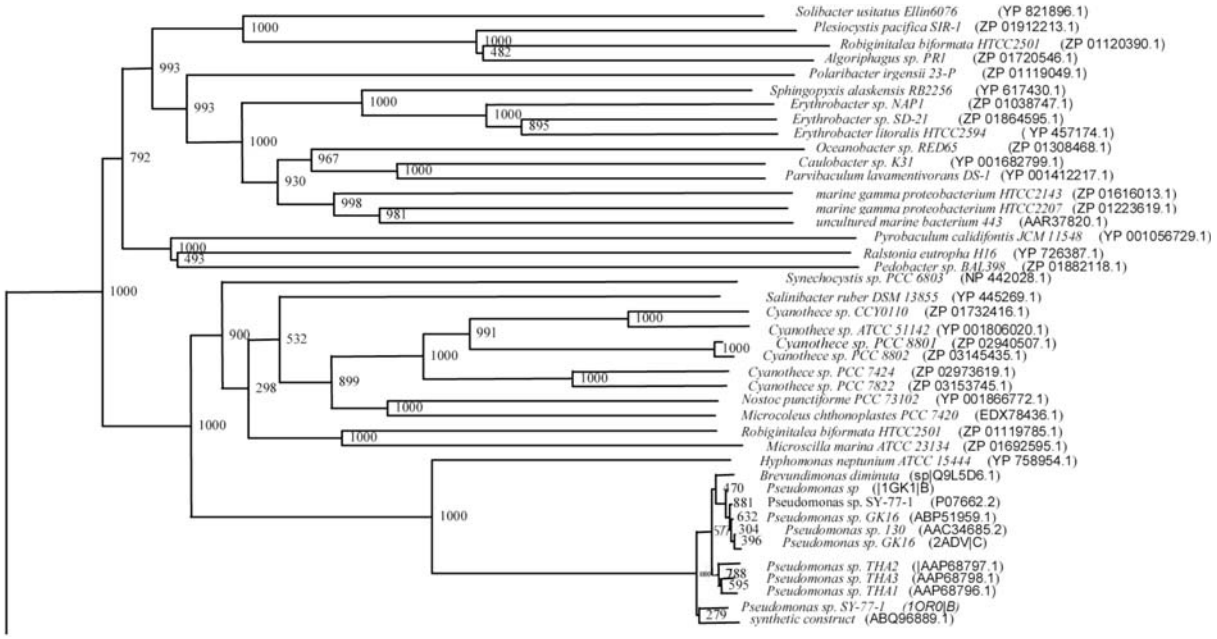


Fig. (4). Phylogenetic tree (Upper half) based on protein sequences of organisms containing conserved domains with respect to *Ralstonia* sp. XJ12B AHL acylase as reference (For Taxonomic details refer to Additional file 3). A Neighbor-Joining analysis with Jukes-Cantor correction and bootstrap support was performed on the protein sequences. Bootstrap values are given at nodes, 1000 bootstrap replicates were run. The leaf contains Accession number and name of the organism.

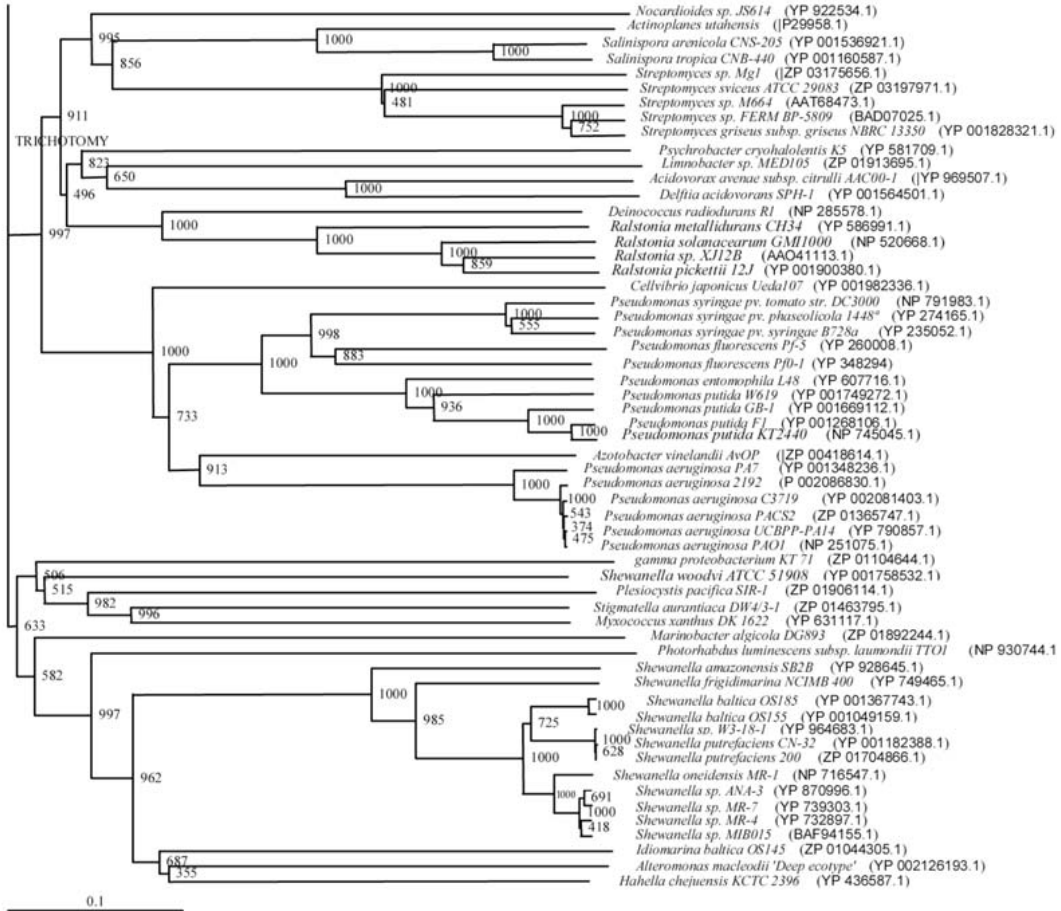


Fig. (5). Phylogenetic tree (Lower half) based on protein sequences of organisms containing conserved domains with respect to *Ralstonia* sp. XJ12B AHL acylase as reference (For Taxonomic details refer to Additional file 3). A Neighbor-Joining analysis with Jukes-Cantor correction and bootstrap support was performed on the protein sequences. Bootstrap values are given at nodes, 1000 bootstrap replicates were run. The leaf contains Accession number and name of the organism.

their 16S rDNA sequences. A few phylogenetic discrepancies were observed for AHL-lactonase (Figs. **2**,**3**, Table **2**), the notable being the associations between i) *Moorella thermoacetica* ATCC 39073 (Firmicutes) and *Burkholderia graminis* C4D1M (β-Proteobacteria) (Boot Strap Value, BV 1000), ii) Actinobacteria (*Mycobacterium* sp. MCS and *Rubrobacter xylanophilus* DSM9941) and members of α-Proteobacteria (*Granulibacter bethesdensis* CGDNIH1, *Acidiphilum cryptum* Jf-5 and *Agrobacterium tumefaciens*) (BV 820), and iii) *Deinococcus radiodurans* R1 (Deinococcus-Thermus) and *Xylella fastidiosa* (γ-Proteobacteria) (BV 1000). Similarly, certain taxonomic discrepancies were also recorded in the phylogenetic tree (Figs. **4**,**5**, Table **2**) drawn with respect to sequences of organisms possessing AHL-acylase: i) *Solibacter usitatus* Ellin 6076 (Acidobacteria) and *Plesiocystis pacifica* SIR-1 (δ-Proteobacteria) (BV 1000), ii) *D. radiodurans* R1 (Deinococcus-Thermus) and *Ralstonia metallidurans* CH34, *R. solanacearum* GMI1000 and *Ralstonia* sp. XJ12B (β-Proteobacteria (BV 1000). Since these associations between widely distributed organisms show very high BVs, these may be interesting cases of horizontal gene transfers.

ORGANISMS POSSESSING GENES FOR AHL-LACTONASE AND AHL-ACYLASE

Sixteen bacterial strains of 5 different genera and a group of 3 strains belonging to marine gamma proteobacterium were found to possess genes for both the enzyme types: AHL-lactonase and AHL-acylase (Table **3**). The diversity of these 19 strains was evident by their distribution among 5 different taxonomic groups: i) *Streptomyces* spp. (Actinobacteria), ii) *Deinococcus* spp. (Deinococcus-Thermus), iii) *Hyphomonas* sp. (α-Proteobacteria), iv) *Ralstonia* spp. (β-Proteobacteria), v) *Photorhabdus* sp. (γ-Proteobacteria), vi) marine gamma proteobacterium. Three out of these 19 strains can be more clearly classified as those which possessed genes for both the enzyme types: i) *D. radiodurans* R1, ii) *Hyphomonas neptunium* ATCC 15444 and iii) *Photorhabdus luminescens* subsp. *laumondii* TTO1. This observation is supported by the presence motifs for lactonase and acylase in these strains.

SIGNATURE ANALYSIS – FREQUENCY AND DISTRIBUTION PATTERN

Ten signatures (nucleotides) were obtained through MEME for different taxonomic groups with reference to: i) AHL-lactonase gene (*aiiA*) of *Bacillus* sp. SB4 Accession No. AAR85482.1 (Figs. **S1** to **S8** and Tables **S2** to **S9**) and ii) AHL-acylase gene (*aiiD*) of *Ralstonia* sp. XJ12B Accession No. AAO41113.1 (Figs. **S9** to **S19** and Tables **S10** to **S17**). (See Additional File 4 for a description of data in Tables **S2** to **S17** and Figs. **S1** to **S17**). Nucleotide signatures found to be present at high frequency within the taxonomic group and absent from all other groups were initially classified as

Table 2. Potential Cases of Horizontal Gene Transfer of Acyl-Homoserine Lactone-Acylase and –Lactonase

Incongruent Phylogenetic Associations		Boot Strap Value
Organism(s)	**Taxonomy**	
***Bacillus* sp. SB4 (AHL-lactonase)[a]**		
Moorella thermoacetica ATCC 39073	Firmicutes	1000
Burkholderia graminis C4D1M	β-Proteobacteria	
Mycobacterium sp. MCS	Actinobacteria	820
Rubrobacter xylanophilus DSM9941		
Granulibacter bethesdensis CGDNIH1	α-Proteobacteria	
Acidiphilum cryptum Jf-5		
Agrobacterium tumefaciens		
Deinococcus radiodurans R1	Deinococcus-Thermus	1000
Xylella fastidiosa	γ-Proteobacteria	
***Ralstonia* sp. XJ12B (AHL-acylase)[a]**		
Solibacter usitatus Ellin 6076	Acidobacteria	1000
Plesiocystis pacifica SIR-1	δ-Proteobacteria	
Deinococcus radiodurans R1	Deinococcus-Thermus	1000
Ralstonia metallidurans CH34	β-Proteobacteria	
R. solanacearum GM1000		
Ralstonia sp. XJ12B		

[a:] Reference organism.

Table 3. Taxonomic Distribution of Organisms (Genera) Possessing Enzymes Acyl-Homoserine Lactone-Acylase as well as – Lactonase

AHL-Lactonase		AHL-Acylase	
***Bacillus* sp. SB4**[a] (GenBank AAR85482.1)	**Motif**[b]	***Ralstonia* sp. XJ12B**[a] (GenBank AAO41113.1)	**Motif**[c]
Actinobacteria			
Streptomyces clavuligerus ATCC 27064	+	*Streptomyces* sp. FERM BP-5809	+ (I_{54}-D_{61})
		Streptomyces sp. M664	+ (I_{54}-D_{61})
		Streptomyces sp. Mg1	+ (I_{54}-D_{61})
		Streptomyces griseus subsp. *griseus* NBRC 13350	+ (I_{54}-D_{61})
		S. sviceus ATCC 29083	+ (L_{54}-D_{61})
Deinococcus-Thermus			
Deinococcus radiodurans R1	+	*Deinococcus radiodurans* R1	+ (I_{58}-S_{65})
D. geothermalis DSM 11300	+		
Alphaproteobacteria			
Hyphomonas neptunium ATCC 15444	+	*Hyphomonas neptunium* ATCC 15444	+ (I_{55}-S_{62})
Betaproteobacteria			
Ralstonia eutropha JMP134	+	*Ralstonia* sp. XJ12B	+ (I_{49}-D_{56}), + (I_{52}-S_{59})
		Ralstonia eutropha H16	+ (I_{62}-D_{69})
		R. metallidurans CH34	+ (I_{51}-S_{58})
		R. pickettii 12J	+ (I_{51}-D_{58})
		R. solanacearum GMI1000	+ (I_{50}-D_{57})
Gammaproteobacteria			
Photorhabdus luminescens subsp. *laumondii* TTO1	+	*Photorhabdus luminescens* subsp. *laumondii* TTO1	+ (I_{57}-S_{64})
marine gamma proteobacterium HTCC2080	+	marine Gamma proteobacterium HTCC2143	+ (I_{43}-D_{53})
		marine Gamma proteobacterium HTCC2207	+ (L_{106}-D_{113})

[a]: Reference organisms
[b]: Motif for AHL-lactonase: His^{106}-X-Asp^{108}-His^{109}-59X-His^{169}-21X-Asp^{191}
[c]: Motif for AHL-acylase: amino acid – Ile (I) /Leu (L)^{50} and Ser(S) /Asp (D)^{57}

unique. The BLAST (against the NCBI database) result validated the uniqueness of these signatures (Table **4**). A high correlation was recorded for signatures M1 and M10 of *Bacillus* sp. SB4 and their occurrence among a large number of organisms possessing AHL-lactonase (BLAST hits with high frequency). Similarly, among the different signatures identified within each taxonomic group for AHL-acylase, those retrieved from *Pseudomonas* spp. within γ-Proteobacteria gave the BLAST hits with high frequency for organisms of this taxonomic group. Sequence homology searches using BLAST for all other signatures showed best hits for the query sequence from the respective taxonomic group but their frequency among the top 50 hits was quite low.

DISCUSSION

Bacteria with Ability to Quench QS Signals

With the information available in the literature on the presence of AHL-lactonase and AHL-acylase activities in *R. erythropolis* W2 [30-32], we also looked for certain other organisms with potential to produce both these enzyme types. Our *in silico* study allowed us to identify organisms from five different genera with potential to produce both types of AHL-degrading enzymes. The three strains i) *D. radiodurans* R1, ii) *H. neptunium* ATCC 15444 and iii) *P. luminescens* subsp. *laumondii* TTO1 are among the top contenders for producing both the enzzyme type from an individual strain. However, more such strains can be tracked among strains belonging to *Streptomyces* spp. (Actinobacteria), ii) *Ralstonia* spp. (β-Proteobacteria), and marine gamma proteobacterium. In order to reduce the efforts needed to search organims, conserved regions can be used for designing primers for amplifying these genes. Signature sequences deduced in this study can be used as an additional tool to assign these sequences to respective taxonomic groups, at least in some cases.

Another interesting features of this *in silico* study is that *R. solanacearum* GMI1000 (β-Proteobacteria) has been

Table 4. Characteristics of Unique Nucleotide Signatures for Acyl-Homoserine Lactone-Acylase and –Lactonase Showing Closest Match to the Respective Taxonomic Group

Taxonomic Group	Signature		*Source Organism*
	Nucleotide Sequence	No.	
AHL-lactonase with reference to Bacillus sp. SB4			
Actinobacteria	CGTGAGCTCGAGTACGGCCTGTCGAATCAC[a]	M8	*Mycobacterium* sp.MCS [gi|108796981: 1991857-1992693]
Firmicutes	TCACTTACATTTTGATCATGCAGGAGGAAA	M1	*Bacillus* sp. SB4 [gi|40388446|gb| AY483161.1|]
	TCCGGTTCAGTTTTATTAACGATTGATGCA	M10	
Alphaproteobacteria	GTTGTGCAGTCGCACCTGCATCTCGATCAT[a]	M1	*Agrobacterium tumefaciens* [gi|17223781: 3565-4356]
	ATCCGCAAGGACTTCGACAAGCCGGGCCT[a]	M4	
Betaproteobacteria	GTGGATACGGAAGACGGCGTGGTGCTGAT[a]	M7	*Burkholderia graminis* [gi|170694799: 130043-130822]
Gammaproteobacteria	AATCCGGTATCAATCAGTACAAGGCCTCGA[a]	M2	*Acinetobacter baumannii AYE* [gi|169794206:627842-628654]
Deltaproteobacteria	ATGGTCGTTATGCAGGTGCGTGTGTATGAC[a]	M1	*Desulfovibrio desulfuricans* G20 [gi|78355047:1350539- 1351285]
Ascomycota	TACACGAGGAAGAGTTCAAGCATGCATGCT[a]	M2	*Aspergillus oryzae* RIB40 [gi|169784499|ref| XM_001826659.1|]
	GATCAATTCCATGTGCGGGAGAACTACGAG[a]	M3	
Euryarchaeota	*All signatures*[a]		
AHL-acylase with reference to Ralstonia sp. XJ12B			
Actinobacteria	CGGGTTCGCCGCCGGGTACAACGCGTGGAT[a]	M2	*Streptomyces* sp. M664 [gi|49781326:185-2599] and Streptomyces sp. FERM BP-5809 [gi|40786372:948-3362]
	GTCATCCGGTACACGGAGTACGGCATTCCG[a]	M3	
Bacteroidetes	ATCGAGATGCTTGAAGGGGATTCCTCGA[a]	M3	*Salinibacter ruber* DSM 13855 [gi|83814055:1438503-1440524-]
	GGCTTCGGGGGGAGATGGTCTTTTTG[a]	M7	
	TTTGACGAGATGGAGGGCGGG[a]	M8	
Cyanobacteria	GCTGGTTCCAATGCTTGGGCGATCGCACCT[a]	M5	*Cyanothece* sp. [gi|126661348:2396-4477-]
Alphaproteobacteria	ATGACTCCGGCAAGCCCGAAGAAGAAAGGC[a]	M7	*Erythrobacter litoralis* HTCC2594 [gi|85372828:319908-322139]
	GATATTGCCCTCGGCATCGCCATAGATGAA[a]	M10	
Betaproteobacteria	CCAGCCGCATGCGCAGGGTTTCCTGGGTCC[a]	M3	*Ralstonia eutropha* H16 [gi|113866031:2083444-2085906]
	TCGGCACGCGCAGGTTGAACAGCGGCGACA[a]	M7	
Gammaproteobacteria: *Pseudomonas* spp.	CTGCCGGTGGTCAACATCGGCTTCAGCCGC	M1	*Pseudomonas aeruginosa* PA7 [gi|152983466:2955521-2957803]
	TTCGTACAGAACTCCAACGACAGCGCCTGG	M2	
	GATGCGTTTCTACCAGATGCACCTGACCAT	M3	
	ATGCTGCTGGCCAACCCGCATTTCCCCTGG	M4	
	CGGCATCGGCTACGCCTACGCGCGCGACA	M6	
	GCGCGATGCCAACCTGGAGAACACCCGGGT	M7	
	CCGCTGGACCGCCTATGGCGTGCCGCACAT	M9	
	ATCCCCTGGGTCAACACCCTGGCCGCGGA	M10	

Table 4. contd….

Taxonomic Group	Signature		*Source Organism*
	Nucleotide Sequence	**No.**	
AHL-acylase with reference to Ralstonia sp. XJ12B			
Gammaproteobacteria: *Shewanella* spp.	CAGATAATAAAGGCAACGCCTTCTACATCG[a]	M1	*Shewanella woodyi* ATCC 51908 [gi|170724370:166858-169239]
	CCCTTTGAGCGTGCCCCCAAAGTGATGCGT[a]	M2	
	GGCCGTGATAAAACCGAATCTGGTAGCGGT[a]	M3	
	TTAATCGGCTTCAATGAACATGTTGCTTGG[a]	M6	
	GGTTATGGGCAAGCCTATGCCCATGCTCAG[a]	M10	
Others Gammaproteobacterial members	TTATCCGCTTCGATGTGCGGCGCGCCGAA[a]	M8	*Hahella chejuensis* KCTC 2396 [gi|83642913:5633846-5636326]

[a:] Sequence homology searches using BLAST for these signatures showed best hits for the query sequence but the frequency among the top 50 hits was quite low.

found to possses gene and motif for AHL-acylase. This observation finds support from the work which demonstrates the presence of AHL-acylase in *R. solanacearum* GMI1000, through cloning and expression of *aac* gene [25]. It led to inactivation of four different AHLs [25]. Another interesting observation was the presence of genes for quenching the QS signals in archaeal member *A. fulgidus.* Although archaea have not been reported so far to be directly involved in causing infectious diseases [48], however, there are evidences of their presence in endodonic infections [49]. The presence of these genes support the possibility of their role in infections.

MOTIFS OF AHL-LACTONASES AND AHL-ACYLASES

AHL-lactonase is a member of metallo-hydrolase superfamily containing the motif: His^{104}-X-His^{106}-X-Asp^{108}-His^{109}, which resembles zinc binding motif of several other metallo-enzymes such as glyoxalase II, aryl sulfatase and β-lactamase. The motif His^{106}-X-Asp^{108}-His^{109}-59X-His^{169}-21X-Asp^{191} has been reported to be essential for AHL-lactonase activity [18, 22].

A very interesting scenario was observed while searching for motifs essential for AHL-lactonase enzyme activities. It was observed to be that motif essential for AHL-lactonases - His^{106}-X-Asp^{108}-His^{109}-59X-His^{169}-21X-Asp^{191} was present in all the organisms having potential for AHL-lactonase except *R. solanacearum* GMI1000, *A. baumannii* AB0057, *A. baumannii* ACICU, *A. baumannii* AYE, *Acinetobacter* sp. ADP1 and *Pseudomonas stutzeri* A1501 (Table **3**). Incidentally, these latter 6 bacteria lacked even the motif specific for metallo-enzymes in genral- His^{104}-X-His^{106}-X-Asp^{108}-His^{109}. Among the organisms found to possess both the motifs – all belonged to *Bacillus* spp.: *B. anthracis* str. Ames, *B. cereus, B. cereus* AH1134, *B. cereus* AH820, *B. cereus* ATCC 10987, *B. cereus* ATCC 14579, *B. cereus* B4264, *B. cereus* E33L, *B. cereus* G9241, *B. cereus* NVH0597-99, *Bacillus* sp. 240B1, *Bacillus* sp. 42, *Bacillus* sp. 91, *Bacillus* sp. A24, *Bacillus* sp. COT1, *Bacillus* sp. CSX-1, *Bacillus* sp. SB4, *B. subtilis, B. thuringiensis, B. thuringiensis serovar alesti, B. thuringiensis serovar canadensis, B. thuringiensis serovar galleriae, B. thuringiensis serovar israelensis* ATCC 35646, *B. thuringiensis serovar jinhongiensis, B. thuringiensis serovar kim, B. thuringiensis serovar konkukian* str. 97-27, *B. thuringiensis serovar kurstaki, B. thuringiensis serovar kyushuensis, B. thuringiensis serovar ostriniae, B. thuringiensis serovar oswaldocruzi, B. thuringiensis serovar pakistani, B. thuringiensis serovar toumanoffi* and *B. weihenstephanensis* KBAB4.

Available literature reveals that AHL-acylases are structurally similar with respect to the two amino-acids at positions 50 and 57 [18] as a) AiiD of *Ralstonia* sp. XJ12B has Ile^{50} and Ser^{57} [26], b) PvdQ of *P. aeruginosa* PAO1 has Leu^{50} and Asp^{57} [27], and c) AhlM of *Streptomyces* sp. has Leu^{50} and Ser^{57} [50]. These share features with Ntn hydrolases including the signal peptide, α- and β-subunits [51].

On the other hand, Cephalosporin-acylase has Gln and Arg at positions 50 and 57 respectively [18]. Our analysis reveals that most of the organisms had amino-acids of AHL acylase in a slightly different positions than those reported in the literature (Table **3**). Unique features were observed in i) *Streptomyces* spp., where the amino acids of motif for acylase resembled either those found in PvdQ or were a combination of Ile of AiiD and Asp of PvdQ, ii) *Ralstonia* spp., where Ser was substituted with Asp and also that *Ralstonia s*p. XJ12B was found to posses two motifs – a) Ile^{52} and Ser^{59} and b) Ile^{49} and Asp^{56}. In view of the fact that in spite of such a large number of organisms, which had the conserved domains for AHL-acylase, only few seem to have retained the property observed at respective positions as reported in literature [18, 26, 27, 50]. It may not be too inappropriate to conclude that amino-acids at positions 50 and 57 might have changed during the course of evolution.

A few other organisms which deserve attention are: i) *Burkholderia* spp. (β-Proteobacteria), which had motifs for

lactonase and cephalosporin acylase, ii) *Pseudomonas* sp. (γ-Proteobacteria), which possessed two motifs for AHL-acylase and one for cephalosporin acylase, and iii) *A. baumannii* AYE (γ-Proteobacteria), which showed motifs for cephalosporin acylase.

USING SIGNATURES TO DESIGN PRIMERS

Bacillus spp. and *Pseudomonas* spp. have been reported to possess repeating elements in the 16S rDNA [2, 45]. We observed such repeats in the AHL-lactonase and -acylase gene in different members of these two genera. Individually, these signatures were validated by using them as query sequences in BLAST search. Since the frequency of repeating elements in this gene was quite high in the case of *Pseudomonas* spp., we also checked them for regular expressions [52]. We observed a very good co-relation between the full length gene sequence and the strings (Fig. **S18a**, **b**). The nucleotide signatures identified in our study for each taxonomic group may enable one to design primers to amplify genes for AHL-lactonase [20, 24, 53] or AHL-acylase and identify a possible taxonomic group within a microbial community.

THE ROLE OF QUORUM QUENCHERS

The role of QQ enzymes *in vivo* is debatable [54]. They may be involved in metabolic pathways where AHL and its products may be used as energy and carbon sources -such as by *Variovorax paradoxus* and *Arthrobacter* spp. [55-57]. AHL-acylase PvdQ from *P. aeruginosa* is thought to be involved in biosynthesis of protein (pyoverdine) necessary for acquisition of iron [28, 54, 58]. The role of the enzymes – lactonase and acylase for QQ has been proposed to prevent food, prevent biofilm growth, surface fouling, treatment of bacterial infections, etc. [59]. It can act as a potential drug, which will selectively block the virulence and pathogenic traits [60]. Heterologous expression of *Bacillus* AiiA lactonase in *Burkholderia thailandensis* [24], in *P. aeruginosa* PAO1 [23], in *Vibrio harveyi* [53], in *E. carotovora* [22] have been found to disrupt QS mediated properties. In fact, certain antibiotics become effective on biofilms which have been exposed to AHL degrading enzymes [61], which imply a combination of the two treatments can be complementary and effective in controlling the pathogenic organism(s). Although there may be barriers to their diffusion within the microbial communities in the soil environment, however, their usage as a drug may not face similar barriers and may prove effective.

CONCLUSION

Using available metabolic and genomic databases databases, we have been able to trace potential organisms such as i) *Deinococcus radiodurans* R1, ii) *Hyphomonas neptunium* ATCC 15444 and iii) *Photorhabdus luminescens* subsp. *laumondii* TTO1 to possess genes for AHL-lactonases and –acylases. In addition, all the cyanobacterial members were found to possess genes only for AHL-acylase whereas members of Acidobacteria, Sphingobacteria, Spirochaetales, Nitrospirales and Planctomycetes were found to possess genes only for AHL-lactonase. It also revealed those bacteria which evolved to produce either of the two enzymes, an indication of the environmental stresses encountered during their evolution. The presence of genes for quenching the QS signals in archaea has further increased the range of organisms which can be exploited for producing these enzymes for preventing food spoilage, bioremediation and design drugs against bacterial infections.

Phylogenetic analyses and multiple sequence alignment of the gene sequences coding for the two enzymes revealed consensus sequences, which can be used to design primers for amplifying these genes even among mixed cultures and metagenomes. Nucleotide signature sequences deduced in this study can be used as an additional tool to assign these sequences to respective taxonomic groups. A few phylogenetic discrepancies observed for AHL-lactonase and –acylase may imply HGT and help us identify those organisms which can be subjected to genetic engineering with a relative ease.

It has been proposed that since lactonase activity is not influenced by the acyl chain length of the AHL signal molecule, hydrolysis of the ring may be more effectve approach for QQ. As a wide range of *Bacillus* spp. have an ability to produce lactonase, it may be the organism of choice in future. The usage of *Bacillus* is quite beneficial since it has been labeled as industrial workhorse and accorded the designation GRAS (Generally Regarded As Safe) by FDA. These organisms can be exploited for producing drugs against pathogens.

SUPPLEMENTARY MATERIAL

Supplementary material is available on the publishers web site along with the published article.

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AUTHOR'S CONTRIBUTIONS

VCK has contributed towards the conceived and designed the experiments, analyzed the data and wrote the paper.

CRS and HJP have performed the experiments, and contributed reagents/ materials/ analysis tools. All authors read and approved the final manuscript.

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